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PRINCIPAL INVESTIGATOR:

Byron Caughey, Ph.D.

CONTRACTING ORGANIZATION:

National Institutes of Health
Rocky Mountain Laboratories
Hamilton, MT 59840

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14. ABSTRACT Transmissible spongiform encephalopathies (TSEs) or prion diseases are fatal, transmissible and untreatable neurodegenerative diseases of humans and animals. To aid in the search for anti-TSE therapeutics, we have 1) developed new relatively high through-put in vitro drug screens, 2) assayed large numbers of previously untested compounds as TSE inhibitors, 3) identified numerous new prion/PrP ^{Sc} inhibitors in vitro and in vivo, and 4) developed a new unified mechanistic model for the activity of various classes of PrP ^{Sc} inhibitors which is consistent with a considerable body of evidence from our laboratory and others.									
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INTRODUCTION

Prion diseases, or transmissible spongiform encephalopathies are neurodegenerative diseases of humans and animals that are transmissible, fatal and, as yet, untreatable. These diseases include Creutzfeldt-Jakob disease (CJD) in humans, bovine spongiform encephalopathy (BSE), scrapie in sheep (and rodent models), and chronic wasting disease (CWD) in cervids. Our goal in this work is to identify new prophylaxes and therapeutics for the transmissible spongiform encephalopathies (TSEs) or prion diseases. The key pathogenic process in prion diseases is the accumulation of misfolded prion protein, e.g. PrP^{Sc}. Although some effective experimental post-exposure prophylactic treatments have been identified that can substantially increase the survival times of prion-infected animals if treatments are initiated well in advance of the onset of clinical signs of disease [reviewed in (Cashman and Caughey, 2004)], no treatments that are known to be effective once clinical signs have appeared. To aid in the search for anti-TSE compounds, we have continued to develop new *in vitro* screens for inhibitors of PrP^{Sc} accumulation and to test new ways of administering such compounds to infected animals to improve their survival times.

BODY

Research Accomplishments

Task 1: To increase the throughput of a screen for anti-PrP^{Sc} activity in the scrapie-infected neuroblastoma cell model: We developed a high-through-put version of the scrapie-infected neuroblastoma cell culture model (ScN2a). See Kocisko *et al.*, New inhibitors of scrapie associated prion protein formation in a library of 2000 drugs and natural products, *J. Virol.*, 2003, in Appendix.

Task 2: Develop a high-throughput cell-free system to measure the ability of compounds to interfere with PrP^C to protease-resistant PrP conversion. Using the ScN2a screening system described above (Task 1) as well as animal studies described below in Task 4, we determined that a variety of non-CpG phosphorothioate oligonucleotides (PS-ONs) had potent anti-TSE activity *in vitro* and *in vivo* (see Kocisko *et al.*, Potent anti-scrapie activities of degenerate phosphorothioate oligonucleotides. *Antimicrob Agents Chemother*, 2006, in Appendix). Furthermore, we found that effective PS-ONs, like several other classes of anti-TSE compounds, could bind to normal prion protein (PrP-sen) and cause it to cluster and be internalized from the surface of cultured cells. In consideration of these and other observations, we developed a new mechanistic model for the mechanism of inhibition of various anti-TSE compounds (Caughey *et al.*, Prions and TSE chemotherapeutics: A common mechanism for anti-TSE compounds? *Accts Chem Res* 2006; see Appendix). Based on this model, we surmised that molecules that can compete with PS-ONs binding to PrP-sen might also have anti-TSE activity. Using a fluorescently tagged PS-ON, recombinant PrP-sen (rPrP-sen), and fluorescence correlation spectroscopy, we developed a competitive binding assay for compounds that block the binding of PS-ONs to PrP-sen as detailed (see Kocisko *et al.*, Identification of anti-prion molecules by a fluorescence polarization-based competitive binding assay. *Anal Biochem*, 2007; in Appendix). This assay provides a new rapid and potentially high-throughput screen for anti-TSE compounds. The predictive accuracy of this cell-free screen rivaled that of scrapie-infected cell-based assays. We have summarized the latter assays in detail in Kocisko and Caughey, Searching for anti-prion compounds: Cell-based high-throughput *in vitro* assays and animal testing strategies. *Meth Enzymol* 2006, (in Appendix).

Task 3: To screen libraries of compounds for anti-PrP^{Sc} activity. In addition to the cell culture and cell-free assays described for Tasks 1 & 2, we developed another TSE cell culture model for drug screening, namely the first cell culture line (from mule deer) that is chronically infected with CWD, to broaden the base of screening for anti-prion compounds (see Raymond *et al.*, Inhibition of protease-

resistant prion protein formation in a transformed deer cell line infected with chronic wasting disease. *J. Virol.*, 2007, in Appendix). These *in vitro* systems were used to screen thousands of compounds for inhibition of PrP^{Sc} formation. Many new classes of inhibitors were identified. For details see the following publications in the Appendix: Kocisko *et al.*, New inhibitors of scrapie associated prion protein formation in a library of 2000 drugs and natural products. *J Virol*, 2003; Kocisko *et al.*, Comparison of protease-resistant prion protein inhibitors in cell cultures infected with two strains of mouse and sheep scrapie. *Neurosci Lett* 2005; Kocisko *et al.*, Potent anti-scrapie activities of degenerate phosphorothioate oligonucleotides. *Antimicrob Agents Chemother*, 2006; Kocisko *et al.*, Identification of anti-prion molecules by a fluorescence polarization-based competitive binding assay. *Anal. Biochem.*, 2007.

Task 4: To test the compounds with the best activity in the anti-PrP^{Sc} screens in TSE-infected animals. A number of the best PrP^{Sc} inhibitors from the *in vitro* screens were tested in scrapie-infected rodents. Although many were found to be ineffective (Kocisko *et al.*, Evaluation of new cell-culture inhibitors of PrP-res against scrapie infection *in vivo*. *J Gen Virol*, 2004; Mefloquine, an anti-malaria drug with anti-prion activity *in vitro*, lacks activity *in vivo*. *J Virol* 2006. Many compounds can profoundly delay scrapie onset in rodents when administered at or near the time of peripheral infection; e.g. see Kocisko *et al.*, Potent anti-scrapie activities of degenerate phosphorothioate oligonucleotides. *Antimicrob Agents Chemother*, 2006, (in Appendix) but few have helped after intracerebral (ic) inoculation. We found that one exception is Fe(III)meso-tetra (4-sulfonatophenyl) porphine (FeTSP), which due to poor blood brain barrier penetration must be administered directly to the brain. (Kocisko *et al.*, A porphyrin increases survival time of mice after intracerebral prion infection. *Antimicrob Agents Chemother* 2006; in Appendix). Also, Doh-Ura and colleagues have shown that PPS, a semi-synthetic carbohydrate polymer approved as an oral therapy for interstitial cystitis (Elmiron®), can also be somewhat effective late in the course of infection if administered intracerebrally (Doh-ura *et al.*, 2004). Based on these observations, we tested the anti-scrapie activity of a combined formulation of PPS and FeTSP as detailed in Kocisko *et al.*, Enhanced anti-scrapie effect using combination drug treatment, *Antimicrob Agents Chemother* 2006; see Appendix. Combination treatments of mice beginning 14 or 28 days after scrapie inoculation significantly increased survival times over those seen with either of the compounds by themselves. The observed effects appeared to be more than additive, implying that these compounds might be acting synergistically *in vivo*. Combination therapies may therefore be more effective for treatment of TSEs and other protein misfolding diseases.

In further structure-function studies of a notable class of PrP^{Sc} inhibitors that we have identified, namely the cyclic tetrapyrroles like FeTSP, we have identified important characteristics of compounds that are effective both *in vitro* and *in vivo*. See Caughey *et al.*, Cyclic tetrapyrrole sulfonation, metals, and oligomerization in anti-prion activity. *Antimicrob Agents Chemother* 2007; and Lee *et al.*, Hemin interactions and alterations of the subcellular localization of prion protein. *J Biol Chem* 2007; in Appendix. Such insights should assist further rational drug design for prion diseases.

KEY RESEARCH ACCOMPLISHMENTS

- Developed a high-throughput, multiwell plate-based, scrapie-infected N2a cell culture assay for inhibitors of PrP^{Sc} accumulation.
- Identified new inhibitors of PrP^{Sc} accumulation in a library of 2000 drugs and natural products.
- Evaluated of new cell-culture inhibitors of PrP-res against scrapie infection in rodent models.
- Compared PrP^{Sc} inhibitors in cell cultures infected with two strains of mouse and sheep scrapie.
- Developed the first transformed deer cell line infected with chronic wasting disease.
- Identified inhibitors of the pathological PrP^{CWD} accumulation in CWD-infected cells.
- Showed that mefloquine, an anti-malaria drug with anti-prion activity *in vitro*, lacks activity *in vivo*.

- Demonstrated that a porphyrin can increase survival time of mice after intracerebral prion infection.
- Demonstrated that combination therapy with a porphyrin and pentosan polysulfate enhanced anti-scrapie effects *in vivo*.
- Showed that degenerate phosphorothioate oligonucleotides have potent anti-scrapie activities.
- Developed a model for a common mechanism for anti-TSE compounds.
- Identified anti-prion molecules by using a new fluorescence polarization-based competitive binding assay.
- Characterized the role of sulfonation, metals, and oligomerization in the anti-prion activity of cyclic tetrapyrroles.
- Showed that the natural cyclic tetrapyrrole hemin interacts with, and alters the subcellular localization of, cellular prion protein

REPORTABLE OUTCOMES

Kocisko DA, Baron GS, Rubenstein R, Chen J, Kuizon S, Caughey B. New inhibitors of scrapie associated prion protein formation in a library of 2000 drugs and natural products. **J Virol** 2003; 77:10288-10294.

Kocisko DA, Morrey JD, Race RE, Chen J, Caughey B. Evaluation of new cell-culture inhibitors of PrP-res against scrapie infection *in vivo*. **J Gen Virol** 2004; 85:2479-83.

Cashman NR, Caughey B. Prion diseases: Close to effective therapies? **Nature Reviews Drug Discovery** 2004; 3:874-884

Kocisko DA, Engel AL, Harbuck K, Arnold KM, Olsen E, Raymond LD, Vilette D, Caughey B. Comparison of protease-resistant prion protein inhibitors in cell cultures infected with two strains of mouse and sheep scrapie. **Neurosci Lett** 2005 388:106-111.

Raymond GJ, Olsen EA, Lee KS, Bryant III PK, Raymond LD, Baron GS, Caughey WS, Kocisko DA, McHolland LE, Favara C, Langeveld JPM, van Zijderveld FG, Mayer RT, Miller MW; Williams ES, Caughey B. Inhibition of protease-resistant prion protein formation in a transformed deer cell line infected with chronic wasting disease. **J Virol** 2006 80:596-604.

Kocisko DA, Caughey B. Mefloquine, an anti-malaria drug with anti-prion activity *in vitro*, lacks activity *in vivo*. **J Virol** 2006 80:1044-1046.

Kocisko DA, Caughey WS, Race RE, Roper G, Caughey B, and Morrey JD. A porphyrin increases survival time of mice after intracerebral prion infection. **Antimicrob Agents Chemother** 2006 50:759-761.

Kocisko DA, Vaillant A, Lee KS, Arnold KM, Bertholet N, Race RE, Olsen EA, Juteau J-M, Caughey B. Potent anti-scrapie activities of degenerate phosphorothioate oligonucleotides. **Antimicrob Agents Chemother** 2006 50:1034-1044.

Kocisko DA, Caughey B. Searching for anti-prion compounds: Cell-based high-throughput *in vitro* assays and animal testing strategies. **Meth Enzymol** 2006 412:223-234.

Caughey B, Caughey WS, Kocisko DA, Lee KS, Silveira JR, Morrey JD. Prions and TSE chemotherapeutics: A common mechanism for anti-TSE compounds? **Accounts Chem Res** 2006 39:646-653.

Kocisko DA, Caughey B, Morrey JD, Race R. Enhanced anti-scrapie effect using combination drug treatment. **Antimicrob Agents Chemother** 2006 50:3447-3449.

Kocisko DA, Bertholet N, Moore RA, Caughey B, Vaillant A. Identification of anti-prion molecules by a fluorescence polarization-based competitive binding assay. **Anal Biochem** 2007 363:154-156.

Caughey WS, Priola SA, Kocisko DA, Raymond LD, Ward A, Caughey B. Cyclic tetrapyrrole sulfonation, metals, and oligomerization in anti-prion activity. **Antimicrob Agents Chemother** 2007 51:3887-3894.

Lee KS, Raymond LD, Schoen B, Raymond GJ, Kett L, Moore RA, Johnson LM, Taubner L, Speare JO, Onwubiko HA, Baron GS, Caughey WS, Caughey B. Hemin interactions and alterations of the subcellular localization of prion protein. **J Biol Chem** 2007 282:36525-36533.

CONCLUSIONS

We have made significant progress toward all of the goals of this project and have published 14 papers based at least in part on this work. We have identified numerous new classes of PrP^{Sc} inhibitors some of which show encouraging prophylactic efficacy against prion disease *in vivo*. One of these compounds, FeTSP, substantially prolonged the survival of rodents even when administered intracerebrally later on in the course of scrapie infections. When such administration of FeTSP was combined with pentosan polysulfate, an even greater, apparently synergistic, beneficial effect was observed. To bolster the rational basis for the search for anti-TSE therapeutics, we have learned about the structure-activity relationships for various PrP^{Sc} inhibitors and developed a new unified mechanistic model for the activity of various classes of PrP^{Sc} inhibitors. Based on this model, we have successfully developed a potentially high-throughput screen for new anti-TSE compounds which is based on monitoring the ability of compounds to compete with the binding of a well-characterized anti-TSE compound (a PS-ON) to PrP-sen. Much progress has been made but much remains to be done to find a drug that is safe and effective in humans with prion diseases.

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APPENDICES (publications, in order of presentation)

1. Kocisko DA, Baron GS, Rubenstein R, Chen J, Kuizon S, Caughey B. New inhibitors of scrapie associated prion protein formation in a library of 2000 drugs and natural products. **J Virol** 2003; 77:10288-10294.

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New Inhibitors of Scrapie-Associated Prion Protein Formation in a Library of 2,000 Drugs and Natural Products

David A. Kocisko,¹ Gerald S. Baron,¹ Richard Rubenstein,² Jiancao Chen,³
Salomon Kuizon,² and Byron Caughey^{1*}

Laboratory of Persistent Viral Diseases, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, Montana¹; Laboratory of Molecular and Biochemical Neurovirology, New York State Institute for Basic Research in Developmental Disabilities, Staten Island, New York²; and Chengdu Jinniu Institute, Food Bureau of Sichuan Province, Chengdu Sichuan, China³

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Transmissible spongiform encephalopathies (TSEs) are fatal, untreatable neurodegenerative diseases associated with the accumulation of a disease-specific form of prion protein (PrP) in the brain. One approach to TSE therapeutics is the inhibition of PrP accumulation. Indeed, many inhibitors of the accumulation of PrP associated with scrapie (PrP^{Sc}) in scrapie-infected mouse neuroblastoma cells (ScN₂a) also have antiscrapie activity in rodents. To expedite the search for potential TSE therapeutic agents, we have developed a high-throughput screening assay for PrP^{Sc} inhibitors using ScN₂a cells in a 96-well format. A library of 2,000 drugs and natural products was screened in ScN₂a cells infected with scrapie strain RML (Chandler) or 22L. Forty compounds were found to have concentrations causing 50% inhibition (IC₅₀s) of PrP^{Sc} accumulation of ≤ 10 μ M against both strains. Seventeen had IC₅₀s of ≤ 1 μ M against both strains. Several classes of compounds were represented in the 17 most potent inhibitors, including naturally occurring polyphenols (e.g., tannic acid and tea extracts), phenothiazines, antihistamines, statins, and antimalarial compounds. These 17 compounds were also evaluated in a solid-phase cell-free hamster PrP conversion assay. Only the polyphenols inhibited the cell-free reaction, and their IC₅₀s were near 100 nM. Several of the new PrP^{Sc} inhibitors cross the blood-brain barrier and thus have potential to be effective after TSE infection reaches the brain. The fact that many are either approved human drugs or edible natural products should facilitate their use in animal testing and clinical trials.

Transmissible spongiform encephalopathies (TSEs) are neurodegenerative diseases that include Creutzfeldt-Jakob disease, chronic wasting disease, scrapie, and bovine spongiform encephalopathy. These diseases are characterized by the accumulation of a form of prion protein (PrP) that is partially resistant to degradation by proteases (23). The infectious TSE agent is not fully understood but is surmised to contain the proteinase K (PK)-resistant aggregate of PrP (8). The occurrence of TSEs is associated with specific mutations in PrP, inoculation with infectious material, or apparently spontaneous onset (23). Currently, there are no therapies for TSEs, and the diseases are invariably fatal. Thus, it is important to identify compounds with therapeutic or prophylactic activity against these diseases.

The conversion of PrP from the normal, protease-sensitive, and nonaggregated form (PrP^C) to the aggregated and protease-resistant form associated with scrapie (PrP^{Sc}) or other TSEs (PrP^{TSE}) is a hallmark of the diseases. While the mechanism of neurodegeneration in TSEs is not clear, interactions between PrP^C and PrP^{TSE} seem to be important in the pathology of TSEs. Thus, the prevention of PrP^{TSE} formation and/or the elimination of existing PrP^{TSE} may be therapeutic (14, 22, 29).

Chronically scrapie-infected neuroblastoma cells (ScN₂a)

have been used extensively as a model for studying TSEs (1). The cells produce PrP^{Sc}, permitting cellular processes associated with PrP^{Sc} production to be examined. ScN₂a cells have been used to study the effect of PrP mutations (16, 30), barriers to interspecies transmission (21, 25), PrP metabolism (5), and inhibitors of PrP^{Sc} formation (11). To expedite the screening of compounds for anti-PrP^{Sc} activity in cell cultures, slot blot and dot blot assays have been developed (24, 31). Many different types of compounds, such as sulfonated dyes (9), sulfated glycols (4), cyclic tetrapyrroles (7), polyene antibiotics (18), curcumin (6), lysosomotropic antimalarial compounds (11), phenothiazines (17), and polyamines (27), can inhibit PrP^{Sc} formation when added to the medium of these cells. In addition, several of these classes of inhibitors have prolonged the survival time of scrapie-infected animals when administered near the time of infection (3, 10, 12, 15, 22). Thus, ScN₂a cells provide a useful in vitro model for screening compounds for anti-TSE activity.

In the present study, we screened a commercially available library of drugs and natural products to find new candidates for therapeutic intervention against TSEs. The inhibition of PrP^{Sc} production was monitored in ScN₂a cells infected with scrapie strain RML (Chandler) (4) or 22L. PrP^{Sc} from cells plated in a 96-well format was assayed with a modification of the dot blot method of Rudyk et al. (24). Of the 2,000 compounds screened, 17 had concentrations causing 50% inhibition (IC₅₀s) of PrP^{Sc} accumulation of ≤ 1 μ M against the RML and 22L strains. A number of these candidates are used for other

* Corresponding author. Mailing address: Rocky Mountain Laboratories, 903 S. 4th St., Hamilton, MT 59840. Phone: (406) 363-9264. Fax: (406) 363-9286. E-mail: bcaughey@niaid.nih.gov.

indications in humans and would therefore be available for immediate clinical trials.

MATERIALS AND METHODS

Compound library. The library tested was The Spectrum Collection (Micro-Source Discovery Inc., Groton, Conn.). The 2,000 compounds in the library are primarily Food and Drug Administration (FDA)-approved compounds or natural products. An alphabetical list of the compounds is available at the Micro-Source Discovery website at www.msdiscovery.com/spect.html. The compounds are supplied as 10 mM solutions in dimethyl sulfoxide (DMSO).

Testing for PrP^{Sc} inhibitory activity in cell cultures. Approximately 20,000 RML (4)-infected or 22L-infected mouse neuroblastoma cells in 100 μ l of medium were added to each well of a Costar 3595 flat-bottom 96-well plate with a low-evaporation lid (Corning Inc., Corning, N.Y.) prior to the addition of test compounds. 22L-infected cells were developed by reinfection of RML-infected mouse neuroblastoma cells cured by seven passages in 1 μ g of pentosan polysulfate/ml of medium (2). The cured cells were reinfected by incubation with PrP^{Sc} purified from mouse brains infected with scrapie strain 22L. Others have reported the susceptibility of mouse neuroblastoma cells to 22L infection (20). Neuroblastoma cells reinfected with 22L have stably expressed PrP^{Sc} for over 100 passages. The cells were allowed to settle for 4 h before test compounds were added.

The 10 mM solutions of test compounds were diluted in DMSO and then in phosphate-buffered saline (PBS) prior to being introduced to the cell medium. Five-microliter solutions were added to the cell medium. DMSO concentrations in the cell medium were never higher than 0.5% (vol/vol). After a compound was added, the cells were incubated for 5 days at 37°C in a CO₂ incubator before being lysed.

Prior to cell lysis, the cells were inspected by light microscopy for toxicity, bacterial contamination, and density compared to controls. After removal of the cell medium, 50 μ l of lysis buffer was added to each well. Lysis buffer was composed of 0.5% (wt/vol) Triton X-100, 0.5% (wt/vol) sodium deoxycholate, 5 mM Tris-HCl (pH 7.4 at 4°C), 5 mM EDTA, and 150 mM NaCl. At 5 min after the addition of lysis buffer, 25 μ l of PK (0.1 mg/ml; Calbiochem) in Tris-buffered saline (TBS) was added to each well and incubated at 37°C for 50 min. A total of 225 μ l of 1 mM Pefabloc (Boehringer Mannheim) was added to each well to inhibit PK activity. A total of 250 μ l of 1 mM Pefabloc was added to samples that were not PK treated.

High-throughput measurement of PrP^{Sc} by a dot blot procedure. The dot blot procedure used is a streamlined version of that developed by Rudyk et al. (24). A 96-well dot blot apparatus (Schleicher & Schuell) was set up with a 0.45- μ m-pore-size polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore), and each dot was rinsed with 500 μ l of TBS. Under vacuum, the lysed and PK-treated samples were added to the apparatus over the PVDF membrane and rinsed with 500 μ l of TBS. The PVDF membrane was removed and covered with 3 M GdnSCN (Fluka) for 10 min at ambient temperature. GdnSCN was removed by five PBS rinses, and the membrane was blocked with 5% (wt/vol) milk-0.05% (vol/vol) Tween 20 (Sigma) in TBS (TBST-milk) for 30 min. An appropriate dilution of monoclonal antibody 6B10, an immunoglobulin G2a antibody reactive against mouse, hamster, elk, and sheep PrP in immunoblotting assays and enzyme-linked immunosorbent assays (data not shown), or 8 μ g of purified anti-PrP mouse monoclonal antibody 6H4 (Prionics) in 15 ml of TBST-milk was incubated with the membrane for 60 min. After TBST rinsing, a solution of ~500 ng of an alkaline phosphatase-conjugated goat anti-mouse antibody (Zymed) in 15 ml of TBST-milk was added and incubated for 45 min. After additional TBST rinsing, the membrane was treated with an enhanced chemifluorescence agent (Amersham) for 10 min, allowed to dry, and then scanned with a Storm Scanner (Molecular Dynamics). The intensity of the PrP^{Sc} signal from each well was quantitated by using ImageQuant software (Molecular Dynamics). Each 96-well plate had six untreated control wells and six wells treated with curcumin, a known PrP^{Sc} inhibitor in RML-infected ScN₂a cells (6).

Solid-phase PrP conversion assay. In brief, for the solid-phase PrP conversion assay (18), a 100-ng suspension of hamster scrapie strain 263K PrP^{Sc} in 40 μ l of PBS was added to wells of a 96-well plate and air dried to promote adherence of the protein to the surface. The wells were then blocked with 2% bovine serum albumin in PBS. This solution was removed, and another solution, containing ~20,000 cpm of hamster ³⁵S-labeled PrP^C with or without potential inhibitors, was added and incubated at 37°C for 48 h. The ³⁵S-labeled PrP^C solution was removed, and the wells were washed. PK (20 μ g/ml) was added to the wells and then removed after 1 h to digest unconverted but bound ³⁵S-labeled PrP^C. The protein in the wells was eluted by boiling in sodium dodecyl sulfate sample buffer and scintillation counted. To obtain the relative percent conversion, the mea-

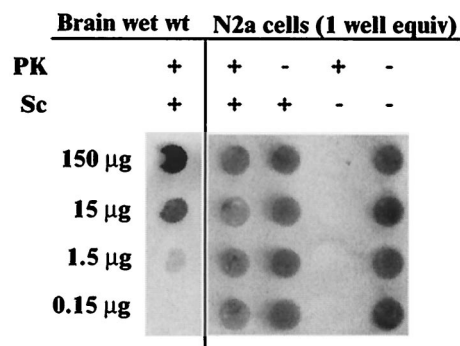


FIG. 1. Dot blot of brain-derived PrP^{Sc} and ScN₂a cell-derived PrP^C and PrP^{Sc}. The wells shown are from a single membrane visualized with primary antibody 6H4. The samples in the first lane contain the indicated brain wet-weight (wet wt) equivalents in a lysate from a hamster clinically ill from infection with scrapie strain 263K. The second and third lanes from the left contain lysates from RML-infected ScN₂a cells (one well equivalent). The fourth and fifth lanes contain lysates from uninfected N₂a cells. PrP^C from uninfected cells was detected without any PK treatment.

sured counts in PrP^{Sc} wells less the counts in bovine serum albumin-blocked wells lacking PrP^{Sc} were compared to the total ³⁵S-labeled PrP^C counts added to the wells.

RESULTS

High-throughput screen for PrP^{Sc} inhibitors. To facilitate the screening of large numbers of compounds for the inhibition of PrP^{Sc} accumulation, we developed a high-throughput test using ScN₂a cell cultures in combination with a rapid dot blot assay for PrP^{Sc} (scrapie cell dot blot [SCDB] assay). PrP^{Sc} from one well of ScN₂a cells in a 96-well plate was readily detectable by the SCDB assay (Fig. 1). Without PK treatment, PrP^C from uninfected N₂a cells was also readily detectable, but PK treatment eliminated this signal. Dilutions of scrapie-infected brain homogenates indicated that the PrP^{Sc} signal intensity from one well of cells fell between that from samples with 1.5 and 15 μ g of brain wet-weight equivalents (Fig. 1) in a linear response range of the PrP^{Sc} SCDB assay (data not shown). Similar results were obtained with anti-PrP monoclonal antibodies 6H4 (Fig. 1) and 6B10 (data not shown). A typical dot blot from the SCDB assay with antibody 6B10 is shown in Fig. 2.

Screening of a 2,000-compound library. The Spectrum Collection, a library of 2,000 drugs and natural products, was screened for PrP^{Sc} inhibitory activity with the SCDB assay. The identities of the compounds were not revealed to the investigator until screening was completed. A flowchart of the screening sequence is shown in Fig. 3. The compounds were screened initially at 10 μ M against RML-infected cells. Approximately 70% of the compounds showed less than 50% inhibition of PrP^{Sc} formation at 10 μ M in these cells and were not evaluated further. Approximately 20% (398) of the compounds were cytotoxic at 10 μ M and were tested again at 1 μ M. A smaller group of 246 compounds inhibited RML PrP^{Sc} accumulation by more than 50% at 10 μ M without observed toxicity. These 246 compounds were tested further at 10 μ M in ScN₂a cells infected with scrapie strain 22L, and 40 of them were found to reduce PrP^{Sc} accumulation by \geq 50% (35 compounds with IC₅₀s of between 1 and 10 μ M are shown in Fig. 4). These 40

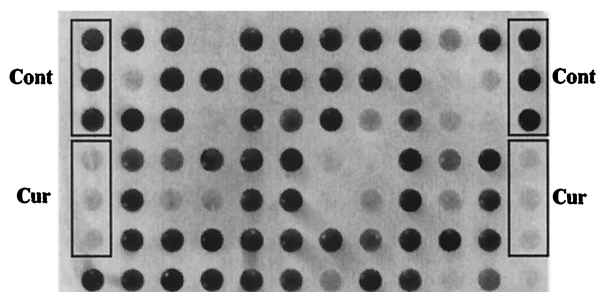


FIG. 2. Partial 96-well dot blot showing the PK-resistant PrP signal visualized with primary antibody 6B10. Signals from untreated control (Cont) cells and curcumin-inhibited (Cur) cells are indicated. The latter were incubated in the presence of 10 μM curcumin, a known inhibitor of PrP^{Sc} in RML-infected cells (6). Other dots represent signals from ScN₂a cells after incubation with 10 μM concentrations of various compounds. Some of these spots have an intensity comparable to that of controls, indicating no inhibition of PrP^{Sc} formation. Others that are less intense were due to compounds with various inhibitory strengths or toxicities.

compounds were then tested at 1 μM against both RML- and 22L-infected cells, revealing 5 compounds with IC₅₀s of ≤ 1 μM against both strains. Twelve additional inhibitors fitting these criteria were discovered when the 398 compounds cytotoxic at 10 μM were tested at 1 μM against both RML- and 22L-infected cells. Thus, of the 2,000 compounds screened, 17 had an IC₅₀ of ≤ 1 μM against both scrapie strains without observed toxicity (Fig. 5).

For compounds to pass through the screen described in Fig.

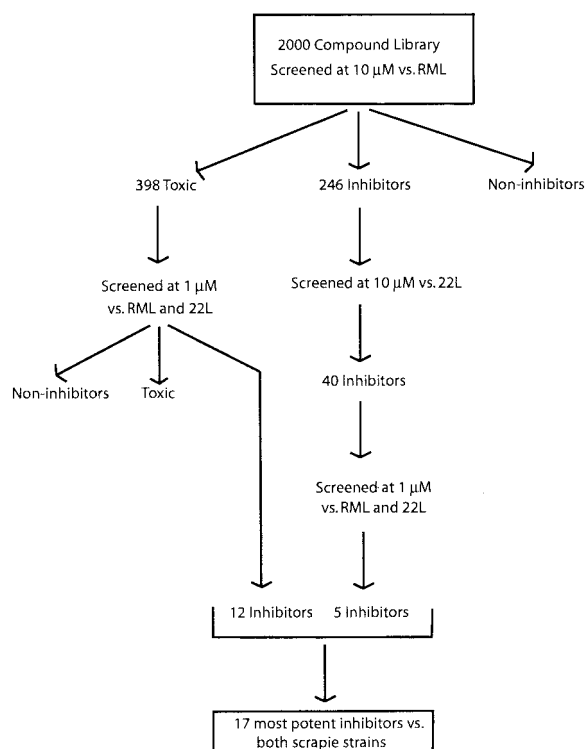


FIG. 3. Flowchart of the screening of The Spectrum Collection compound library.

3, the cells had to grow from low density to confluence and thrive in the presence of test compounds. However, as an additional test for potential cytotoxicity, a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) cell viability assay was performed with the 17 most potent inhibitors (19). The assay was done in duplicate after 3 and 4 days of incubation with the 17 compounds at 1 μM . The percentage of cell viability with all compounds tested in the MTT assay was in the range of 90 to 129% of controls on both days. In contrast, with aklavine and celastrol, which had visually obvious cytotoxicity, 2 to 9% cell viability was obtained on both days. The inhibition of PrP^{Sc} accumulation by these 17 compounds in the SCDB assay was confirmed in six separate experiments. Subsequent testing of these 17 compounds at 500, 100, and 10 nM revealed that tannic acid had an IC₅₀ of ~ 100 nM, whereas the other 16 compounds had IC₅₀s of between 100 nM and 1 μM (Fig. 5).

Of the 17 most potent inhibitors in The Spectrum Collection that were active against both scrapie strains, two, quinacrine and lovastatin, were identified previously as PrP^{Sc} inhibitors (11, 28). The remaining 15 compounds are novel inhibitors representing multiple classes of drugs or natural products, including polyphenols (e.g., tea and tree gall extracts), antimalarial compounds, antihistamines, phenothiazine analogs (e.g., antipsychotics), statins (hepatic hydroxymethyl glutaryl coenzyme A reductase inhibitors), and others, as indicated in Fig. 5.

Test of inhibition of cell-free PrP conversion. To test for direct effects on PrP conversion, the 17 most potent inhibitors were added to a solid-phase cell-free conversion (SP-CFC) reaction in which hamster PrP^C is used to induce the conversion of radiolabeled hamster PrP^C to a PrP^{Sc}-like PK-resistant state (18a). Three polyphenols, tannic acid, katechin, and 2',2''-bisepigallocatechin digallate, inhibited the SP-CFC reaction, with an IC₅₀ of approximately 100 nM (Fig. 6). The other 14 compounds were not inhibitory in the SP-CFC reaction at concentrations up to 100 μM (data not shown).

Test for destabilization of preexisting PrP^{Sc}. To search for compounds that can destabilize preexisting PrP^{Sc}, compounds with IC₅₀s of ≤ 10 μM were incubated at 250 μM with ScN₂a cell lysates for 24 h at 37°C to determine whether they could increase the PK sensitivity of PrP^{Sc}. However, even at a concentration at least 25 times its IC₅₀, no compound was able to increase the PK sensitivity of PrP^{Sc} (data not shown).

DISCUSSION

The high-throughput SCDB assay has greatly expedited our search for new, potentially therapeutic inhibitors of PrP^{Sc} accumulation. Clearly, one cannot expect that all compounds selected as potent inhibitors with this *in vitro* screen will prove to be effective anti-TSE drugs *in vivo*. However, given that a majority of the different classes of compounds that were previously identified as potent inhibitors by use of RML-infected ScN₂a cells have prophylactic efficacy against scrapie *in vivo*, we expect the SCDB assay will be valuable in the initial screening of potential drugs from large compound libraries. The additional use of ScN₂a cells infected with the 22L strain of scrapie in the SCDB assay helps to identify compounds with broader, less strain-dependent inhibitory activity. Adaptations of the SCDB assay for use with TSE-infected cell cultures from

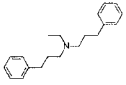
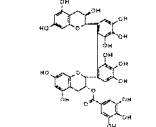
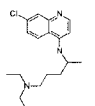
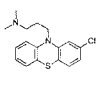
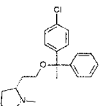
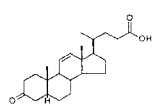

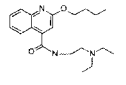
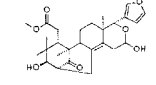

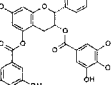
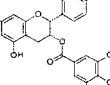
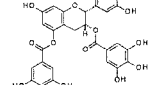
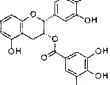
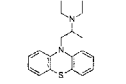
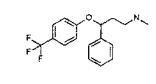

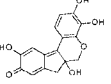
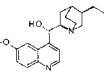
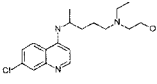

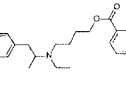
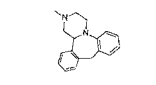

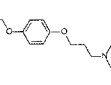
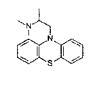
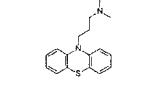
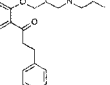
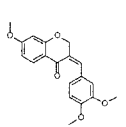
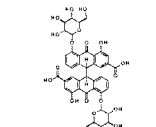
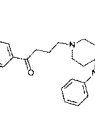
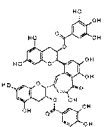
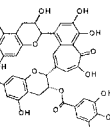
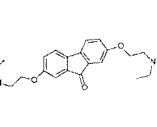
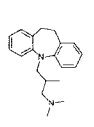
Alverine 	2',2''-bisepigallocatechin monogallate 	Chloroquine 	Chlorpromazine 	Clemastine 	Chol-11-enic acid 	Cyclobenzaprine 
Dibucaine 	3,16-dideoxymexicanolide-3 αdiol 	Drofenine 	Epicatechin 3,5-digallate 	Epicatechin monogallate 	Epigallocatechin 3,5-digallate 	Epigallocatechin-3-monogallate 
Ethopropazine 	Fluoxetine 	Haloperidol 	Hematein 	Hydroquinidine 	Hydroxychloroquine 	Imipramine 
Mebeverine 	Mianserin 	2,3,5,7,3',4'-penta- 	Pramoxine 	Promethazine 	Promazine 	Propafenone 
Sappanone A 	Senoside B 	Spiperone 	Theaflavin digallate 	Theaflavin monogallate 	Tilorone 	Trimipramine 

FIG. 4. Structures of compounds in The Spectrum Collection with IC₅₀s of >1 and ≤10 μM against both the RML and 22L scrapie strains, listed in approximate alphabetical order. 2,3,5,7,3',4'-penta-, 2,3,5,7,3',4'-pentahydroxyflavan.

other species should increase the value of the assay for predicting efficacy against TSE diseases of humans and livestock.

In our screening of the 2,000 compounds of The Spectrum Collection, both new and old inhibitors were identified. Of the 17 most potent inhibitors in the library with activity against the RML and 22L mouse scrapie strains (Fig. 5), 15 were new, whereas quinacrine and lovastatin were already known as PrP^{Sc} inhibitors in scrapie-infected cell cultures (11, 28). Other previously identified inhibitors, such as chloroquine (11) and promazine, promethazine, and chlorpromazine (17), also inhibited PrP^{Sc} accumulation in the SCDB assay screening (Fig. 4) but were not among the 17 most potent and strain-independent compounds in the library. The fact that several previously known inhibitors were selected by our blind screening of a large compound library inspires confidence in the utility of the SCDB assay.

Polyphenol inhibitors. Numerous polyphenols were selected as PrP^{Sc} inhibitors against both strains of mouse scrapie in the SCDB assay. Tannin (tannic acid), the most potent inhibitor found, is a relatively nontoxic constituent of foods such as tea, red wine, beer, and nuts. 2',2''-Bisepigallocatechin digallate is also a component of tea, and katechin is another naturally occurring polyphenol antioxidant. Relatively few studies have been done on the bioavailability of the polyphenols from tea extracts, but significant oral absorption has been shown in humans (32). While at first glance these water-soluble com-

pounds might not be considered likely to cross the blood-brain barrier, radiolabeled epigallocatechin gallate, another tea extract polyphenol, has been detected in mouse brains after oral administration (26). A number of other polyphenols, including epigallocatechin 3,5-digallate and epicatechin monogallate, were included in the group with IC₅₀s of between 1 and 10 μM (Fig. 4). The naturally occurring polyphenols represent a part of the normal human diet and are relatively nontoxic. Even if the ability of these compounds to cross the blood-brain barrier is questionable, they may be useful as prophylactic agents against peripheral infections or as TSE decontaminants.

Not all polyphenols tested were PrP^{Sc} inhibitors. Epicatechin and epigallocatechin, with molecular weights of about 300, were ineffective, although they represent portions of larger polyphenol molecules that were effective, such as epigallocatechin 3,5-digallate. While most of the polyphenol inhibitors were larger than 350 Da, a similar polyphenol with a molecular weight of 304, 2,3,5,7,3',4'-pentahydroxyflavan, was an inhibitor. This molecule is more conjugated and planar than epicatechin (Fig. 7). Although these results indicate that minor structural differences can have dramatic effects on polyphenol efficacy, further study is needed to clarify the structure-activity relationships.

Malaria drugs. Quinacrine and other antimalarial compounds have been reported to inhibit PrP^{Sc} formation in cell-based assays (11, 17). Quinacrine was reported to have an IC₅₀

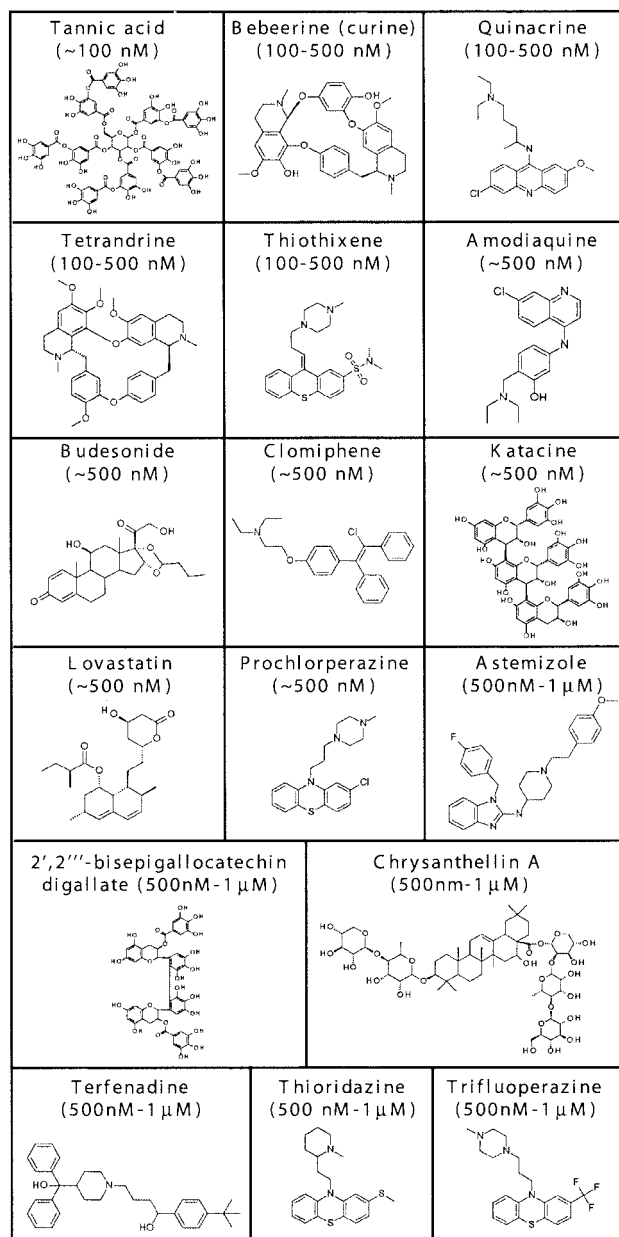


FIG. 5. Structures of compounds in The Spectrum Collection with IC_{50} s of $\leq 1 \mu$ M against both the RML and 22L strains of scrapie. Compounds are arranged from low to high approximate IC_{50} s.

of 400 nM, in good agreement with our present results. However, quinacrine has not shown any long-term benefit against Creutzfeldt-Jakob disease in preliminary clinical trials in humans (13). Since drug bioavailability, transport, and metabolism can depend markedly on structural details, it is possible that the other antimalarial compounds that were identified in this study are more effective than quinacrine in vivo. For instance, amodiaquine and bebeerine were among the 17 most potent inhibitors. Amodiaquine is a 4-aminoquinoline analog of chloroquine that is currently used as an antimalarial drug, but it was a stronger inhibitor of PrP^{Sc} formation in the SCDB assay. Chloroquine had an IC_{50} between 1 and 10 μ M, in good

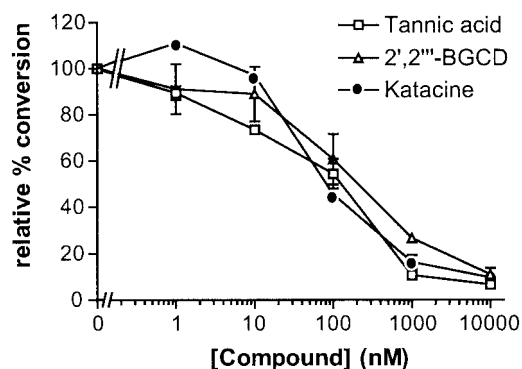


FIG. 6. Inhibition of solid-phase cell-free PrP conversion by polyphenols. The conversion relative to that in control reactions is plotted against the concentration of polyphenol added to the reaction. 2',2'''-BGCD, 2',2'''-biseipigallocatechin digallate.

agreement with a previous study (11). The closely related compounds hydroxychloroquine and hydroquinidine had similar IC_{50} s. Bebeerine (curine) is a bisbenzylisoquinoline alkaloid naturally produced from the root bark of *Chondrodendron platyphyllum*. Another naturally produced bisbenzylisoquino-

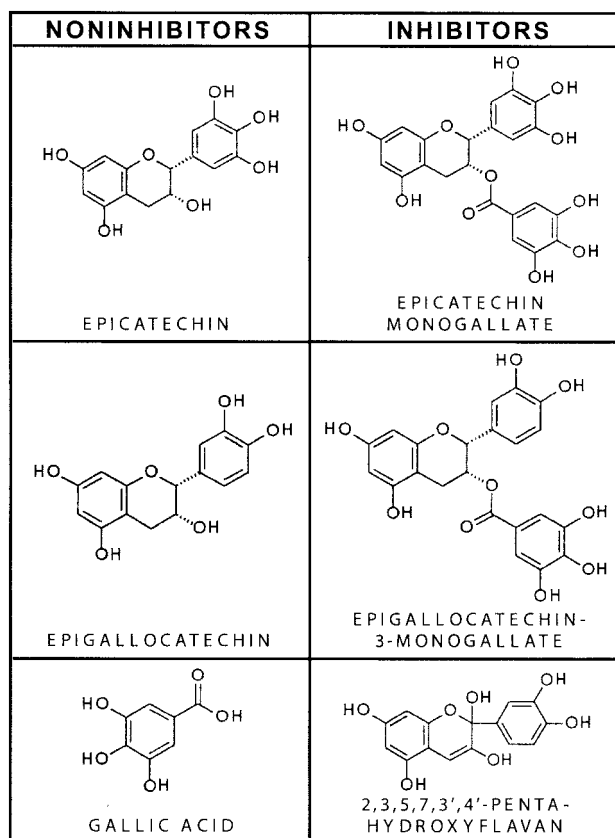


FIG. 7. Structural comparisons of inhibitory and noninhibitory polyphenols. Epicatechin and epigallocatechin were not inhibitors until the addition of a gallate, which was not an inhibitor on its own. Compared to epicatechin, the inhibitor 2,3,5,7,3',4'-pentahydroxyflavan has one additional conjugated double bond and an additional hydroxyl group. The double-ring system in the flavan should be more planar than the corresponding rings in epicatechin.

line alkaloid, tetrandrine, was also a potent inhibitor. Although not known to be antimalarial, this compound is a nonselective Ca²⁺ channel blocker derived from a Chinese medicinal herb, *Stephania tetrandra* S. Moore, and has been used to treat hypertension and autoimmune disorders in traditional Chinese medicine.

Antihistamines. The antihistamines astemizole and terfenadine were both among the most potent PrP^{Sc} inhibitors. These compounds are known to be poor at crossing the blood-brain barrier, a fact which may limit their therapeutic usefulness against TSEs. These antihistamines have been used extensively in humans but are currently not marketed in the United States because of a concern for serious, but rare, cardiovascular toxicity and the availability of safer alternatives.

Phenothiazine derivatives and analogs. The phenothiazine derivatives chlorpromazine, promazine, and promethazine inhibited PrP^{Sc} accumulation, in agreement with another study (17). However, our screen identified several more potent phenothiazine inhibitors, including the FDA-approved antipsychotics thioridazine, trifluoperazine, and prochlorperazine. The most potent group of 17 inhibitors identified in this study also included the FDA-approved antipsychotic thiothixene, which is a phenothiazine structural analog. These phenothiazine derivatives and analogs penetrate the blood-brain barrier, a feature that should be beneficial in treating TSEs.

Other inhibitors. Lovastatin is an FDA-approved hepatic hydroxymethyl glutaryl coenzyme A reductase inhibitor that reduces blood cholesterol levels and is known to cross the blood-brain barrier. Its inhibition of PrP^{Sc} accumulation at 500 nM agrees with a previous study (28) and places it among the best inhibitors. Budesonide is a steroid derivative approved by the FDA to treat asthma, chrysanthellin A is a naturally produced steroidal glycoside, and clomiphene is the FDA-approved treatment of choice for anovulatory infertile women with polycystic ovary syndrome.

Inhibition of cell-free PrP conversion. The SP-CFC reaction monitors direct hamster PrP interactions. Because there presumably are therapeutic targets besides PrP conversion for the TSEs in vivo, a compound could be effective in scrapie-infected cells and animals without being effective in the SP-CFC assay. For example, quinacrine was an effective PrP^{Sc} inhibitor in the SCDB assay but was not effective at inhibiting the SP-CFC reaction. Quinacrine is a lysosomotropic amine and may function by altering endosomal or lysosomal microenvironments (11). Another example is lovastatin, which is thought to inhibit PrP^{Sc} formation indirectly by depleting cellular cholesterol (28), consistent with its inability to block the SP-CFC reaction. Indeed, a majority of the 17 most potent inhibitors in the SCDB assay were unable to block the SP-CFC reaction. Another possible explanation for the discordance between the SCDB and SP-CFC assays is the species specificity of interactions with PrP isoforms. The SCDB and SP-CFC assays involve mouse and hamster PrP molecules, respectively. Regardless, the three polyphenols were potent inhibitors in both types of assays and thus appear to be direct inhibitors of PrP conversion. Although we do not anticipate that the screening of compound libraries with the SP-CFC assay alone would be as predictive of in vivo efficacy as the SCDB assay, we have shown that the cell-free assay can be used to obtain mechanistic in-

sights into whether inhibitors identified in the SCDB assay act via direct or indirect mechanisms.

Conclusion. This screening has identified new compounds and classes of compounds that are effective PrP^{Sc} inhibitors against two scrapie strains in cell cultures. The naturally occurring polyphenols were also effective inhibitors of cell-free PrP conversion. Barring hamster and mouse PrP species differences, these results suggest that the polyphenols inhibit PrP^{Sc} formation through direct PrP interactions, whereas the other inhibitors may work indirectly. Among the list of the 17 best inhibitors are FDA-approved compounds and dietary constituents that should be acceptable for testing in infected animals and humans. The fact that a number of the new inhibitors are known to cross the blood-brain barrier makes them attractive as potential anti-TSE therapeutic agents and distinguishes them from many previously identified PrP^{TSE} inhibitors.

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ERRATUM

New Inhibitors of Scrapie-Associated Prion Protein Formation in a Library of 2,000 Drugs and Natural Products

David A. Kocisko, Gerald S. Baron, Richard Rubenstein, Jiancao Chen,
Salomon Kuizon, and Byron Caughey

Laboratory of Persistent Viral Diseases, Rocky Mountain Laboratories, NIAID, NIH, Hamilton, Montana; Laboratory of Molecular and Biochemical Neurovirology, New York State Institute for Basic Research in Developmental Disabilities, Staten Island, New York; and Chengdu Jinniu Institute, Food Bureau of Sichuan Province, Chengdu Sichuan, China

Volume 77, no. 19, p. 10288–10294, 2003. Page 10292, Fig. 7: The structures for epicatechin and epigallocatechin should be interchanged. The structures for epicatechin monogallate and epigallocatechin-3-monogallate should be interchanged.

Short Communication

Evaluation of new cell culture inhibitors of protease-resistant prion protein against scrapie infection in mice

David A. Kocisko,¹ John D. Morrey,² Richard E. Race,¹ Jiancao Chen³ and Byron Caughey¹

Correspondence

David A. Kocisko
DKocisko@niaid.nih.gov

¹Laboratory of Persistent Viral Diseases, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, MT 59840, USA

²Institute for Antiviral Research, Animal, Dairy, and Veterinary Sciences Department and Biotechnology Center, Utah State University, Logan, UT 84322-4700, USA

³Chengdu Jinniu Institute, Food Bureau of Sichuan Province, Chengdu Sichuan, China

In vitro inhibitors of the accumulation of abnormal (protease-resistant) prion protein (PrP-res) can sometimes prolong the lives of scrapie-infected rodents. Here, transgenic mice were used to test the *in vivo* anti-scrapie activities of new PrP-res inhibitors, which, because they are approved drugs or edible natural products, might be considered for clinical trials in humans or livestock with transmissible spongiform encephalopathies (TSEs). These inhibitors were amodiaquine, thioridazine, thiothixene, trifluoperazine, tetrandrine, tannic acid and polyphenolic extracts of tea, grape seed and pine bark. Test compounds were administered for several weeks beginning 1–2 weeks prior to, or 2 weeks after, intracerebral or intraperitoneal 263K scrapie challenge. Tannic acid was also tested by direct preincubation with inoculum. None of the compounds significantly prolonged the scrapie incubation periods. These results highlight the need to assess TSE inhibitors active in cell culture against TSE infections *in vivo* prior to testing these compounds in humans and livestock.

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Transmissible spongiform encephalopathies (TSEs) or prion diseases are neurodegenerative diseases that affect mammals. Examples of TSEs include sheep scrapie, bovine spongiform encephalopathy, chronic wasting disease of cervids and Creutzfeldt–Jakob disease (CJD) in humans. TSEs are associated with the accumulation of an abnormal and protease-resistant aggregate of prion protein (PrP) known as PrP-res or PrP^{Sc} (Caughey & Lansbury, 2003). The exact makeup of the infectious particle of the TSEs is not clear, but infectious preparations are composed mainly of PrP-res, which accumulates primarily in the brains of affected individuals. All TSEs are fatal and there is no known cure for these diseases.

Because PrP-res is associated with disease and infectivity, it has been a target of therapeutic intervention for TSEs (Aguzzi *et al.*, 2001; Dormont, 2003; Brown, 2002). Murine N2a cells chronically infected with the RML (Chandler) strain of scrapie (Race *et al.*, 1988) have been used widely to test compounds for their ability to inhibit PrP-res formation (Caughey *et al.*, 1999; Beranger *et al.*, 2001; Kocisko *et al.*, 2003). Many compounds that inhibit PrP-res in cell culture have also delayed the onset of TSEs in animal models, but none has been curative. These compounds include porphyrins and phthalocyanins (Caughey

et al., 1998; Priola *et al.*, 2000), polyene antibiotics (Dormont, 2003), Congo red (Caughey & Race, 1992; Ingrosso *et al.*, 1995), suramin (Gilch *et al.*, 2001), sulfated glycans and other polyanions (Ehlers & Diring, 1984; Kimberlin & Walker, 1986; Farquhar & Dickinson, 1986; Caughey & Raymond, 1993; Birkett *et al.*, 2001; Schonberger *et al.*, 2003; Gabizon *et al.*, 1993). We recently used N2a cell cultures infected with either the RML or the 22L strains of scrapie to screen PrP-res inhibitors from a library of 2000 drugs and natural products (Kocisko *et al.*, 2003).

In this study we tested a number of the most potent of these new cell culture PrP-res inhibitors against scrapie infection in transgenic mice (Tg7). Tg7 mice produce no mouse PrP, but express approximately 4- to 8-fold higher levels of hamster PrP than do hamsters. They have a short disease incubation period of ~45–50 days after intracerebral (i.c.) inoculation with a high dose of the 263K strain of hamster scrapie (Race *et al.*, 2000; Priola *et al.*, 2000).

Compounds were administered either to treat an established infection or to test for prophylaxis. To test for activity against an established infection, compound administration started 2 weeks after i.c. scrapie inoculation and continued for 5–6 weeks. The 2-week period after i.c. inoculation

allowed time for the disease to progress before the compound was administered. To test for prophylaxis, administration of a compound began 2 weeks before and continued for 4 weeks after intraperitoneal (i.p.) scrapie inoculation. The rationale was to have a compound approaching a steady-state level in the mouse, enabling it to block a peripheral inoculation of scrapie infectivity from being established in the brain. The treatment following inoculation would allow time for the animal potentially to clear infectious material while the compound prevented further formation of PrP-res.

Compounds were administered either as an i.p. injection or in the drinking water. For i.p. injections, compounds were dissolved or suspended in an appropriate buffer and the dose volume was 10 ml kg⁻¹. Injections were given three times per week, on Monday, Wednesday and Friday. Solutions of compounds in drinking water were made to yield the desired dose based on the mean daily consumption of water by mice, 15 ml (100 g body wt)⁻¹. A solution of compound in the drinking water was the sole source of water for the mice during the dosing period. All 263K scrapie brain homogenates made up for inoculation in these studies were in physiological buffer supplemented with 2% fetal bovine serum. Different control groups are presented because testing was not done all at once and mice were inoculated with different homogenate preparations. In these studies, Tg7 mice were euthanized when clinical signs of scrapie were present, which included ruffled fur, lethargy, ataxia and weight loss. All procedures were approved by the Institution's Animal Care and Use Committee and were designed to minimize the animals' pain and distress. Animals that died from causes other than scrapie, such as from inoculation, dosing and anaesthetizing procedures, have been excluded from the data.

Compounds evaluated in animals had IC₅₀ (concentration of a compound inhibiting half of the production of PrP-res) values of ≤1 μM against both the RML and the 22L scrapie strains in cell culture. Since the *in vivo* testing involved hamster 263K scrapie, it was felt that compounds that inhibited multiple strains of mouse scrapie had a better chance of showing efficacy against PrP from another species. The inhibitors tested had been identified previously (Kocisko *et al.*, 2003) except for polyphenolic extracts of grape seed and pine bark (data not shown). In addition to their history of use in humans, the anti-psychotic drugs thioridazine, thiothixene and trifluoperazine were also selected for testing because they are known to cross the blood-brain barrier of humans. Amodiaquine is an inexpensive anti-malarial drug that has been used extensively in humans. The polyphenol tannic acid, which is contained in many foods, was the most potent inhibitor in our test set with an IC₅₀ of ~100 nM in both the scrapie-infected neuroblastoma cells and a solid-phase cell-free hamster 263K conversion assay (Kocisko *et al.*, 2003). A tea extract containing ~55% epigallocatechin monogallate and other polyphenols was also tried because of its relatively

low toxicity and use as a human food. Finally, tetrandrine, a Chinese herbal medicine with anti-malarial activity, was tested. Generally, the highest known tolerated dose of a compound in mice was given to maximize the chance of seeing an effect. For instance, 5 mg thioridazine kg⁻¹ dosed i.p. was used in this trial because 10 mg kg⁻¹ i.p. is not tolerated (Burke *et al.*, 1990). In our experiments, 10 mg trifluoperazine kg⁻¹ was mildly toxic but was tolerated, and 4500 mg tannic acid kg⁻¹ per day was not tolerated but 3000 mg kg⁻¹ per day had no apparent toxicity.

Table 1 contains the incubation period of each individual Tg7 mouse after i.c. inoculation of 263K scrapie brain homogenate and administration of compounds. No compound used as a treatment against established infection after i.c. inoculation significantly extended incubation periods. Nor was any compound protective when administered for a week prior to i.c. inoculation.

Prophylaxis tests with a number of compounds were also done on animals infected by i.p. inoculation to test for inhibition of the spread of infection from the periphery, where most natural infections initiate. A lack of effect against i.c. inoculation may be due to low brain penetration for some compounds such as tannic acid, epigallocatechin monogallate in tea, and other naturally occurring polyphenols from pine bark or grape seed. In addition, one of the anti-psychotic drugs that does cross the blood-brain barrier, trifluoperazine, was also tried in this type of test to see if it would perform better against an i.p. inoculation compared with an i.c. inoculation. None of these drug treatments showed any efficacy against an i.p. inoculation (Table 2).

In addition to being a potent PrP-res inhibitor *in vitro*, tannic acid is appealing as a potential drug because of its relatively low oral toxicity and low cost. However, with a molecular mass of ~1700 Da, tannic acid would be unlikely to cross the blood-brain barrier in significant quantity and its bioavailability via the oral route in the mouse may not be high enough to be effective. In agreement with this, previously mentioned prophylaxis and therapeutic tests in scrapie-infected Tg7 mice with orally administered tannic acid demonstrated no benefit. Therefore, we tried direct incubation of tannic acid with infectious brain homogenate. Solutions of tannic acid at 10 mM, 1 mM and 10 μM in the presence of 5% scrapie-infected brain homogenate were tested for their ability to reduce infectivity. After incubation at 37 °C for 2 h, the solutions were diluted 5000-fold to 0.001% brain homogenate and then inoculated i.c. into Tg7 mice to assess infectivity. The results in Table 3 indicate that incubation of infectious material with tannic acid had no significant effect on scrapie incubation period.

There are many reasons why a given compound that is effective *in vitro* might not show efficacy in an *in vivo* test against scrapie infection. The pharmacokinetics and metabolism of these compounds in mice might be unfavourable. The drug concentration attained at an active site using the highest tolerated dose may not be high enough for long

Table 1. Treatment of Tg7 mice inoculated with 263K scrapie

M, Monday; W, Wednesday; F, Friday; inoc., inoculation; homog., homogenate; wk, week.

Compound	Dose (mg kg ⁻¹)	Dosing regimen	Scrapie inoculation	Incubation periods (days)	Mean ± SD
None			50 µl 0.01 % brain homog. i.c.	59, 63, 63, 63, 63, 56, 63, 64	61.8 ± 2.8
Amodiaquine	50 i.p.	M, W, F for 5 wks starting 2 wks after inoc.	50 µl 0.01 % brain homog. i.c.	56, 63, 59, 60, 63	60.2 ± 2.9
Thioridazine	5 i.p.	M, W, F for 5 wks starting 2 wks after inoc.	50 µl 0.01 % brain homog. i.c.	59, 63, 63, 58, 56, 59, 63, 63, 59	60.3 ± 2.7
Thiothixene	5 i.p.	M, W, F for 5 wks starting 2 wks after inoc.	50 µl 0.01 % brain homog. i.c.	58, 56, 58, 58, 66, 58, 63, 65, 63, 58, 56, 63	60.2 ± 3.6
Trifluoperazine	10 i.p.	M, W, F for 5 wks starting 2 wks after inoc.	50 µl 0.01 % brain homog. i.c.	65, 63, 65, 72, 59, 63, 63, 63, 64	64.0 ± 3.3
None			50 µl 0.001 % brain homog. i.c.	62, 69, 65, 66, 69, 69, 71, 66, 71, 65, 62, 62	66.4 ± 3.4
Tetrandrine	50 i.p.	M, W, F for 6 wks starting 2 wks after inoc.	50 µl 0.001 % brain homog. i.c.	64, 77, 69, 72, 64, 61	67.8 ± 6.0
Tannic acid	1500 per day in drinking water	Continuously for 6 wks starting 2 wks after inoc.	50 µl 0.001 % brain homog. i.c.	66, 63, 64, 68, 63, 64, 62, 64	64.3 ± 1.9
Polyphenolic tea extract	1500 per day in drinking water	Continuously for 6 wks starting 2 wks after inoc.	50 µl 0.001 % brain homog. i.c.	76, 79, 66, 73, 69, 57	70.0 ± 7.9
None			50 µl 0.01 % brain homog. i.c.	71, 71, 71, 77, 71, 71, 79, 71	72.8 ± 3.3
Amodiaquine	50 i.p.	M, W, F for 6 wks starting 1 wk prior to inoc.	50 µl 0.01 % brain homog. i.c.	74, 74, 77, 74, 77, 81, 79, 74	76.3 ± 2.7
Thioridazine	5 i.p.	M, W, F for 6 wks starting 1 wk prior to inoc.	50 µl 0.01 % brain homog. i.c.	74, 77, 71, 71, 77, 74, 71, 71	73.3 ± 2.7
Thiothixene	5 i.p.	M, W, F for 6 wks starting 1 wk prior to inoc.	50 µl 0.01 % brain homog. i.c.	84, 77, 71, 74, 84, 71, 80	77.3 ± 5.6
Trifluoperazine	10 i.p.	M, W, F for 6 wks starting 1 wk prior to inoc.	50 µl 0.01 % brain homog. i.c.	74, 71, 71, 77, 84, 77, 71	75.0 ± 4.8

Table 2. Prophylaxis of Tg7 mice against 263K scrapie

All mice were infected with scrapie by i.p. inoculation with 50 µl 1 % brain homogenate. M, Monday; W, Wednesday; F, Friday; wk, week.

Test compound/preparation	Dose (mg kg ⁻¹)	Dosing regimen	Incubation periods (days)	Mean ± SD
None			101, 81, 127, 88, 97, 83, 88, 99	95.5 ± 14.7
Tannic acid	1500 per day in drinking water	Continuously for 6 wks starting 2 wks prior to inoc.	89, 94, 87, 90, 89, 90, 96, 73	88.5 ± 6.9
Tannic acid	3000 per day in drinking water	Continuously for 6 wks starting 2 wks prior to inoc.	81, 102, 87, 92, 88, 93	90.5 ± 7.1
Polyphenolic tea extract	1500 per day in drinking water	Continuously for 6 wks starting 2 wks prior to inoc.	77, 105, 90, 77, 96, 77, 81, 75	84.8 ± 11.0
Polyphenolic grape seed extract	2250 per day in drinking water	Continuously for 6 wks starting 2 wks prior to inoc.	80, 91, 100, 95, 73, 108, 97, 98, 90, 71	90.3 ± 12.1
Polyphenolic pine bark extract	2250 per day in drinking water	Continuously for 6 wks starting 2 wks prior to inoc.	90, 87, 95, 91, 87, 71, 73, 91, 90, 83	85.8 ± 7.9
Trifluoperazine	10 i.p.	M, W, F for 6 wks starting 2 wks prior to inoc.	90, 91, 90, 104, 99, 101, 103	96.9 ± 6.3

Table 3. Tannic acid as a potential 263K scrapie disinfectant

[Tannic acid], tannic acid concentration incubated with brain homogenate (homog.).

[Tannic acid]	Scrapie inoculation (i.c.)	Incubation periods (days)	Mean \pm SD
None	50 μ l 0.001 % brain homog.	62, 64, 63, 68, 69, 64	65.0 \pm 2.8
10 μ M	50 μ l 0.001 % brain homog. in 2 nM tannic acid	72, 69, 59, 60, 61, 62, 61, 62	63.3 \pm 4.7
1 mM	50 μ l 0.001 % brain homog. in 200 nM tannic acid	63, 66, 64, 67, 69, 66, 67	66.0 \pm 2.0
10 mM	50 μ l 0.001 % brain homog. in 2 μ M tannic acid	64, 67, 66, 70, 70, 72, 71, 72	69.0 \pm 3.0

enough to be efficacious. Also, the mechanism by which these particular molecules inhibit PrP-res formation in infected N2a cells is unclear and may not be replicated sufficiently *in vivo*. These results showed that inhibition in the infected N2a cells may not always correlate with anti-scrapie activity *in vivo*. Nonetheless, the scrapie-infected N2a assay remains a valuable initial screen for potential drugs because numerous compounds that have been identified as PrP-res inhibitors in scrapie-infected cells have proven to have at least some anti-scrapie activity *in vivo*.

Since many potent inhibitors of PrP-res formation *in vitro* are not efficacious against scrapie in animals, it is important to consider animal testing of inhibitors prior to clinical trials, especially in cases in which there might be negative side effects for the patient. Quinacrine, an anti-malarial drug used extensively in humans, was found to inhibit RML (Doh-ura *et al.*, 2000; Korth *et al.*, 2001) and later 22L (Kocisko *et al.*, 2003) mouse scrapie strains in cell culture. Based on its PrP-res inhibitory activity in cell culture, as well as its previous human use, testing of quinacrine in human TSE trials was strongly advocated, even in the absence of any supportive animal data (Korth *et al.*, 2001). However, in subsequent *in vivo* testing, quinacrine was ineffective in scrapie-infected mice (Collins *et al.*, 2002; Barret *et al.*, 2003) and, unfortunately, only transiently beneficial in some CJD patients, albeit with some liver toxicity (Kobayashi *et al.*, 2003; Nakajima *et al.*, 2004). Thus, although many inhibitors of PrP-res in cell culture are known, testing in TSE-infected rodents should help to select those with the most promise for human clinical trials.

When testing the efficacy of compounds against scrapie infection in an *in vivo* system there are many complex variables. Some of these include the dose, the vehicle, the dosing regimen, when to initiate and terminate treatment, the routes of compound administration and scrapie inoculation, the animal model and the TSE strain. Part of the difficulty in deciding on these variables is a lack of understanding of TSE pathogenesis. We have tried to select reasonable options from among these variables, but many others might be considered. Although none of the inhibitors tested herein was effective in our *in vivo* tests, we report the results of these expensive and time-consuming experiments in the hope that future work with potential anti-TSE therapeutics and prophylactics can advance beyond our particular approaches rather than repeat them.

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PRION DISEASES — CLOSE TO EFFECTIVE THERAPY?

Neil R. Cashman* and Byron Caughey‡

Abstract | The transmissible spongiform encephalopathies could represent a new mode of transmission for infectious diseases — a process more akin to crystallization than to microbial replication. The prion hypothesis proposes that the normal isoform of the prion protein is converted to a disease-specific species by template-directed misfolding. Therapeutic and prophylactic strategies to combat these diseases have emerged from immunological and chemotherapeutic approaches. The lessons learned in treating prion disease will almost certainly have an impact on other diseases that are characterized by the pathological accumulation of misfolded proteins.

TRANSMISSIBLE SPONGIFORM ENCEPHALOPATHY

A class of infectious diseases characterized by neuronal degeneration, spongiform change, gliosis and accumulation of amyloid protein deposits.

SPONGIFORM CHANGE

Microvacuolization of brain tissue, which typically accompanies prion disease.

*Centre for Research in Neurodegenerative Diseases, University of Toronto, 6 Queen's Park Crescent West, Toronto, Ontario M5S3H2, Canada.

‡Rocky Mountain Laboratories, National Institutes of Health, 903 South Fourth Street, Hamilton, Montana 57840, USA.

e-mails:

neil.cashman@utoronto.ca;
bcaughey@niaid.nih.gov
doi:10.1038/nrd1525

TRANSMISSIBLE SPONGIFORM ENCEPHALOPATHIES (TSEs) are rapidly progressive, fatal and untreatable neurodegenerative syndromes that are neuropathologically characterized by SPONGIFORM CHANGE (microcavitation of the brain), neuronal loss, glial activation and accumulation of an abnormal amyloidogenic protein. Human TSEs include classical CREUTZFELDT–JAKOB DISEASE (CJD), which has sporadic, iatrogenic and familial forms. Since 1996 (REF. 1), a new VARIANT CJD (vCJD) has been identified in the United Kingdom, France, the Republic of Ireland, Hong Kong, Italy, the United States and Canada that is characterized by young age of onset, a stereotypical pattern of illness progression and distinctive neuropathological features². This disease, which probably derives from the consumption of cattle neural tissues contaminated with the BOVINE SPONGIFORM ENCEPHALOPATHY (BSE) agent, has afflicted ~150 individuals to date. Although some studies suggest that the 'primary' vCJD epidemic is waning³, the report of two probable cases of blood-borne transmission⁴ raises concerns about a secondary vCJD epidemic resulting from iatrogenic transmission through donation of blood, tissues or organs, and contaminated surgical instruments⁵. Notably, in contrast to classical forms of CJD, vCJD infectivity is more likely to accumulate in peripheral tissues and organs to levels that could represent a substantial transmission hazard⁶.

The timing of the vCJD epidemic, similarities in transmission characteristics in experimental animals

(mice and primates)⁷, and similar biochemical features indicate that vCJD almost certainly represents interspecies transmission of the agent responsible for BSE⁸. In the United Kingdom alone between December 1986 and 31 March 2003, BSE was confirmed in 179,973 cattle⁹, with up to 3 million infected cattle entering the human food supply undetected¹⁰. The UK BSE epidemic, initially amplified by the now-proscribed supplementation of cattle feed with meat-and-bone meal, is clearly in decline. The possibility that BSE has entered the UK sheep population cannot be ruled out at present^{11–13}. In 1993, Canadian authorities reported North America's first case of BSE, in a steer imported from the United Kingdom. The discovery of a case of BSE in an Alberta cow fed locally rendered feed in May 2003, and a second case in Washington State in December 2003, has drastically altered awareness of this disease in North America. Yet another animal TSE concern in North America is CHRONIC WASTING DISEASE (CWD) of captive and wild cervids (deer and elk), which has been insidiously emerging since initial reports in Colorado in the 1960s¹⁴. CWD, arguably the most contagious of TSEs, has now been reported in 12 US states as far east as Illinois, and in the Canadian provinces of Saskatchewan and Alberta. Unlike sheep SCRAPIE, with which humans have coexisted for at least 300 years¹⁵, CWD represents an uncertain threat whose impact on human health is as yet unknown¹⁶.

CREUTZFELDT–JAKOB DISEASE (CJD). The most common human prion disease, classically comprising sporadic, familial and iatrogenic forms, and now including variant CJD (vCJD).

VARIANT CJD (vCJD). A newly emergent form of human prion disease, initially linked to consumption of cattle afflicted with bovine spongiform encephalopathy.

BOVINE SPONGIFORM ENCEPHALOPATHY (BSE). A naturally acquired transmissible spongiform encephalopathies of cattle, epidemically amplified by feed supplementation with rendered products from afflicted bovines.

CHRONIC WASTING DISEASE (CWD). A relatively contagious prion disease of captive and wild cervids (elk and deer).

SCRAPIE
A naturally acquired prion disease of sheep, clinically recognized for ~300 years. Mouse-adapted scrapie agent strains are now used in many paradigms to identify therapies to prion disease.

PRION HYPOTHESIS
The proposal that transmissible spongiform encephalopathies infectivity comprises the conformational conversion of the host-encoded cellular prion protein PrP^C into the disease-associated isoform PrP^{Sc}.

PrP^C
The native, protease-sensitive, normally expressed form of PrP, named for its 'cellular' location.

PrP-SEN
A generic operational term referring to forms of PrP that are protease-sensitive. This includes native PrP^C as well as various non-native or abnormal forms of PrP (for example, recombinant or mutant forms) that are protease-sensitive.

PrP^{Sc}
The native disease-associated isoform of PrP, originally named for its association with scrapie. Now often used generically to refer to the disease-associated form of PrP in prion diseases other than scrapie.

Novel form of infectivity

Clearly, there is an urgent need for effective and efficient animal prophylactic therapies for prion diseases, to prevent the spread of BSE, CWD and sheep scrapie throughout the world. Moreover, successful therapies for human TSEs need development, particularly in view of the uncertainty surrounding the extent of primary and iatrogenic vCJD in the United Kingdom and other countries. However, the development of prophylactic and therapeutic agents will not be a trivial challenge because of the unusual biology of prions. The agents that transmit TSEs differ from viruses and viroids in that no evidence for an agent-specific nucleic-acid component has been reproducibly detected in infectious materials¹⁷. According to the 'protein only' or PRION HYPOTHESES^{18–21}, infectivity resides in an abnormal isoform of the host-encoded cellular prion protein (PrP^C or PrP-SEN). The abnormal isoform (PrP^{Sc} or PrP-RES) is β -sheet rich^{22–24}, insoluble and partially protease resistant, whereas PrP^C is α -helix-rich^{24,25}, soluble in mild detergents and protease sensitive^{26,27}. PrP^{Sc} is the most prominent macromolecule in preparations of prion infectivity and a reliable marker of most TSE infections. PrP^C is a widely distributed glycosylphosphatidylinositol (GPI)-linked cell-surface protein with a molecular mass of 33–35 kDa^{28,29}, and is non-infectious.

Currently favoured versions of the prion hypothesis posit that PrP^{Sc} propagates itself as an infectious agent by causing PrP^C to convert into PrP^{Sc} in a template-directed process catalysed by physical contact with PrP^{Sc}^{18,21,30,31} (FIG. 1). It has been shown that PrP^C can be converted to a protease-resistant form by contact with PrP^{Sc} *in vitro* in highly species- and strain-dependent reactions^{32–34}. This conversion can be made more continuous in crude brain homogenates by protein misfolding cyclic amplification (PMCA), a process analogous to the polymerase chain reaction for nucleic-acid amplification³⁵. Although the conversion mechanism is not fully understood, most available evidence is consistent with the idea that ordered PrP^{Sc} aggregates serve as templates or catalysts for the conformational change and ordered aggregation of PrP^C. However, the formation of protease-resistant PrP species *in vitro* has not yet been associated with the generation of increased TSE infectivity³⁶. Although PrP^{Sc} in most infected tissues and cells is partially protease-resistant and insoluble, these properties can vary with experimental handling, TSE strain and host species and, therefore, should not be considered absolute prerequisites for infectivity even if they are likely to help stabilize it^{37–39}. Highly protease-sensitive molecules (sPrP^{Sc}) co-purify with protease-resistant PrP^{Sc} (rPrP^{Sc}) and infectivity^{40–42}, which raises questions as to whether sPrP^{Sc} has a role in infectivity or neuropathogenesis. Attempts to separate sPrP^{Sc} and rPrP^{Sc} have shown that infectivity fractionates with the latter⁴³. Other studies indicate that accumulation of weakly protease-resistant and PrP^{Sc}-like PrP can cause neurodegenerative disease without infectivity⁴⁴. Moreover, high levels of infectivity and neurological disease have been reported in the absence of measurable PrP^{Sc}^{39,162}. Collectively, the available evidence indicates that several disease-associated forms of PrP exist, and

that neurotoxic forms are not necessarily infectious. Conversely, the most infectious forms need not be the most neurotoxic.

Should the protein-only hypothesis of TSE infectivity be considered 'proven'? The most direct proof — that is, *de novo* conversion of PrP^C alone into a high-titred, serially transmissible infectious agent — has been extremely difficult to achieve. However, synthetic fibrils of mutant PrP peptides were recently reported to cause serially transmissible diseases when inoculated into transgenic mice overexpressing related mutant PrP^C molecules^{45,46}. These results provide tantalizing support for the protein-only prion model, but with certain caveats. One is that the apparently synthetic prions seem to be many orders of magnitude lower in infectivity per unit PrP than is genuine TSE infectivity, raising the question of what is necessary to produce reasonably potent prions. Second, additional controls are needed to rule out the possibility of spontaneous prion formation and neuropathology in the transgenic mice without the inoculation of synthetic fibrils. Nonetheless, considerable, but less direct, experimental data seem to support the prion model. Perhaps most tellingly, PrP^{Sc}-null mice do not support the replication of TSE infectivity⁴⁷. Moreover, antibodies directed against PrP^C (see below) and chemical inhibitors of PrP conversion⁴⁸ can block propagation of TSE infectivity *in vitro* and *in vivo*. Whether or not various aberrant forms of PrP are solely responsible for TSE infectivity and/or neuropathology, their central role in the TSE pathogenesis provides a cogent framework to approach drug discovery in TSEs.

Immunopathogenesis

In addition to approaching TSEs as a protein chemical problem of conformational conversion, the role of the immune system in prion infection provides other avenues for therapy (FIG. 2). Prion infection typically occurs by the oral route in sheep scrapie, BSE of cattle and human vCJD, and is necessarily followed by replication in a peripheral compartment prior to brain invasion. Non-human primates can also be experimentally infected orally⁴⁹. CWD is probably also orally contracted⁵⁰. In orally transmitted TSEs, prion infectivity and/or protease-resistant PrP can be identified in gut Peyer's patches^{51,52}. Prion propagation to splanchnic lymphoid tissue and spleen has been also demonstrated for natural and experimental scrapie infection^{52,53}, and humans with vCJD display high levels of infectivity in gut-associated lymphoid tissue (GALT; including tonsil) and spleen⁵⁴. From GALT and other lymphoid tissue, prions are transported by splanchnic innervation to the brainstem and spinal cord^{52,53}. This means that in early infection, replication occurs in compartments accessible to immunotherapy and antibody neutralization.

A cell of particular importance in the peripheral propagation of prions is the follicular dendritic cell (FDC), which resides in immune follicles in the gut, lymph nodes and spleen. These cells acquire a large burden of PrP^{Sc} in lymph nodes and spleens of scrapie-infected mice⁵⁵. Moreover, FDCs seem to be crucial for the propagation of prion infectivity in these tissues⁵⁶.

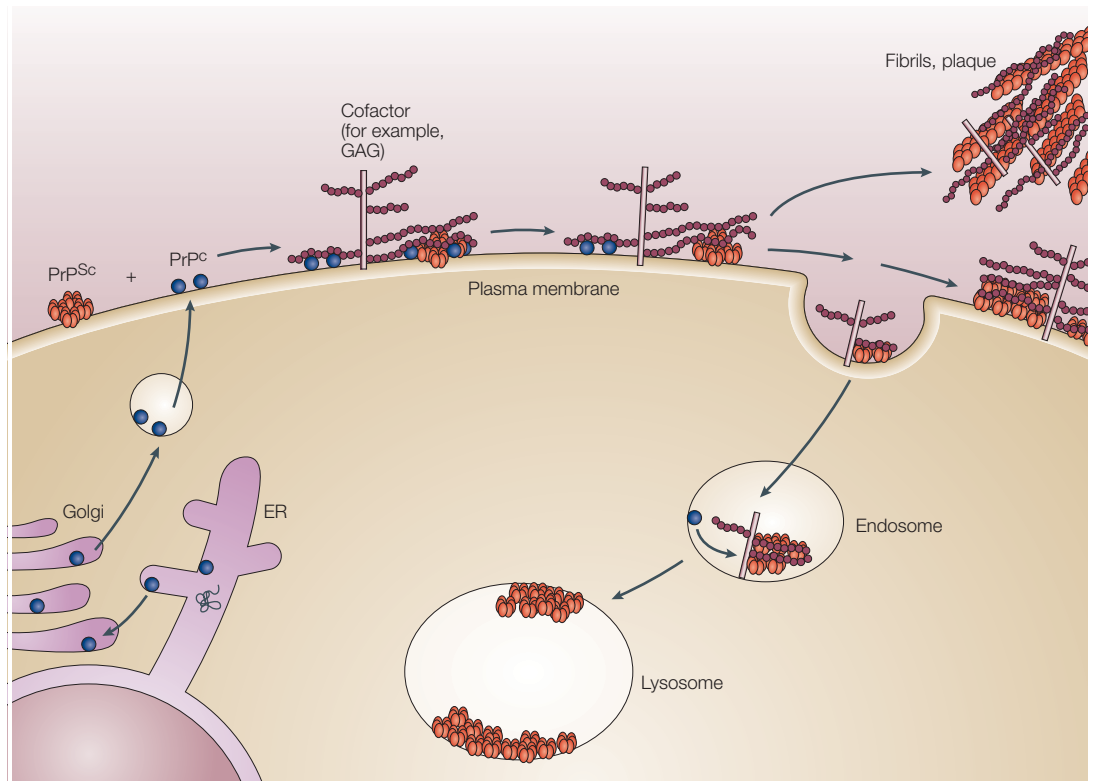


Figure 1 | PrP^{Sc} formation in scrapie-infected cells. Cellular prion protein (PrP^C; blue dots) follows the secretory pathway through the endoplasmic reticulum (ER) and Golgi apparatus to the plasma membrane, where it is anchored by a glycosylphosphatidylinositol anchor. According to the prion hypothesis, the abnormal prion isoform (PrP^{Sc}; shown as ordered red oligomeric clusters) binds to PrP^C and causes it to undergo a conformational change while being incorporated into the PrP^{Sc} oligomer^{18,20}, as has been suggested experimentally^{32,46}. This occurs primarily on the cell surface or in endocytic vesicles. The conversion is affected by association with raft membrane microdomains⁸⁶ and cofactors such as sulphated glycosaminoglycans (GAGs; maroon beads) or proteoglycans^{120–123} (rods with attached maroon beads). PrP^{Sc} can then accumulate in lysosomes, in the plasma membrane or in extracellular deposits such as amyloid fibrils and plaques. It is notable that the sites of PrP^{Sc} formation are accessible to potential inhibitors in the extracellular medium without having to cross cellular membranes.

Mutant or knockout mice lacking functional B cells, tumour-necrosis factor- α (TNF- α), TNF receptor-1, lymphotoxin (LT) $\alpha + \beta$ and LT β -receptor, which are all deficient in maturation and activation of FDCs, have also been shown to be poorly permissive for peripheral inoculation of scrapie prions for many experimental scrapie strains^{57–60}. PrP^C expression in FDCs is required for efficient infection of spleen^{55,59}, whereas PrP^C expression restricted to non-FDC lymphoid cells does not permit scrapie replication in this organ. The mutation or depletion of the complement component C3 also blocks peripheral prion propagation, a result that supports a proposed role for FDC complement receptors in prion infection of these cells⁶¹. PrP^C expression, which is proven to be crucial for prion replication in knockout mice⁴⁷, is readily detected even in resting FDCs not infected with prions⁶². FDCs can therefore efficiently support prion replication because they are long-lived cells that express high levels of PrP^C (similar to neurons), and are specialized to trap, retain and present unprocessed antigens⁵². Other cells in the gut and follicle have also been implicated in prion propagation in orally transmitted disease, including M cells⁶³. Accessory-cell-dependent replication of prions in the brain is less understood than that in the

lymphoid follicle. However, brain microglial cells express many FDC and myeloid markers, including receptors for TNF, immunoglobulin G and C3 (REF 45), and have been implicated in neurotoxicity of prions⁶⁴.

Immune-active therapies for TSEs

It therefore seems possible that immune manipulation might affect lymphoid prion replication, to block or slow neuro-invasion, as has been shown with experimental depletion of TNF- α and complement^{56,58,61,65}. Moreover, prions that replicate in peripheral compartments might be vulnerable to circulating anti-prion protein antibodies; unfortunately, however, prions do not naturally elicit protective immune responses (reviewed in REF 66). Several strategies have emerged to test the possibility that immune recognition of prion protein isoforms could prove to be of therapeutic importance in treating prion infection. Several recent publications have indicated that antibodies predominantly directed against PrP^C can clear scrapie-infected cells of PrP^{Sc} *in vitro*, and presumably scrapie infectivity as well^{67–69}. In addition, Aguzzi and colleagues have found that the transgenic expression of the antibody 6H4, which is non-selective for prion protein isoforms

PrP^{RES}
A generic term referring to the disease-associated form of PrP that is recognizable by its partial resistance to proteolytic digestion, and therefore partially synonymous with PrP^{Sc} when the latter is applied generically. Also commonly used to denote the highly protease-resistant 27–30 kDa product of partial proteolysis of native disease-associated PrP.

PRNP
The human gene that encodes PrP. Also used to designate the prion protein gene in other species, although the mouse nomenclature is *Prnp*.

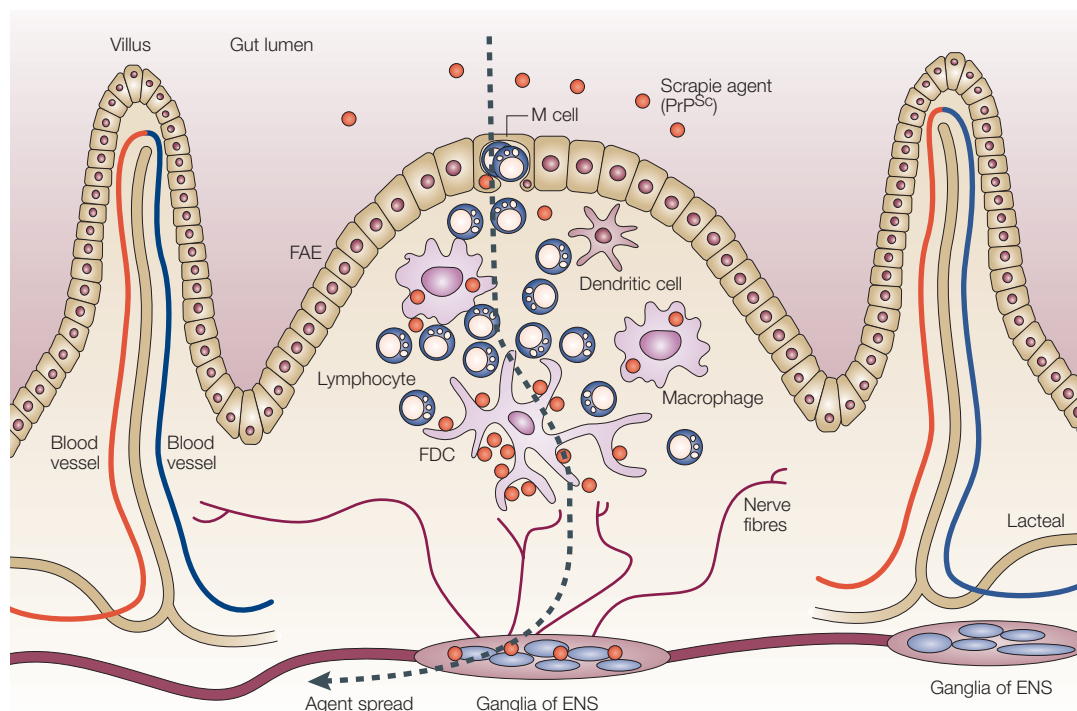


Figure 2 | Possible spread of scrapie infectivity from the gut lumen to the nervous system following oral infection (route indicated by dotted line). Soon after ingestion, the abnormal prion isoform (PrP^{Sc}) is detected readily within Peyer's patches on follicular dendritic cells (FDCs), within macrophages, within cells with morphology consistent with that of M cells and within ganglia of the enteric nervous system (ENS). These observations indicate that, following uptake of scrapie infectivity from the gut lumen, infectivity accumulates on FDCs in Peyer's patches and subsequently spreads via the ENS to the central nervous system. FAE, follicle-associated epithelium. Adapted, with permission, from REF. 55 © Elsevier Ltd (2000).

can block experimental scrapie in mice⁷⁰, and Hawke and colleagues have shown that anti-PrP antibody infusion can generate a similar effect with peripherally inoculated prions⁷¹. These data indicate that interference with the intermolecular interactions of PrP^{C} , or changes in compartmental cycling of this protein, disrupt the conversion of PrP^{C} to PrP^{Sc} .

However, antibodies directed against PrP^{C} , a normal cell-surface protein, could have adverse consequences if used as immunotherapies *in vivo*. PrP^{C} is ubiquitously expressed^{28,29}, and therefore circulating antibodies against PrP^{C} could trigger widespread complement-dependent lysis of many cells. Moreover, it is possible that anti- PrP^{C} antibodies would cause a breakdown of immunological tolerance of this molecule, with the consequent induction of autoimmune disease. Furthermore, antibodies directed against PrP^{C} might impair its normal function, thereby triggering apoptosis in the brain⁷² and causing inappropriate activation of signalling cascades⁷³. Antibody-mediated cell-surface ligation of PrP^{C} can also suppress T-cell activation of human lymphocytes⁷⁴. PrP^{Sc} -specific immune recognition would circumvent problems of autoimmune recognition and impaired function of PrP^{C} . A PrP^{Sc} -specific immune response would be expected to opsonize infectious prions for degradation in the reticulo-endothelial system, and could block the production of PrP^{Sc} by impairing PrP^{C} - PrP^{Sc} interactions that are considered a prerequisite for the recruitment process⁷⁵. As the conversion of prion

isoforms occurs at the cell surface, or a compartment close to cell surface⁷⁶⁻⁷⁹, PrP^{Sc} -specific antibodies are likely to interfere with the infectious process, as does 6H4 (REF. 67) and recombinant PrP^{C} -specific antibodies and fragments⁶⁸. It is also possible that anti- PrP^{Sc} antibodies might participate in the immune recognition and destruction of prion-infected cells, possibly by targeting them for antibody-dependent cellular cytotoxicity.

A recent report has shown that the prion protein repeat motif Tyr-Tyr-Arg is accessible to antibody binding in the misfolded PrP^{Sc} isoform, but not on the molecular surface of native PrP^{C} ⁸⁰ (FIG. 3). The incubation of scrapie-infected ScN2a neuroblastoma cells with Tyr-Tyr-Arg monoclonal antibodies has also been shown to reduce the cell content of PrP^{Sc} in a concentration- and time-dependent manner⁸¹, similarly to PrP^{C} -directed antibodies⁶²⁻⁶⁶. PrP^{Sc} -specific antibodies have been generated that do not have toxic effects through Tyr-Tyr-Arg peptide immunization using conventional adjuvants in animals expressing endogenous PrP^{C} ⁸⁰. Moreover, PrP^{Sc} -specific monoclonal and polyclonal antibodies do not recognize antigens at the cell surface of normal dissociated splenocytes and brain cells, despite the presence of Tyr-Tyr-Arg motifs in PrP^{C} and in other non-prion proteins. Tyr-Tyr-Arg motifs in the prion protein, and in non-prion proteins, are therefore sequestered from antibody recognition on normal cells that have not been infected by prions or exposed to denaturing agents. The lack of immunological

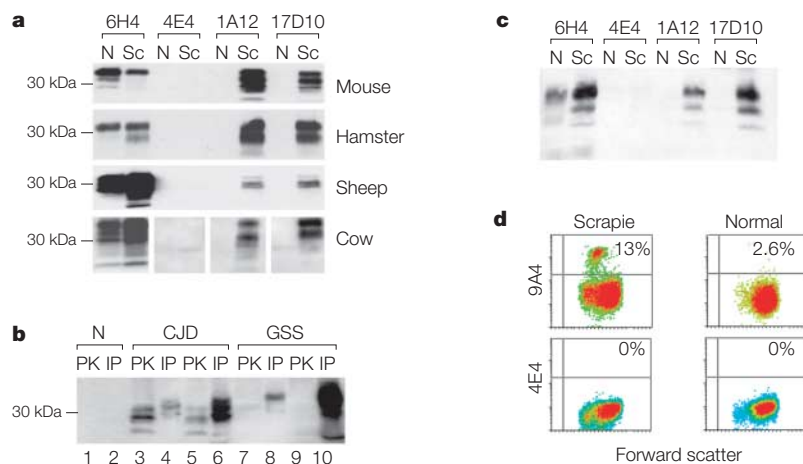


Figure 3 | Tyr-Tyr-Arg antibodies selectively recognize PrP^{Sc}. **a** | Monoclonal antibodies 1A12 and 17D10 selectively immunoprecipitate the abnormal prion isoform (PrP^{Sc}) from experimentally and naturally infected prion disease brain, but not normal prion (PrP^C) from uninfected brain. **b** | Efficiency comparison of proteinase K resistance and Tyr-Tyr-Arg immunoprecipitation (monoclonal antibody 16A18) from equivalent samples of frontal (1–4, 7, 8) and cerebellar (5, 6, 9, 10) regions of a Creutzfeldt–Jakob disease (CJD) and a GERSTMANN–STRAUSSLER SYNDROME (GSS) brain. **c** | Tyr-Tyr-Arg antibodies recognize low concentrations of PrP^{Sc} in ME7-infected mouse spleen. All panels: 6H4 (**a** and **c**) or 3F4 (**b**) immunoblot detection of immunoprecipitated PrP. **d** | Tyr-Tyr-Arg monoclonal antibody 9A4 recognizes a population of dendritic cells from scrapie-infected sheep lymph nodes. CD58⁺ CD45RO⁻ retropharyngeal lymph node cells from scrapie-infected and normal sheep stained with 9A4, or monoclonal antibody control 4E4. Adapted, with permission, from REF. 80 © Macmillan Magazines Ltd.

recognition of PrP^C or other native-structured cell-surface proteins by PrP^{Sc}-specific Tyr-Tyr-Arg antibodies indicates that specific prion immunoprophylaxis and/or immunotherapy could ultimately prove possible in animals and humans.

Notably, vaccine therapies are also now being organized with another neurotoxic amyloid peptide, the Aβ fragment of **amyloid precursor protein of Alzheimer's disease**. Preclinical and clinical data seem to demonstrate that immune recognition of Aβ could provide an effective therapy for Alzheimer's disease⁸². Human Aβ immunization trials have been halted because of the development of acute encephalopathy in some patients⁸³, which presumably results from T-cell immune recognition of brain plaque Aβ, but further human strategies are being explored at present. It is likely that antibodies directed against PrP^{Sc} in pre-symptomatic cattle and humans would prevent neuro-invasion without causing encephalopathy, as the cognate antigen is not yet present in the brain.

Chemotherapeutic targets

Depending on the type of TSE and the circumstances, a number of different modes of intervention are possible: decontamination of sources of infection; prophylaxis against initial infections; inhibition of agent propagation in the periphery; blockade of invasion of central nervous system (CNS) from the periphery; inhibition of pathogenic PrP accumulation; destabilization of pathogenic PrP; blockade of direct or indirect neurotoxic effects of pathogenic PrP; and compensation for damage to brain cells. The first four of these modes will probably

only apply to TSEs that result from infections by peripheral routes. For these situations, it would be worthwhile to identify compounds that are protective against at least low-level sources of infections. Ideally, they would be safe enough to be used prophylactically in foodstuffs or other potentially contaminated materials that are consumed or inoculated peripherally into humans or animals. Such compounds need not cross the blood–brain barrier. However, once TSE infections have penetrated the CNS, it will probably be necessary to target drugs directly to the CNS (that is, the last four modes of intervention). This will be true in the clinical and late preclinical phases of any TSE, with iatrogenic transmissions into the CNS, and with sporadic CJD and familial TSEs in which PrP^{Sc} formation might occur spontaneously in the CNS. These scenarios will usually require drugs that can cross the blood–brain barrier; however, it might also be possible — although cumbersome, expensive and potentially risky — to inject or infuse drugs directly into the brain.

At the molecular level, one primary chemotherapeutic target is the PrP conversion reaction, and this has been the focus of the majority of TSE drug discovery efforts to date. PrP conversion inhibitors can act directly by binding to PrP^C or PrP^{Sc}, and by affecting their interactions with themselves or other influential ligands. Indirect PrP^{Sc} inhibition mechanisms are also possible, such as those that affect PrP expression⁸⁴, turnover, trafficking⁸⁵, membrane associations⁸⁶ or ligand binding. One might also block initial infections or the spread of infection by blocking interactions between PrP^{Sc} and as-yet-unidentified receptor(s) on various cell types that might carry the infection to the CNS.

Screens for potential anti-TSE drugs

In vivo testing of potential anti-TSE compounds tends to be very slow and expensive. Most of the initial screening for potential anti-TSE chemotherapies has therefore been done with surrogate *in vitro* tests. The most common approach has been to test for inhibition of PrP^{Sc} accumulation in scrapie-infected tissue culture cells. Several chronically infected cell lines have been developed, including murine neural (N2a^{87,88}, SMB⁸⁹, GT1⁹⁰) and fibroblast⁹¹ cell lines, and sheep scrapie-infected rabbit Rov epithelial cells expressing sheep PrP^{C92}. Unfortunately, little progress has been reported in developing human, bovine or cervid cell lines infected with CJD, BSE or CWD, respectively. Such cell lines would probably be helpful, because striking TSE strain- and species-dependence has been observed with a few of the known antiscrapie compounds and it cannot always be assumed that what works against one TSE strain will be effective against another. Nonetheless, the identification of antiscrapie therapeutics should provide at least proof of principle that compounds of a given class can be beneficial against TSE diseases. Of course, as is true with drug discovery in general, not all compounds with *in vitro* activity are effective *in vivo*. However, the fact that numerous classes of PrP^{Sc} inhibitors in scrapie-infected cell cultures have also shown some antiscrapie activity in animals (TABLE 1)

GERSTMANN–STRAUSSLER SYNDROME
A familial prion disease, linked with mutations in the PRNP open reading frame.

Table 1 | **Anti-transmissible spongiform encephalopathy compounds**

Class	Example(s)	Activity <i>in vitro</i>	Prophylactic activity <i>in vivo</i>	Therapeutic activity <i>in vivo</i>	Comments	Reference(s)
Sulphonated dyes	Congo red and suramin	+	+	–	Potential toxicity	126,127,133
Sulphated glycans	Pentosan polysulphate and heparan sulphate	+	+	–	Human trials	108,109,120,121
Polyoxometalates	HPA23	+	+	–		120,161
Cyclic tetrapyrroles	Porphyrins and phthalocyanines	+	+	–		100,111,135
Polyene antibiotics	Amphotericin B, MS8209 and filipin	+	+	+/-	Human drugs, but toxic	136,137,139,140
Quinacrine, quinoline, acridines, phenathiazines and related molecules	Quinacrine, quinine, biquinoline and chlorpromazine	+	+/-	+/-	Human trials	112,113,117,141
Metal chelators	Penicillamine	+	+	?		142
Dimethyl sulphoxide		+	+	?		107,111
Flupirtine		+	?	+	Human trials	155
Branched polyamines	Polyamidoamine	+	?	?		95
Statins	Lovastatin and squalastatin	+	?	?	Human drugs	86,94,118

indicates the utility of these cultures as initial high-throughput screening tools. Recent progress in adapting mouse scrapie-infected N2a cells^{93,94} (FIG. 4) and sheep scrapie-infected Rov cells (D. Kocisko, A. Engel, K. Harbuck, D. Villette and B. C., unpublished data) to higher-throughput formats has allowed the screening of hundreds of compounds per week by a single person. In the few examples that have been tested, long-term inhibition of PrP^{Sc} accumulation in cultured cells has resulted in elimination of scrapie infectivity as assayed by injecting cell lysates into animals^{48,95,96}.

Other types of *in vitro* tests for potential anti-TSE compounds have been devised as well. Cell-free PrP binding^{97–99}, conversion^{94,97,100} and polymerization assays comprising purified PrP molecules, or fragments of such molecules (FIG. 5) (reviewed in REF. 101), can provide evidence of whether or not PrP^{Sc} inhibitors act directly on PrP molecules. PrP^{Sc} amplification reactions in crude brain or cellular extracts^{35,102,103} provide more continuous, but less defined, cell-free assays that might be adapted to high-throughput formats. PrP^{Sc} destabilization assays can identify compounds that disinfect potential sources of infection or aid infected hosts in reducing their burden of PrP^{Sc}^{104–107}. Cell-culture assays of cytotoxicity induced by PrP^{Sc} or peptide fragments of them can be used to screen for compounds that might protect against the neuropathological consequences of TSE infections¹⁰¹. Some caution should be used in interpreting such assays, as it is not yet clear which abnormal form or forms of PrP are the primary cytotoxic species in TSE diseases.

Rodent models for testing anti-TSE drugs

For practical reasons, most *in vivo* testing has been done in mice and hamsters inoculated with rodent-adapted TSE strains^{5,108–113}. The rodent models allow for much faster and less expensive screening than is possible in the natural, large-animal host species. Whereas incubation periods tend to be years in sheep, cattle, deer

and elk, rodents can become ill within as little as 40–45 days after intracerebral inoculation with high doses of an appropriate scrapie strain^{114,115}. The latter scenario is presumably suitable for testing potential therapeutic activities in hosts with established CNS infections. However, it is often also desirable to test for pre- or post-exposure prophylactic effects against lower-dose TSE infections by peripheral routes, such as oral exposure. However, under the latter circumstances, rodent incubation periods can be much longer, and, in extreme cases, extend nearly to the lifespan of the animal. Researchers are therefore often torn between the conflicting goals of minimizing the duration of experiments and reducing the infectious challenge to increase the likelihood of detecting drug efficacy. To abbreviate the testing of compounds against peripheral scrapie inoculations, some investigators have opted to assay the accumulation of PrP^{Sc} in the spleen part way through the incubation period, rather than waiting for the appearance of clinical illness¹¹⁶.

Pre- and post-exposure chemoprophylaxis

A growing list of compounds can prolong the lives of scrapie-infected rodents, provided that drug treatment is initiated prior to or near the time of infection (TABLE 1). The prophylactic agents fall into several different chemical classes and are usually among the most potent inhibitors of PrP^{Sc} accumulation in scrapie-infected cell cultures. Hundreds of other PrP^{Sc} inhibitors have been identified in *in vitro* tests that await further testing *in vivo*, including branched polyamines⁹⁵, polyphenols, antipsychotics, antidepressants, analgesics and statins^{93,94,117,118}.

Sulphated glycans and other polyanions. In the 1980s, various compounds with antiviral and/or immunological effects were tested against scrapie in animals. Surprisingly, given that no virus or conventional immune response has been associated with TSE infections, a number of large polyanions, such as dextran

sulphate, pentosan polysulphate and heteropolyanion 23, were effective in protecting rodents against scrapie infections^{108,109}. In some cases, protection was observed by short treatments months before peripheral scrapie infection^{108,119}. This seemed to be related to sequestration of the polyanion in cells of the lymphoreticular system. More recent studies showed that these polyanionic compounds were potent inhibitors of PrP^{Sc} formation, which provides a likely explanation for their prophylactic efficacy^{120,121}. A number of lines of evidence indicate that sulphated glycans and other polyanionic drugs act by affecting interactions between PrP molecules and endogenous sulphated glycosaminoglycans that seem to be important in PrP^C trafficking and PrP^{Sc} formation^{85,120–123} (FIG. 1). Alternatively, polyanionic drugs might also interfere with PrP interactions with RNA molecules (also large polyanions) that were observed to support PrP conversion in brain homogenates and proposed to be physiological cofactors^{124,125}. Limitations of these large polyanionic drugs include potential anti-coagulant activity and poor bioavailability to the CNS (see below).

Sulphonated dyes. The first identified inhibitor of PrP^{Sc} accumulation, Congo red¹²⁶, is a sulphonated amyloid stain that has modest prophylactic activity against scrapie in rodents¹²⁷. *In vitro* studies have shown that Congo red can compete with sulphated glycans for binding to PrP^C, which is consistent with an inhibitory mechanism that is similar to that of the large polyanions¹²⁸. At high concentrations relative to those required to inhibit PrP^{Sc} formation, Congo red can also overstabilize PrP^{Sc}, and this effect has also been suggested as an aspect of its inhibitory mechanism¹²⁹. Interest in Congo red as a potential drug was diminished by fears of teratogenic and/or carcinogenic activity related to its benzidine moiety. Since then, numerous analogues of Congo red and related sulphonated dyes have also proven to be potent PrP^{Sc} inhibitors *in vitro*^{93,130–132} and, in the case of suramin¹³³, to have modest prophylactic activity as well. Structure–activity analyses of Congo red analogues indicate that the central benzidine moiety can be altered without neutralizing its inhibitory activity^{93,130,132}. These observations raise hopes that safer and more effective analogues of these types of inhibitors can be discovered. Curcumin — the major yellow pigment in the spice turmeric, which has a structure that lacks sulphonates and a benzidine moiety but is otherwise reminiscent of Congo red — was recently shown to also be a very potent, yet non-toxic (edible), inhibitor of PrP^{Sc} formation¹³⁴. Unfortunately, efforts to show *in vivo* efficacy of curcumin have so far failed.

Cyclic tetrapyrroles. Porphyrins and phthalocyanines are another diverse group of PrP^{Sc} inhibitors¹⁰⁰ that can delay the onset of disease in mice inoculated intraperitoneally with scrapie, but only if treatment is initiated within several weeks of infection^{111,135}. Cyclic tetrapyrroles tend to have highly conjugated planar aromatic ring systems that bind transition metal ions and can be

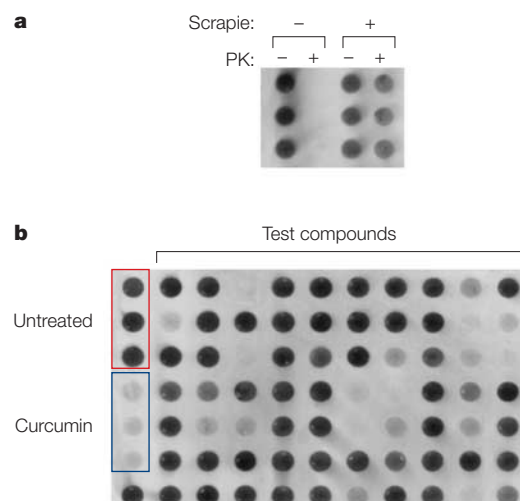


Figure 4 | High-throughput cell-based screen for inhibitors of PrP-res formation. Scrapie-infected murine N2a neuroblastoma cells are grown in 96-well plates for several days in the presence or absence of test compounds. The cultures are lysed, treated with proteinase K (PK) to eliminate the normal prion protein isoform (PrP^C), and assayed for accumulated PrP-res content by a dot blot immunoassay. **a** | Dot blot of PrP content with and without PK treatment of lysates of scrapie-infected or uninfected N2a cells. **b** | Representative dot blot screen of multiple (unidentified) test compounds for inhibition of PrP-res accumulation. The turmeric component curcumin (10 μ M), a known inhibitor¹³⁴, was included as a positive control. Adapted, with permission, from REF. 94 © American Society for Microbiology (2003).

circumscribed by anionic, cationic or uncharged peripheral constituent groups. Several cyclic tetrapyrroles have been shown to directly block cell-free PrP conversion reactions, so it is assumed that their inhibitory mechanism involves direct interaction with PrP molecules. None of the antiscrapie tetrapyrroles that have been tested *in vivo* are likely to cross the blood–brain barrier, and this presumably limits the therapeutic window of opportunity. However, other tetrapyrrole inhibitors (*in vitro*) are thought to penetrate the blood–brain barrier and could prove to be efficacious later in the TSE incubation period.

Polyene antibiotics. Another promising group of compounds that inhibit PrP^{Sc} formation and delay the onset of experimental scrapie in rodents is the anti-fungal drug amphotericin B and its analogues^{136,137}. The antiscrapie mechanism of action of the polyene antibiotics could result from a perturbation of the raft membrane domains with which PrP-sen is associated^{138,139}. Unfortunately, although treatment can be initiated well after the point of infection, it must begin before the onset of clinical disease. Other drawbacks of the prototypic amphotericin B are its toxicity and scrapie strain-specificity. However, less toxic and more broadly active analogues have now been identified, strengthening hopes that more effective therapies based on this type of compound might be possible¹⁴⁰.

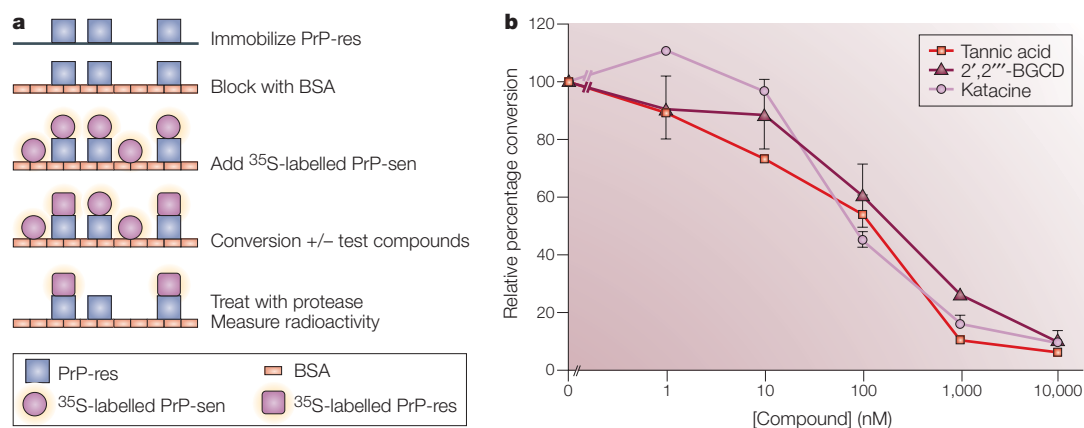


Figure 5 | **Solid-phase cell-free PrP conversion assay for inhibition of PrP-res formation.** **a** | Outline of assay as described previously^{94,97}. **b** | Inhibition curves of three PrP-res inhibitors. Adapted, with permission, from REF. 94 © American Society for Microbiology (2003). BSA, bovine serum albumin; PrP-res, protease-resistant prion protein; PrP-sen, protease-sensitive prion protein.

Quinacrine, quinoline, acridines, phenanthiazines and related molecules. Quinacrine, chlorpromazine, quinine and related molecules have been shown to be PrP^{Sc} inhibitors *in vitro*^{112,117,141}. Although quinacrine has not delayed the onset of disease in rodents infected intracerebrally^{5,113}, quinine and biquinoline have shown some efficacy when administered intraventricularly through osmotic pumps¹¹². Quinacrine, the antimalarial drug, has nevertheless been tried extensively with little success in human CJD patients (see below).

Dimethylsulphoxide. The organic solvent dimethylsulphoxide (DMSO) inhibits the aggregation of PrP^{Sc}, reduces PrP^{Sc} accumulation, promotes PrP^{Sc} excretion in the urine and modestly prolongs the lives of scrapie-infected hamsters^{107,111}. Accordingly, it has been suggested that DMSO might be useful therapeutically, especially in combination with other potential drugs.

Copper chelators. Early treatment of scrapie-infected mice with the copper chelator D-(–)-penicillamine delays the onset of clinical disease¹⁴². As the proteinase K resistance of PrP^{Sc} was enhanced *in vitro* by increasing copper in a dose-dependent manner, it is possible that the *in vivo* effect of penicillamine relates to a decrease in the amount of copper available to bind to PrP^{Sc}.

Incompatible PrP^C molecules and PrP peptides. One clearly demonstrated strategy for reducing susceptibility to TSE diseases is to express PrP^C molecules that are incompatible with conversion driven by particular TSE strains^{143–145}. This effect has been demonstrated by the natural resistance of certain host species (for example, dogs), or PrP genotypes of host species (for example, ARR sheep, or humans heterozygous at PrP codon 129), to specific TSE diseases^{146,147}, and by manipulation of the susceptibility of mice by transgenic expression of various PrP genes¹⁴⁸. *In vitro* experiments indicate that incompatible PrP^C molecules both resist conversion themselves and block conversion of compatible PrP^C molecules that might also be

present^{144,145}. Collectively, these data indicate that the introduction of incompatible PrP^C molecules or fragments of them^{149,150} — either directly, or indirectly via gene therapy methods — would be a useful approach for prophylaxis against TSE diseases. In any case, the breeding of scrapie-resistant genotypes into sheep flocks is moving ahead quickly.

Therapeutic agents

As noted above, no chemotherapeutic treatments are known to be effective against TSEs once the clinical symptoms have developed. A number of compounds have been tested, with little success in clinically ill patients^{137,151}. Recently, following its discovery as a PrP^{Sc} inhibitor^{117,141}, the antimalarial drug quinacrine was administered to rodents^{5,112,113,152} and human CJD patients^{153,154}, but there is little evidence of anything more than transient benefit. Flupirtine treatments of human CJD patients reduced their deterioration in dementia tests¹⁵⁵. Intracerebral treatments of at least one CJD patient with pentosan polysulphate have been reported by the media, but it is unknown whether this constitutes a safe and effective therapy. Similar pentosan polysulphate intracerebral treatments of scrapie-infected rodents showed promising effects¹⁵².

Non-immune neutralization of TSE infectivity

Extensive studies of harsh methods for decontaminating TSE infectivity have been reviewed elsewhere¹⁵⁶. Here we will restrict our discussion to compounds that can be mixed with infectious inocula just before inoculation to neutralize or destabilize infectivity. Such compounds might be useful as additives to edible or injectable substances at risk of being contaminated with TSE infectivity. One type of compound that fits that description are the ‘β-sheet breaker’ peptides which Soto and colleagues have shown can destabilize PrP^{Sc} and reduce infectivity titres in brain homogenates¹⁰⁴. Effective infectivity titres have also been reduced by mixing scrapie inocula with 4'-iodo-4'-deoxy-doxorubicin¹⁰⁵, tetracycline¹⁰⁶ and phthalocyanine tetrasulphonate¹³⁵.

GLIOSIS

Activation and proliferation of astrocytes and microglial cells in the brain.

Box 1 | **Testing for prion infection**

The treatment of human prion diseases, and the prevention of prion contamination of the food supply, will be crucially dependent on the sensitive and specific detection of prion infectivity. Unfortunately, there is, to date, no universally accepted test for the ante-mortem detection of prion infection, despite the availability of numerous methods to detect prion infection in brain samples. The ‘gold standard’ for the definitive diagnosis of prion disease in humans and animals depends on the detection of histological features of prion infection in affected brains, such as degeneration of specific populations of neurons, regional spongiform change, gliosis and abnormal deposits of prion protein. Biochemical tests for protease-resistant prion protein can be conducted by enzyme-linked immunosorbent assay (such as that marketed by BioRad) or immunoblotting (as commercially pioneered by Prionics). The conformation-dependent immunoassay (CDI; InPro) can sensitively distinguish between misfolded and cellular isoforms of the prion protein. Affinity reagents for the abnormal prion protein isoform (PrP^{Sc}) (for example, from Idexx Laboratories) are also making the transition from the lab to the field. However, the definite diagnosis of human prion disease is contingent on post-mortem (or biopsy) analysis of brain, despite a number of clinical and paraclinical laboratory features that can establish a ‘probable’ diagnosis during life. Similarly, diagnosis of bovine spongiform encephalopathy, scrapie and chronic wasting disease is dependent on access to brain samples, although research for a non-invasive, high-throughput, inexpensive test (for blood or other accessible biofluids) is a goal sought by at least 100 academic and commercial laboratories.

Future prospects for chemotherapeutics

In the absence of effective TSE therapeutics, it is pertinent to ask why the numerous compounds that have prophylactic activity tend to have so little effect after the infection has spread within the CNS. For many compounds, such as the large polyanions and highly charged members of the cyclic tetrapyrrole class, it is obvious that the drugs have little if any ability to cross the blood–brain barrier when administered peripherally. It might therefore be helpful to find ways to improve the delivery of such compounds to the brain either by formulating them with carriers that improve bioavailability to the brain or by pumping them into the brain as Doh-Ura and colleagues have done with osmotic pumps^{112,152}.

At the same time, high-throughput screens have accelerated the identification of new PrP^{Sc} inhibitors that can cross the blood–brain barrier^{94,117,134,137,141}. However, some apparently brain-permeable PrP^{Sc} inhibitors, such as the polyene antibiotics¹³⁷, curcumin¹³⁴, quinacrine and quinoline^{5,112,113,152} and others¹⁵⁷, have

already been tested *in vivo* and are unable to halt or substantially modify pathogenesis late in the course of disease. Although the inhibition of new PrP^{Sc} formation is likely to be a major goal in TSE therapeutics, this might not be sufficient in the clinical phase, once significant PrP^{Sc} has accumulated and neuropathology has occurred. At that stage, it might be important to also destabilize PrP^{Sc} and block or reverse the neuro-pathological effects of the infection using other drugs. For instance, compounds that reduce oxidative stress, apoptosis, aberrant signal transduction or other pathological responses of neurons and support cells might be helpful.

As the understanding of TSE pathogenesis and the functions of normal and abnormal PrP isoforms improves, new therapeutic targets should be revealed. The recent report that ablating PrP^C expression in adult scrapie-infected mice prolongs their lives and even reverses pathology⁸⁴ indicates that it might be rewarding to search for compounds that can downregulate PrP^C expression. Such compounds might include small interfering RNAs¹⁵⁸ or antisense RNAs. Furthermore, advances in basic brain biology, neural stem-cell biology and neural differentiation might ultimately suggest treatments — for example, with stem cells and/or neurotrophic and differentiation factors — that could aid the recovery of lost brain functions or prevent further neurological decline in the clinical phase of disease. In the meantime, it will remain important to develop practical preclinical diagnostic tests so that potential therapeutic treatments can begin as early as possible in the pathogenic process (BOX 1).

Conclusion

The prion diseases could provide a prototype for other disorders of protein misfolding, including Alzheimer’s disease, amyotrophic lateral sclerosis and Parkinson’s disease^{159,160}. It is likely that a fuller understanding of the pathogenesis and treatment of prion diseases will provide novel diagnostic and therapeutic approaches to other diseases accompanied by neural accumulation of misfolded proteins, and perhaps additional currently unrecognized post-translational disorders of the proteome.

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The authors declare competing financial interests: see Web version for details.

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Comparison of protease-resistant prion protein inhibitors in cell cultures infected with two strains of mouse and sheep scrapie

David A. Kocisko^{a,*}, Abbi L. Engel^a, Kristin Harbuck^a, Kevin M. Arnold^a, Emily A. Olsen^a,
Lynne D. Raymond^a, Didier Vilette^b, Byron Caughey^{a,**}

^a *Laboratory of Persistent Viral Diseases, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 903 S. 4th Street, Hamilton, MT 59840, USA*

^b *Unité Virologie et Immunologie Moléculaires, Institut National de la Recherche Agronomique, 78350 Jouy-en-Josas, France*

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Abstract

The transmissible spongiform encephalopathies (TSEs) are fatal neurodegenerative diseases. A primary therapeutic target for TSE intervention has been a protease-resistant form of prion protein known as PrP^{Sc} or PrP-res. In vitro testing of mouse scrapie-infected cell cultures has identified many PrP-res inhibitors that also have activity in vivo. Here we identify 32 new inhibitors of two strains of mouse scrapie PrP-res. Furthermore, to investigate the species-specificity of these and other PrP-res inhibitors, we have developed a high-throughput cell culture assay based on Rov9 cells chronically-infected with sheep scrapie. Of 32 inhibitors of murine PrP-res that were also tested in the Rov9 cells, only six showed inhibitory activity against sheep PrP-res. The three most potent inhibitors of both murine and ovine PrP-res formation (with 50% inhibition at $\leq 5 \mu\text{M}$) were tannic acid, pentosan polysulfate and Fe(III) deuteroporphyrin 2,4-bisethyleneglycol. The latter two have anti-mouse scrapie activity in vivo. These results identify new inhibitors of murine and ovine PrP-res formation and reinforce the idea that compounds effective against PrP-res from one species or strain cannot be assumed to be active against others.

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Keywords: Sheep scrapie; Mouse scrapie; Prion; Inhibitors; Therapeutics

The transmissible spongiform encephalopathies (TSEs) are related fatal neurodegenerative diseases that occur in many mammalian species. There is no effective treatment or cure for any of these diseases after the onset of clinical disease. The most common human TSE is sporadic Creutzfeldt–Jakob disease (CJD). A variant form of CJD (vCJD) occurs more rarely as a result of consumption of bovine spongiform encephalopathy (BSE)-infected cattle products. Other TSEs include scrapie in sheep and chronic wasting disease in restricted populations of North American deer and elk.

Prion protein (PrP) is a 23–35 kDa glycoprotein anchored to the cell surface by a glycosylphosphatidylinositol anchor, and its normal function is unclear. One usual feature of the

TSEs is the formation of partially protease-resistant prion protein (PrP-res or PrP^{Sc}) from the normal, protease-sensitive form (PrP-sen or PrP^C), primarily in the nervous and lymphoreticular systems [7]. A consistent difference between PrP-res and PrP-sen is their conformation.

While the precise nature of the infectious agent of the TSEs has not been fully defined, it is clear that PrP-res is usually associated with infectivity and co-localized with neuropathological changes [4]. Thus, PrP-res seems to be a major contributor to TSE pathogenesis and is a primary target in fighting these diseases [2,6,8,9,15].

The use of compounds that inhibit the formation of PrP-res in infected cell culture as TSE prophylactics and therapeutics has been extensively explored. Using mouse neuroblastoma cells that can be chronically-infected with mouse scrapie (N2a) [5,25,26], numerous PrP-res inhibitors have been found and many of these have demonstrated prophylactic activity in vivo (reviewed in [3,6]). Examples of cell-culture

* Corresponding author. Tel.: +1 406 375 9692; fax: +1 406 363 9286.

** Corresponding author. Tel.: +1 406 363 9264; fax: +1 406 363 9286.

E-mail addresses: DKocisko@niaid.nih.gov (D.A. Kocisko),
BCaughey@niaid.nih.gov (B. Caughey).

PrP-res inhibitors with in vivo activity include pentosan polysulfate [9,13,20], cyclic tetrapyrroles [11,23,24], and amphotericin B [1,12,22]. Pentosan polysulfate was also shown to have modest late-stage therapeutic activity in mice when administered via intraventricular infusion [14]. However, no PrP-res inhibitor has proven to be therapeutic in humans.

We recently developed a high-throughput PrP-res cell culture assay to screen for PrP-res inhibitors [18]. This assay measures PrP-res accumulation in N2a cells chronically-infected with either RML or 22L mouse scrapie strains. Numerous inhibitors of PrP-res accumulation were identified, many of which showed some scrapie strain dependence in their activities. These scrapie strain-dependent differences, as well as species-dependent differences noted recently for the in vivo anti-scrapie activity of an amyloid imaging agent [17], encouraged us to investigate further the strain- and species-specificities of PrP-res inhibitors. Unfortunately, other than various murine scrapie-infected cells, the only other currently available TSE-infected cell lines are ovine scrapie-infected Rov9 cells, rabbit epithelial cells that express ovine PrP [27]. Here we have adapted the sheep scrapie-infected Rov9 cells to a high-throughput PrP-res inhibition assay. We also identify 32 new inhibitors of PrP-res accumulation in both RML- and 22L-infected N2a cells, and compare the activity of 23 of these and 9 other previously identified murine PrP-res inhibitors with their effects against sheep scrapie PrP-res in Rov9 cells.

The creation and characterization of the sheep scrapie-infected Rov9 cells used in this assay are reported elsewhere [27]. Rov9 cells were grown in the presence of 1 $\mu\text{g}/\text{mL}$ doxycycline ($\sim 1 \mu\text{M}$) (to maintain expression of ovine PrP) at 37 °C in 5% CO₂ and passaged at a 1–4 dilution weekly. Because cells chronically-infected with TSEs can maintain infections better when grown with OPTIMEM, the Rov9 cells were adapted from MEM supplemented with 10% FBS to OPTIMEM (Invitrogen) supplemented with 10% FBS. This adaptation was accomplished over the course of three passages by using 50, 75, and finally 100% OPTIMEM. The procedure to test for sheep PrP-res inhibition in Rov9 cells was adapted from that used to assay inhibitory activity in the RML and 22L mouse scrapie strains in chronically-infected mouse neuroblastoma cells [18]. Rov9 cells were plated in 96-well plate wells in 100 μL medium. After several hours, appropriate dilutions of potential inhibitors in DMSO or PBS solutions were added and the cells were allowed to grow to confluence during the next 7 days. A maximum of 0.5% (v/v) of DMSO in the medium was used in these studies without effect on cell growth or morphology (not shown). At confluence, cells were carefully examined by light microscopy for any morphological changes or toxicity due to test compounds. The cell medium was then removed and 50 μL of lysing buffer [18] was added. Five minutes after lysis, 25 μL of 0.2 U/ μL benzonase (Sigma) was added and the lysates were incubated for 30 min at 37 °C. The benzonase treatment reduced clumps of nucleic acids and yielded more homogeneous signals in subsequent dot-blots.

After benzonase treatment, 25 μL of 100 $\mu\text{g}/\text{mL}$ proteinase K (PK) was added to give a final concentration of 25 $\mu\text{g}/\text{mL}$, and the plates were incubated at 37 °C for 1 h. The treatment with PK eliminates PrP-sen and most other proteins in the lysate, but only has a limited effect on PrP-res, which can then be more easily detected. After protease treatment, 200 μL of 1 mM Pefabloc was added to each well to inhibit further proteolysis.

The dot-blot procedure was identical to that used for the measurement of PrP-res from scrapie-infected mouse neuroblastoma cells [18]. Briefly, the PK-treated cell lysates were transferred onto a PVDF membrane through a dot-blot apparatus. The membrane was removed, treated with 3 M guanidine thiocyanate for 10 min, blocked in 5% milk, and then incubated with an anti-PrP monoclonal antibody, 6B10 (kindly supplied by Richard Rubenstein). 6B10, which was effective in the mouse PrP-res dot-blot assay [18], also detected sheep PrP-res in this assay with low background (Fig. 1C). The membrane was then incubated with an alkaline phosphatase-conjugated goat anti-mouse secondary antibody in 5% milk and then after rinsing an enhanced chemifluorescence agent (Zymed) was applied. PrP-res was quantified by scanning the membrane with a Storm Scanner (Molecular Dynamics) and ImageQuant software. As the cells were initially plated at $\sim 25\%$ confluent density prior to addition of potential inhibitors, the amount of input PrP-res in the seeded cells needed to be subtracted from all wells for more accurate results. This is in contrast to the situation with N2a cells where the input PrP-res was virtually undetectable. To assess the amount of pre-existing PrP-res in seeded Rov9 cells, a cytotoxic compound such as 20 μM thiothixene was added to at least three wells per 96-well plate to prevent new PrP-res formation while cells in other wells were growing to confluency. New PrP-res accumulation during growth to confluency was calculated as the difference between the total PrP-res signal intensity and the average signal intensity from the wells containing the cytotoxic compound. The approximate IC₅₀ values (concentration inhibiting 50% of PrP-res production) were estimated by graphing the amount of PrP-res formed in the presence of various concentrations of inhibitor compared to control. The average value or approximate range of values for at least 3 independent determinations was reported in Table 1.

During development of this assay, we found that the amount of PrP-res signal from the Rov9 cells increased about four-fold after adaptation to OPTIMEM from MEM medium (Fig. 1A). A Western blot of the PrP-res from Rov9 cells treated with tannic acid showed inhibition of sheep PrP-res (Fig. 1B). Similar inhibition was seen using the dot-blot assay (Fig. 1C). Mock infected cells gave a negligible background in this assay with the 6B10 monoclonal antibody. Tannic acid and pentosan polysulfate, known inhibitors of 22L and RML PrP-res production in N2a cells, also inhibited sheep PrP-res accumulation (Fig. 1C) and were used as positive control inhibitors on subsequent test plates. Fe(III) deuteroporphyrin 2,4-bisethyleneglycol (DPG₂-Fe³⁺) also inhibited

Table 1

Relative activity of inhibitors of PrP-res formation in N2a cells infected with murine scrapie strains and Rov9 cells infected with sheep scrapie^a

Compound	PrP-res inhibition (approx. IC ₅₀)			Scrapie-infected Rov9 cytotoxicity
	RML	22L	Sheep	
Curcumin	10 nM	None ^b	None ^c	20 μM
Thioridazine	500 nM–1 μM	500 nM–1 μM	None ^d	10 μM
Thiothixene	100 nM–500 nM	100 nM–500 nM	None ^d	5 μM
Tetrandrine	100 nM–500 nM	100 nM–500 nM	None ^d	5 μM
Amodiaquine	500 nM	500 nM	None ^d	10 μM
Trifluoperazine	500 nM–1 μM	500 nM–1 μM	None ^d	10 μM
Tannic acid	100 nM	100 nM	1–5 μM	>10 μM
Pentosan polysulfate	10 ng/mL	500 ng/mL	500 ng/mL	>5 μg/mL
DPG ₂ -Fe ³⁺	1–5 μM	1–5 μM	1–5 μM	>20 μM
Acacetin	<1 μM	1–10 μM	NT	NT
Acacetin diacetate	<1 μM	1–10 μM	None ^c	20 μM
Alfalone methyl ether	<1 μM	1–10 μM	NT	NT
Benzo[a]pyrene	<1 μM	1–10 μM	None	>40 μM
Biochanin A diacetate	<1 μM	1–10 μM	NT	NT
Biochanin A, monomethylether	<1 μM	1–10 μM	NT	NT
Dibenzothiophene	<1 μM	1–10 μM	None	>40 μM
6,3'-Dimethoxy flavone	<1 μM	1–10 μM	None ^d	10 μM
6,4'-Dimethoxy flavone	<1 μM	1–10 μM	None ^c	20 μM
7,2'-Dimethoxy-4,5'-methylenedioxyisoflavone	<1 μM	1–10 μM	NT	NT
Diosmetin	<1 μM	1–10 μM	None ^e	40 μM
Domperidone	<1 μM	1–10 μM	NT	NT
Durlettone	<1 μM	1–10 μM	NT	NT
Flavanone	<1 μM	1–10 μM	40 μM ^f	>40 μM
Formononetin	<1 μM	1–10 μM	None ^c	20 μM
Genistein	<1 μM	1–10 μM	None ^c	20 μM
Harmine	<1 μM	1–10 μM	None ^c	20 μM
12a-Hydroxy-5-deoxydehydromunduserone	<1 μM	1–10 μM	40 μM	>40 μM
3-Hydroxyflavone	<1 μM	1–10 μM	None ^d	10 μM
Ipraflavone	<1 μM	1–10 μM	None ^d	10 μM
Maackiain	<1 μM	1–10 μM	None ^c	20 μM
Methoxyvone	<1 μM	1–10 μM	None ^d	10 μM
3-Methylcholanthrene	<1 μM	1–10 μM	None	>40 μM
Metochalcone	<1 μM	1–10 μM	NT	NT
Obliquin	<1 μM	1–10 μM	None ^c	20 μM
Prunetin	<1 μM	1–10 μM	None	>40 μM
Pseudo-baptigenin, methyl ether	<1 μM	1–10 μM	NT	NT
Quercetin tetramethyl (5,7,3',4') ether	<1 μM	1–10 μM	None ^c	20 μM
Resveratrol	<1 μM	1–10 μM	None ^e	40 μM
Rhamnetin	<1 μM	1–10 μM	None	>40 μM
Tetramethyl haematoxylene	<1 μM	1–10 μM	None ^c	20 μM
Triacetyl resveratrol	<1 μM	1–10 μM	40 μM ^f	>40 μM

^a RML data for curcumin and RML and 22L data for the next six listed compounds have been reported [10,18] and are included for comparison.

^b No inhibition at 5 or 10 μM, but slight cytotoxicity apparent at 10 μM.

^c No inhibition at 10 μM.

^d No inhibition at 1 μM.

^e No inhibition at 20 μM.

^f Slightly under 50% inhibition at 40 μM. DPG₂-Fe³⁺ = Fe(III) deuteroporphyrin 2,4-bisethyleneglycol. NT = not tested.

PrP-res production in Rov9 cells with an IC₅₀ value between 1 and 5 μM (Fig. 1D). Dilutions of untreated cell lysates showed that the signals from typical control and inhibitor-treated wells of Rov9 cells fell in an approximately linear range of detection for this assay (Fig. 1E).

As ~1 μM doxycycline is required for production of ovine PrP in Rov9 cells, we tested for the possibility that it affected PrP-res production. Doxycycline has been reported to render PrP-res purified from infected brain tissue sensitive to

PK digestion in vitro [16]. One micromolar doxycycline has no effect on PrP^{vCJD} whereas 10 μM causes ~20% reduction in PK resistance. However, with the sheep scrapie Rov9 cultures, doubling the doxycycline concentration from 1 to 2 μM did not reduce PrP-res production. In 6 μM doxycycline, Rov9 cells showed minor morphological changes but still had the same PrP-res production as those growing in 1 μM doxycycline. At 11 μM, doxycycline was lethal to Rov9 cells. Finally, the addition of 1 μM doxycycline had

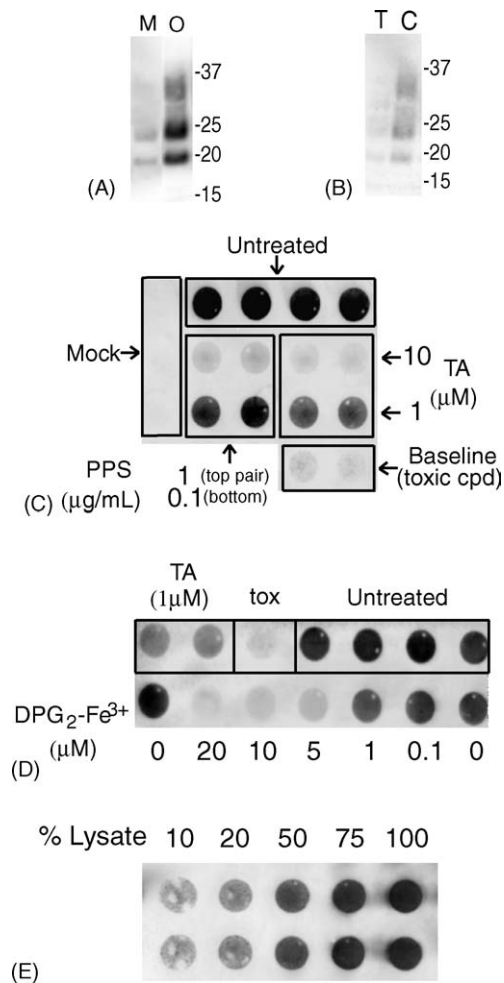


Fig. 1. Characterization of sheep-scrapie dot-blot assay. (A) Western blot of PK-treated sheep PrP-res from Rov9 cells that were cultured either in MEM (M) or OPTIMEM (O) medium developed using 6B10 antibody. Molecular weight markers are indicated on the right in kilodaltons. (B) Western blot of PK-treated sheep PrP-res from Rov9 cells that grew in the presence (T) or absence (C) of 10 μ M tannic acid. Molecular weight markers are indicated on the right in kilodaltons. (C) Dot-blot showing PK-treated sheep PrP-res from Rov9 cells. The left column shows the minimal background signal from mock infected Rov9 cells which produce no PrP-res. The top right box shows the PrP-res from four untreated wells of Rov9 cells. The lowest right box contains a pair of dots that are the signal from the input sheep PrP-res when a cytotoxic compound was added soon after initial cell plating. This amount of signal is subtracted from all wells when determining percent inhibitions. The dots in the center right box are the sheep PrP-res signal from cells treated with 10 μ M (top pair) or 1 μ M (bottom pair) of tannic acid. The most center box shows the sheep PrP-res signal after treatment with 1 μ g/mL (top pair) or 100 ng/mL (bottom pair) of pentosan polysulfate. (D) Dot-blot of PK-treated sheep PrP-res from Rov9 cells incubated with 1 μ M tannic acid (top left pair of dots), untreated cells (four dots at upper right) and various concentrations of DPG₂-Fe³⁺ (bottom row). The one dot labeled "tox" was treated with 20 μ M thiothixene to kill the seeded cells and is a measure of the amount of input PrP-res. (E) Dilution series in duplicate of PK-treated sheep scrapie-infected Rov9 cell lysates put through the dot-blot apparatus onto a PVDF membrane.

no effect on RML or 22L PrP-res production in N2a cells (data not shown). Thus, there was no evidence that doxycycline influenced PrP-res production in either scrapie-infected Rov9 or N2a cells at concentrations required to sustain PrP-res production in the Rov9 cells.

Screening of compounds from a library using scrapie-infected N2a cells and the dot-blot assay revealed 32 new inhibitors with IC₅₀ values of <1 μ M against RML PrP-res accumulation and 1–10 μ M against 22L PrP-res (Table 1, lower 32 entries). Most of these new inhibitors, as well as some previously known inhibitors of at least one strain of mouse PrP-res accumulation (Table 1, top nine entries) were tested for inhibition in sheep PrP-res from Rov9 cells at concentrations up to 40 μ M or until cytotoxicity or PrP-res inhibition was observed. Cytotoxicity detected initially by light microscopy was confirmed using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) cell viability assays [21]. A drop of more than 20% in cell viability detected by MTT assay was also apparent by light microscopy as lower cell densities or changes in cellular morphology. Overall, the inhibitory activity of the compounds against sheep PrP-res was low relative to their activities against the mouse scrapie strains in N2a cells. Clearly there are many compounds exhibiting strong RML PrP-res inhibition and weaker 22L PrP-res inhibition that were not effective against sheep PrP-res in infected Rov9 cells. Interestingly, there were also compounds that had strong inhibitory activity against both RML and 22L PrP-res in N2a cells with no inhibitory activity against the sheep PrP-res in infected Rov9 cells.

Curcumin, a potent inhibitor of RML PrP-res infected N2a cells [10], did not inhibit PrP-res accumulation in 22L-infected N2a cells or sheep scrapie-infected Rov9 cells. To test if curcumin inhibition of 22L PrP-res accumulation might be possible in another type of cell, two chronically 22L-infected mouse fibroblast cell lines, NIH/3T3 and ψ 2C2 [28], were also tested with up to 10 μ M curcumin, 1000 times higher than its IC₅₀ value against PrP-res in RML-infected N2a cells. No inhibition of 22L PrP-res production was detected in either cell type (Fig. 2).

Cell-culture-based PrP-res inhibition assays have been useful initial screens for potential in vivo anti-scrapie activity, especially because testing for anti-TSE activity in animals is expensive and the incubation periods of TSE infections are long. However, since not all cell culture inhibitors work well against TSE diseases in vivo, it remains important to test inhibitors in TSE-infected animals. The first nine compounds listed in Table 1 have been tested for in vivo anti-scrapie activity [10,19] and only pentosan polysulfate [13,14,20] and DPG₂-Fe³⁺[24] were active. Pentosan polysulfate, DPG₂-Fe³⁺, and tannic acid were also the only compounds tested that had anti-PrP-res activity in all three scrapie-infected cell models with IC₅₀ values of 5 μ M or lower. Besides the TSE strain, there are other differences between the sheep-scrapie-infected Rov9 and the RML and 22L scrapie-infected N2a cell assays that could contribute to differences in inhibitor activity. The Rov9 epithelial cells grow more slowly and are

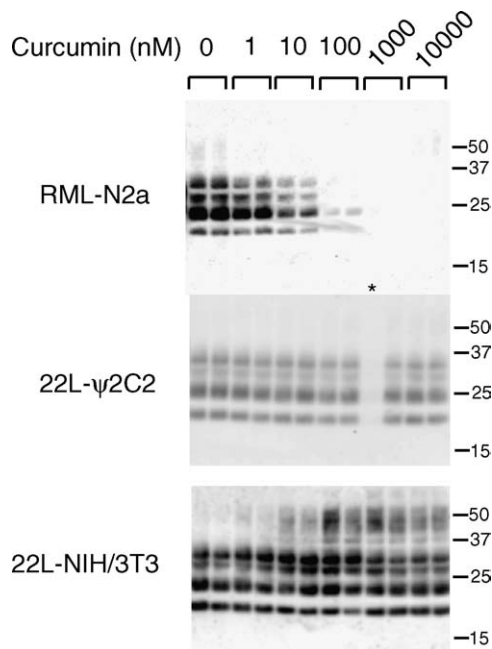


Fig. 2. Curcumin, a potent inhibitor of RML PrP-res in N2a cells [10] (top panel), fails to inhibit 22L production in chronically-infected NIH/3T3 (middle panel) and ψ 2C2 mouse fibroblast cells (lower panel). The concentration of curcumin used to treat the cells is shown at the top. All PrP-res was detected by Western blotting using polyclonal anti-PrP serum R30 [10] as the primary antibody. Molecular weight markers are indicated at the right in kilodaltons. (*) Empty lane on gel.

more dense when confluent. They may also metabolize, bind, or internalize compounds differently. Because of these and other potential factors, a direct comparison of IC_{50} values may not be a straightforward indicator of relative *in vivo* anti-scrapie activity in infected mouse or sheep. However, in the case of curcumin, no inhibition was seen in three different 22L-infected cell types, suggesting that the infecting TSE strain, independent of the cell type, can be important in determining inhibition.

Although high-throughput cell culture assays provide a means of screening large numbers of compounds for PrP-res inhibitory activity, they do not give insight into inhibitory mechanisms. It is likely that inhibition can occur by a number of different mechanisms. Some compounds are known to bind PrP-sen or PrP-res and may directly block PrP-res–PrP-sen interactions or the resulting conformational change. Other inhibitors do not seem to bind to PrP and are therefore likely to influence some cellular process that is important in PrP-res formation. Curcumin and many other compounds strongly inhibit RML PrP-res from infected N2a cells, while having little activity against 22L PrP-res from infected N2a cells or sheep scrapie from infected Rov9 cells. Discovering the reasons for these differences in inhibitory activities may help to answer fundamental questions on the nature of strains.

In developing therapeutic and prophylactic TSE treatments, it would be ideal to have compounds that show broad activity against PrP-res of different strains and species. Given the strain- and species-specificities already exhibited

by various known inhibitors, it will be valuable in the future to develop additional inhibitor screening assays based on cells chronically-infected with TSEs of particular importance such as CJD, bovine spongiform encephalopathy, and chronic wasting disease. Such assays should allow *in vitro* screening data to be more highly predictive of *in vivo* activity against TSEs of humans, livestock and wild animals.

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Inhibition of Protease-Resistant Prion Protein Formation in a Transformed Deer Cell Line Infected with Chronic Wasting Disease‡

Gregory J. Raymond,¹ Emily A. Olsen,¹ Kil Sun Lee,¹ Lynne D. Raymond,¹ P. Kruger Bryant III,² Gerald S. Baron,¹ Winslow S. Caughey,¹ David A. Kocisko,¹ Linda E. McHolland,² Cynthia Favara,¹ Jan P. M. Langeveld,³ Fred G. van Zijderveld,³ Richard T. Mayer,² Michael W. Miller,⁴ Elizabeth S. Williams,^{5†} and Byron Caughey^{1*}

Laboratory of Persistent Viral Diseases, NIAID, NIH, Rocky Mountain Laboratories, Hamilton, Montana 59840¹; USDA/ARS/ABADRL, Laramie, Wyoming 82071²; Department for Bacteriology and TSEs, CIDC-Lelystad, 8203 AA 2004, Lelystad, The Netherlands³; Colorado Division of Wildlife, Wildlife Research Center, Fort Collins, Colorado 80526-2097⁴; and Department of Veterinary Sciences, University of Wyoming, Laramie, Wyoming 82070⁵

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Chronic wasting disease (CWD) is an emerging transmissible spongiform encephalopathy (prion disease) of North American cervids, i.e., mule deer, white-tailed deer, and elk (wapiti). To facilitate *in vitro* studies of CWD, we have developed a transformed deer cell line that is persistently infected with CWD. Primary cultures derived from uninfected mule deer brain tissue were transformed by transfection with a plasmid containing the simian virus 40 genome. A transformed cell line (MDB) was exposed to microsomes prepared from the brainstem of a CWD-affected mule deer. CWD-associated, protease-resistant prion protein (PrP^{CWD}) was used as an indicator of CWD infection. Although no PrP^{CWD} was detected in any of these cultures after two passes, dilution cloning of cells yielded one PrP^{CWD}-positive clone out of 51. This clone, designated MDB^{CWD}, has maintained stable PrP^{CWD} production through 32 serial passes thus far. A second round of dilution cloning yielded 20 PrP^{CWD}-positive subclones out of 30, one of which was designated MDB^{CWD2}. The MDB^{CWD2} cell line was positive for fibronectin and negative for microtubule-associated protein 2 (a neuronal marker) and glial fibrillary acidic protein (an activated astrocyte marker), consistent with derivation from brain fibroblasts (e.g., meningeal fibroblasts). Two inhibitors of rodent scrapie protease-resistant PrP accumulation, pentosan polysulfate and a porphyrin compound, indium (III) meso-tetra(4-sulfonatophenyl)porphine chloride, potently blocked PrP^{CWD} accumulation in MDB^{CWD} cells. This demonstrates the utility of these cells in a rapid *in vitro* screening assay for PrP^{CWD} inhibitors and suggests that these compounds have potential to be active against CWD *in vivo*.

Chronic wasting disease (CWD) is a transmissible spongiform encephalopathy (TSE) or prion disease similar to scrapie of sheep and goats, bovine spongiform encephalopathy (BSE) of cattle, and Creutzfeldt-Jakob disease (CJD) of humans. In North America, CWD is contagious among mule deer (*Odocoileus hemionus*), white-tailed deer (*Odocoileus virginianus*), and Rocky Mountain elk (wapiti, *Cervus elaphus nelsoni*) (42). CWD can be transmitted via environmental contamination (27), although the natural mechanisms of spread are not well understood.

As is true for TSEs generally, CWD is characterized by the conversion of the host's normal protease-sensitive prion protein (PrP^C or PrP^{sen}) to a partially protease-resistant form (generically "PrP-res" or specifically "PrP^{CWD}"). In the wake of the BSE epidemic and the transmission of BSE to humans, CWD is of concern due to its apparent spread among free-

ranging and farmed cervids in the United States and Canada. Indeed, CWD has the distinction of being the only TSE that is known to be endemic to locations with wild, free-ranging animal populations. It is not clear whether CWD poses a threat to humans or other species with potential exposure to CWD infectivity. Direct experimental transmissions to ferrets (2), cattle (17), and "cervidized" transgenic mice (5) have been reported. Cross-species cell-free prion protein (PrP) conversion assays have suggested that the rank order of susceptibilities to CWD is cervids > sheep > cattle > humans (33).

One important experimental model that has been lacking in CWD research is a CWD-infected cell line. Several scrapie-infected cell lines have been established, including the SMB (13), N2a (7, 32), GT1 (36), Rov9 (35), and fibroblast (41) cell lines. A CJD-infected human cell line was reported (24), but apparently this cell line was unstable and has been lost (M. Pocchiari, personal communication). Otherwise, we know of no cell lines chronically infected with BSE, CWD, or any human TSE not previously adapted to rodents. Such cell lines would be critical not only for basic studies of the cellular and molecular biology of these TSE strains but also for the screening of potential drugs and treatments. Numerous inhibitors of

* Corresponding author. Mailing address: Rocky Mountain Labs, 903 S. 4th St., Hamilton, MT 59840. Phone: (406) 363-9264. Fax: (406) 363-9286. E-mail: bcaughey@niaid.nih.gov.

† Deceased.

‡ We dedicate this paper to the memory of Elizabeth S. Williams, a pioneer of CWD research.

PrP-res accumulation have been identified initially using scrapie-infected cell lines, and many of these inhibitors have proven to have at least prophylactic activity against experimental scrapie in rodents. Nonetheless, striking TSE strain and species dependence has been observed with some antiscrapie compounds, and thus, it cannot be assumed that a compound that works against one TSE strain will be effective against others, such as CWD (10, 19, 23).

To help refine the search for possible treatments of CWD and to facilitate other aspects of CWD research, we have developed a cell line that is chronically infected with CWD. Using this cell line, we have identified the first two inhibitors of PrP^{CWD} formation, pentosan polysulfate (PPS) and indium (III) meso-tetra (4-sulfonatophenyl)porphine chloride (In-TSP). PPS is a well known anti-TSE compound in other experimental models and is currently being tested to treat human CJD patients (39). In-TSP is a newly identified inhibitor and a member of the well-established cyclic tetrapyrrole class of anti-TSE compounds (11, 30, 31).

MATERIALS AND METHODS

Primary cultures from mule deer brain. Primary cultures were derived from a hunter-harvested mule deer brain that was determined to be negative for CWD using an immunohistochemical assay (26). All of the following steps were done aseptically. Within 8 h of harvest, the thalamus and cerebellum of the brain were removed, and excess meninges and other extraneous tissues were discarded. Approximately 5 g of tissue was put into 100 ml medium 199 with Hank's salts (Sigma) supplemented with 10% fetal bovine serum (FBS), 200 U/ml penicillin, 200 µg/ml streptomycin, and 0.5 µg/ml amphotericin B (Sigma) and processed following the method of Cole and deVellis (15). Briefly, the tissue was rinsed with sterile calcium- and magnesium-free saline, dissociated mechanically by mincing, and pressed through tissue sieves first using a no. 60 mesh screen followed by a no. 100 mesh screen. The sieve was rinsed, and the filtrate was centrifuged for 8 min in a Beckman JS 5.2 rotor at 1 000 rpm (250 × g). The pellet was resuspended in 45 ml of high-glucose Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% gamma-irradiated FBS (DF) growth medium (Sigma), with 100 U/ml penicillin G, 100 µg/ml streptomycin sulfate, and 0.5 µg/ml amphotericin B. The cells were plated into two 75-cm² Primaria flasks (BD Biosciences) at approximately 2 × 10⁷ cells per flask and incubated at 37°C in a humidified 5% CO₂ incubator. A day later, nonadherent cells were removed (>90% of the cells) and the growth medium was changed. At weekly intervals thereafter, half the medium was exchanged with fresh DF without antibiotics. Actively growing and surviving cells were grown using standard techniques (16) until there were ~2 × 10⁷ cells per flask; ~5 × 10⁹ of these cells, suspended in 7.5% dimethyl sulfoxide in DF, were frozen per vial in liquid nitrogen. For all cell passes in this study, flasks were rinsed once with 3 to 5 ml growth medium without FBS, followed by one rinse of 3 to 5 ml 1× trypsin-EDTA (Invitrogen), and then cells were dissociated by incubation for 5 to 10 min at 37°C in the residual trypsin-EDTA liquid and seeded into new flasks as specified.

Transformation of brain cells. For transformation, cells were thawed rapidly at 37°C, diluted into 10 ml of 45% Dulbecco's modified Eagle's medium–45% OptiMEM (Invitrogen)–10% FBS (DOF), centrifuged at 500 × g, resuspended in the DOF growth medium, and plated into six-well plates at ~7.5 × 10⁵ cells/well. After 24 h, the cells were rinsed and the medium was replaced with OptiMEM without FBS. The cells were then transfected with an expression plasmid carrying the simian virus 40 genome (pBRSV, ATCC 45019) using Lipofectamine 2000 reagent (Invitrogen) per the manufacturer's instructions. The medium was changed 48 h later to OptiMEM and 10% FBS (OF), and the cells were grown for an additional 1 to 2 weeks, when clusters of cells showing loss of contact inhibition and an increased rate of cell division were selected using cloning cylinders. The selected transformed cell line (MDB) was expanded and prepared as described above.

Preparation of CWD brain microsomes. The microsomes were prepared aseptically. A section of the medulla oblongata at the level of the obex from an experimentally CWD-infected and clinically affected mule deer was dissected using new tools. The tissue was immediately frozen, except for a portion that was used for histopathological examination. The latter was formalin fixed and sub-

sequently determined to be CWD positive (26). For the microsomes used in the cell infections, 1.1 g of the frozen tissue was prepared using a previously described method (1), except that the low-speed pellet was not reextracted. The final volume of the microsome preparation was 0.55 ml in phosphate-buffered saline (PBS).

Infection of transformed brain cells with CWD microsomes. A frozen vial of the MDB cells was thawed rapidly at 37°C, diluted in OF, centrifuged at 250 × g, resuspended in OF growth medium and seeded into a 25-cm² flask. The specific lots of OptiMEM and FBS (certified grade; Invitrogen) used were independently pretested for the ability to sustain RML scrapie infection in mouse N2a cells for five passes as measured by detection of PrP-res by immunoblotting (data not shown). This pretesting procedure may be critical because, for unknown reasons, RML scrapie infections in mouse N2a cells can be rapidly lost in some lots of OptiMEM and occasional lots of Invitrogen-certified FBS (data not shown). For adaptation to the pretested OF, the cells were passed serially when near confluent at 1:10 dilution seven times. At pass 8, the cells were passed into 24-well plates at 1:8 and grown for 2 days prior to infection, when the cultures were ~60% confluent. The medium was removed, and the cells were washed twice with prewarmed OptiMEM without FBS. Immediately before addition to the cells, the CWD microsome preparation was sonicated in a cup horn at maximum power for 1.5 min and then diluted in additional PBS to a total volume of 100 µl containing either 750 or 2,500 ng PrP^{CWD}. Each of these microsome suspensions was mixed with 100 µl OptiMEM, added to triplicate wells of cells, and incubated at 37°C for 4 h, at which time 0.5 ml of OF with 100 units/ml penicillin and 100 µg/ml streptomycin was added. After 40 h, the cells were trypsinized and passed, as described above, into 25-cm² flasks (designated pass 1) in OF. After 3 serial passes at ~1:10, cultures originally exposed to both concentrations of CWD microsomes were cloned using dilution. Wells containing one or two colonies of cells were expanded, serially passed at ~1:10, and analyzed for PrP^{CWD} by immunoblot assay at the eighth pass, resulting in a screen of 51 colonies. The single PrP^{CWD}-positive clone (MDB^{CWD}) originated from a well with a single colony. Additional rounds of dilution subcloning increased the probability of clonality and generated the subclone MDB^{CWD2} (see Results).

DNA sequencing and determination of amino acid sequences. The sequences of the PrP genes from samples of mule deer brain and brain-derived cell lines were determined from PCR-amplified DNA of open reading frames from genomic DNA as described using primer set 1 (33). DNA sequencing was done using an ABI 3700 DNA sequencer and the same primer set.

Antibody generation. Mouse monoclonal antibody 12B2 was produced from PrP-knockout mice, generously provided by Charles Weissmann (6), by immunization with a synthetic peptide corresponding to ovine PrP amino acid residues 89 to 107 that was conjugated to Keyhole limpet hemocyanin as described previously (40). The R521 polyclonal antibody was raised against an ovine PrP peptide, residues 94 to 105 (40), the sequence of which is conserved in cervid PrP (33).

To detect the linear epitope specificities of 12B2, Pepsan analysis of solid-phase synthetic peptides bound as described previously was performed in an enzyme-linked immunosorbent assay-like system as previously described (38). A set of overlapping 15-mer peptides covering the complete amino acid sequence of ovine PrP were synthesized (GenBank accession number AJ000739). The epitope of 12B2 was found to require at least the residues ⁹³WGQGG⁹⁷, which are conserved in the mule deer and Syrian hamster PrP molecules analyzed in these studies.

Immunoblot assays for PrP-sen and PrP^{CWD} in cell cultures. For detection of PrP-sen, cells in a nearly confluent 25-cm² flask were lysed with 1 ml 0.5% (wt/vol) Triton X-100, 0.5% (wt/vol) sodium deoxycholate, 50 mM Tris-HCl, pH 8.0 at 4°C, 5 mM EDTA, and 150 mM NaCl (LB) and centrifuged at 5,000 rpm in a microcentrifuge for 5 min to remove nuclei. Lysate supernatant proteins were methanol precipitated and solubilized in a detergent-phospholipid solution by sonication (3, 8). PrP was immunoprecipitated from the solution by incubation with 2 µl of R521 at 4°C overnight, precipitation of antibody-PrP complexes with 25 µl of a 50% vol/vol slurry of protein A–Sepharose CL-4B beads (Amersham-Pharmacia) in LB for 1 to 2 h at 4°C, and elution of the PrP from the beads by boiling for 5 min in 15 µl of 2× loading buffer containing 25 mM dithiothreitol (8). Samples were separated on 10% Bis-Tris NuPAGE sodium dodecyl sulfate (SDS) gels (Invitrogen), electroblotted onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore), and immunostained with 12B2 at 0.34 µg/ml. The secondary antibody was an alkaline phosphatase-conjugated anti-mouse immunoglobulin G (Zymed) diluted 1:5,000. The immunoblot was developed with AttoPhos solution (Promega), air dried, and scanned on a STORM fluorescent detection system (Amersham). Relative band intensities were quantitated using ImageQuant software (Amersham).

For detection of PrP^{CWD}, lysates were prepared as described above for the

detection of PrP-sen and frozen at -20°C . After thawing rapidly at 37°C , 0.5-ml aliquots were treated with 10 $\mu\text{g}/\text{ml}$ Proteinase K (PK; Calbiochem) at 37°C for 30 min, and then 2.5 μl of 0.1 M Pefabloc (Roche Diagnostics) was added to stop PK activity. After 5 min on ice, the samples were ultracentrifuged in a Beckman TL120.1 at 90,000 rpm ($350,000 \times g$) for 60 min at 4°C . After thorough removal of the supernatants, pellets were air-dried for 5 min, and then 10 μl of SDS-polyacrylamide gel electrophoresis (PAGE)-dithiothreitol loading buffer was added, followed by sonication in a cup horn for 2 min at maximum power and then boiling for 5 min to solubilize.

Immunofluorescence of MDB^{CWD2} cell line. MDB^{CWD2} cells were seeded at 1:10 in 35-mm glass-bottom culture dishes (MatTek Corp.) and grown to ~ 50 to 60% confluence. All of the following steps were done at room temperature. Cells were fixed with 4% paraformaldehyde in PBS for 10 min and washed twice with PBS. Then the cells were permeabilized with 0.01% saponin in PBS for 5 min. To block nonspecific antibody binding, cells were incubated with PBS containing 10% normal goat serum and 0.01% saponin for 10 min. The following antibodies were diluted in blocking solution and added to separate dishes of cells: rabbit polyclonal antibody against human fibronectin (1:200; Dakocytomation), rabbit polyclonal anti-bovine glial fibrillary acidic protein (GFAP, 1:2,000; Dakocytomation), chicken polyclonal anti-bovine microtubule-associated protein 2 (MAP2, 1:5,000; EnCor Biotechnology). After 1 h of incubation, the cells were washed three times with PBS containing 0.01% saponin and incubated with an appropriate secondary antibody conjugated with Alexa Fluor 488 (1:1,000) for 1 h. Cells were washed three times with PBS containing 0.01% saponin and observed by confocal microscopy.

To immunostain PrP-res with 12B2, the same procedure was performed, except that the cells were incubated with 3 M guanidinium thiocyanate (GdnSCN) for 5 min between the permeabilization and blocking steps and the antibody was used at 5 $\mu\text{g}/\text{ml}$.

Assay of compounds for inhibition of PrP^{CWD}. Sodium PPS (Sigma) and In-TSP (Mid-Century, Inc.) were tested for their ability to block PrP^{CWD} accumulation in MDB^{CWD2} cells. Cells were passed 1:10 in replicate 25-cm² flasks with OF growth medium containing various concentrations of the test compounds. When nearly confluent, half of the cell lysate from each flask was assayed for PrP^{CWD} by immunoblotting using the 12B2 antibody. Total proteins in inhibitor-treated and untreated cell lysates (without PK digestion) were compared by SDS-PAGE on 10% Bis-Tris NuPAGE SDS gels stained with GelCode blue (Pierce).

RESULTS

Transformed mule deer brain cells. Primary brain cell cultures were derived from a wild, CWD-negative mule deer homozygous for the PrP genotype encoding residues G⁹⁶M¹³²S¹³⁸S²²⁵Q²²⁶. The cells were transformed with a plasmid carrying the simian virus 40 genome. Clusters of cells exhibiting phenotypes of transformation, e.g., rapid cell division and a lack of contact inhibition, were selected and combined to give a transformed brain-derived cell line (MDB for *mule deer brain*). Because PrP-sen expression is required for susceptibility to TSE infection, we analyzed the MDB cell cultures for the presence of PrP-sen. Immunoblot analysis of MDB cell lysates using monoclonal antibody 12B2 showed PrP-immunoreactive molecules migrating between 25- and 37-kDa markers that are typical of the multiple PrP-sen glycoforms (Fig. 1). As expected, these bands were fully sensitive to digestion with PK, and when not PK treated, migrated with a higher apparent molecular mass than bands from PK-treated PrP^{CWD} isolated from CWD-infected mule deer (MD CWD) brain tissue (33).

CWD infection of immortalized brain cell culture. To obtain a source of CWD infectivity that was potentially more concentrated and less cytotoxic than crude brain homogenates, microsomes were prepared using tissue isolated from a section of the medulla oblongata (at the level of the obex) of an experimentally infected mule deer with clinical symptoms of CWD. Like the donor of the uninfected brain cells, this deer was

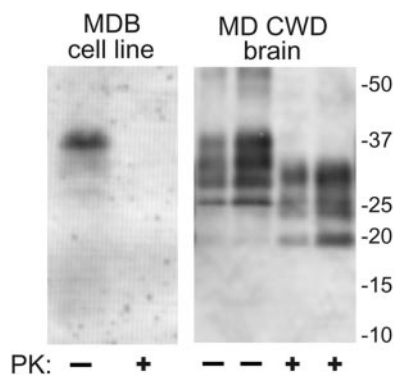


FIG. 1. Expression of PrP-sen by the transformed cell line derived from mule deer brain (MDB). The image is an immunoblot of PK-treated (+) and untreated (-) cell extracts using antibody 12B2 to detect PrP. PK-treated and untreated PrP^{CWD} purified (34) from CWD-affected MD brain (20- and 60-ng samples of each) is shown for comparison. The migration of molecular mass standards in kilodaltons is shown on the right.

homozygous for the PrP genotype encoding amino acid residues G⁹⁶M¹³²S¹³⁸S²²⁵Q²²⁶. This deer was confirmed to be CWD positive by immunohistochemical staining of the brain tissue (26; data not shown) and immunoblotting of the microsome preparation for the detection of PrP^{CWD} (Fig. 2). Treatment of the CWD microsomes with PK resulted in partial truncation of the PrP^{CWD} molecules, which is typical of PrP^{CWD} from animals infected with CWD (33) and other TSEs. The amount of PrP^{CWD} in the microsome preparation

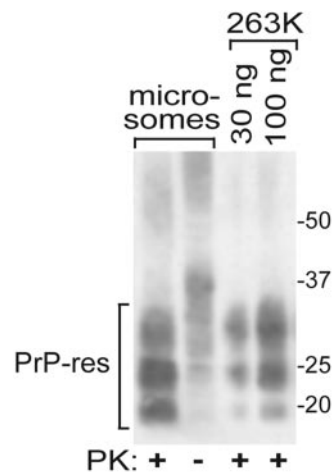


FIG. 2. PrP^{CWD} in microsomes isolated from the CWD-affected MD brain used to infect the MDB cell line. Aliquots of the microsome preparations were incubated with PK (+) or without PK (-) and analyzed on an immunoblot using antibody 12B2 to detect PrP molecules. In this blot, the upper glycoform bands in the PK-treated microsome and 263K lanes were underrepresented relative to the other bands compared to similar samples used in experiments for which results are shown in other figures; this is likely artifactual due to differences in sample protein content or electroblotting conditions. The designated amounts of purified hamster (263K) PrP-res (34) were used to estimate the amounts of PrP^{CWD} in the microsome preparations. The migration of molecular mass standards in kilodaltons is shown on the right.

was estimated by comparison to 263K hamster scrapie PrP-res standards.

The transformed mule deer brain cell culture was exposed to CWD brain microsomes containing approximately 25, 75, 250, 750, or 2,500 ng of PrP^{CWD} and a buffer-only negative control and then passed serially. Immunoblot analyses of cells from each of these CWD-treated bulk cultures at the first pass did not reveal any detectable PrP^{CWD} (data not shown). Considering that only a small subset of cells may have become infected, cells exposed to microsome preparations containing 750 or 2,500 ng PrP^{CWD} were cloned by dilution to isolate and expand possible infected cells. Out of 51 clones analyzed, only one, designated MDB^{CWD}, produced detectable PrP^{CWD} after expansion from a single colony and seven serial passages (Fig. 3). Figure 3A shows a subset of eight of these primary clones, one of which was PrP^{CWD} positive. The positive MDB^{CWD} clone was isolated from the cell culture exposed to microsomes containing 2,500 ng of PrP^{CWD}. As expected, in the MDB^{CWD} clone, the characteristic PrP glycoforms were reduced in size when treated with PK due to the truncation from the amino termini of the PrP molecules (Fig. 3B). The PK-treated PrP glycoform pattern from the MDB^{CWD} cells was clearly distinct from those of MD CWD brain and scrapie-infected N2a cells (RML), with higher molecular masses, most notably for the upper diglycosylated form. These bands were also recognized on immunoblots by R505, a distinct polyclonal anti-PrP antiserum (33; data not shown). Since glycan biosynthesis can vary significantly between cell types, the higher molecular masses of the upper glycosylated bands in both the PK-treated and untreated MDB^{CWD} samples likely reflects differences in the size and nature of the glycans added to PrP molecules in these cells relative to MD brain tissue and N2a cells. The unique PrP^{CWD} glycoform pattern provides evidence that MDB^{CWD} cells were not derived from inadvertent contamination of MDB cultures with scrapie-infected N2a cells or any other scrapie-infected cell line in our facility. Furthermore, genotyping of the MDB^{CWD} cell line confirmed that like their mule deer source, their PrP genes encode the G⁹⁶M¹³²S¹³⁸S²²⁵Q²²⁶ cervid PrP sequence. MDB^{CWD} cells from multiple passes between 7 and 32 (the latest pass tested as of this writing) were clearly positive for PrP^{CWD}, demonstrating persistent CWD infection in this cell line.

Clonal analysis of the MDB^{CWD} cell line. To increase the likelihood that the MDB^{CWD} cell line was clonal, a second round of dilution subcloning was done at the ninth pass after the first round. Immunoblot analyses revealed that 20 of 30 of these MDB^{CWD} subclones were positive for PrP^{CWD} and that the amount of PrP-res produced in the PrP^{CWD}-positive subclones was variable (Fig. 4). One of the PrP^{CWD}-positive subclones, designated MDB^{CWD2}, was selected for cell lineage and inhibitor studies. In addition, a third round of dilution subcloning was done to a PrP^{CWD}-positive subclone at the fifth pass after the second round. From this subcloning, 11 viable clones were obtained, 8 of which were PrP^{CWD} positive (data not shown). The PrP^{CWD} signal among positive subclones of the third round was less variable compared to second-round subclones. These results provided evidence that PrP^{CWD} levels varied between individual cells in apparently clonal MDB^{CWD} cell lines, even though these lines maintained relatively con-

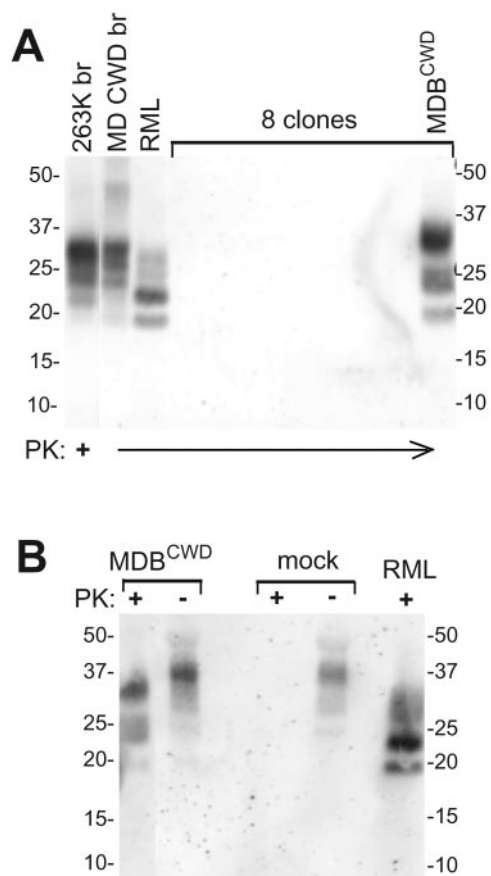


FIG. 3. Immunoblot analysis of mule deer brain cell clones after exposure to CWD microsomes. The MDB-transformed cell line was incubated with microsomes prepared from a CWD-positive mule deer brain. At the third pass, selected lines (see Materials and Methods) were dilution cloned and expanded. At the eighth pass after cloning, cell lysates were analyzed for PrP^{CWD}. (A) PK-treated lysates of a PrP^{CWD}-positive clone (MDB^{CWD}) and 7 representative negative clones. Also shown are PK-treated (+) samples of PrP-res purified from scrapie-infected hamster brain (263K br), CWD mule deer brain (MD CWD br), and a mouse N2a cell line infected with the RML scrapie strain (RML) to compare the glycoform patterns and mass differences among these different TSE strains. (B) Comparison of PK sensitivities of PrP present in lysates of MDB^{CWD} cells versus mock-infected cultures of the transformed MDB cells from which MDB^{CWD} was derived. For comparison, RML cell extracts (PK treated) are shown. The immunoblots shown in panels A and B were developed using the 12B2 antibody to detect PrP. The migration of molecular mass standards in kilodaltons is shown on the sides. -, not PK treated.

sistent PrP^{CWD} production overall through many in vitro passes.

Analysis of PrP^{CWD} by immunofluorescence. The PrP^{CWD} produced by the MDB^{CWD2} cell line was analyzed using immunofluorescence staining (Fig. 5). For in situ staining of PrP^{CWD}, MDB^{CWD2} cells were fixed and treated with GdnSCN; this denaturing treatment was necessary to expose the 12B2 epitope (residues 93 to 97), as has been observed with other conformationally occluded epitopes located in this region of PrP-res molecules (25, 29, 37). About half of the cells had extensive punctate intracellular structures that were immunostained with 12B2 (Fig. 5A). Several observations were

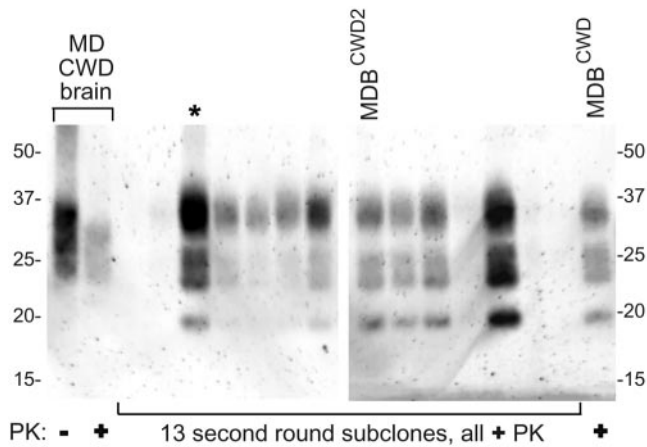


FIG. 4. Immunoblot analysis of MDB^{CWD} subclones from the second-round dilution subcloning. Subclone lysates were PK treated for detection of PrP^{CWD} . The original MDB^{CWD} clone is shown in the last lane on the right. This blot shows 13 representatives of the 30 total second-round subclones that were analyzed. PrP^{CWD} samples in the lanes labeled "MD CWD brain" were purified from a CWD-affected mule deer brain and were PK digested (+) or not (-) (34). The lane labeled " $\text{MDB}^{\text{CWD}2}$ " represents the subclone used in the inhibitor and cell lineage studies. The lane marked with an asterisk contained the subclone used for the third round of dilution subcloning. The left and right panels are from different blots, so it is unclear whether the subtle differences in the relative glycoform band intensities between the clones in these two panels are real or artifactual. The antibody 12B2 was used to detect PrP. The migration of molecular mass standards in kilodaltons is shown on the sides of both panels.

consistent with the punctate staining being due to the presence of PrP^{CWD} . When the GdnSCN treatment was omitted, only a few faintly stained punctate structures were observed (Fig. 5B). No punctate staining was observed in mock-infected transformed mule deer brain cell cultures (Fig. 5C, MDB-MOCK). When the primary antibody was omitted, no staining was observed in the $\text{MDB}^{\text{CWD}2}$ cell line (Fig. 5D). The PrP^{CWD} accumulation pattern observed in $\text{MDB}^{\text{CWD}2}$ cells suggested that PrP-res accumulates in intracellular compartments like those observed in other types of TSE-infected cell lines (25, 37).

Lineage characterization of the $\text{MDB}^{\text{CWD}2}$ cell line. To assess the lineage of the $\text{MDB}^{\text{CWD}2}$ cell line, fixed and permeabilized cells were stained with antibodies to specific cell type marker proteins. Due to the lack of antibodies raised specifically against mule deer cell marker proteins, antibodies against proteins of other species were used. Antibodies raised against human fibronectin showed extensive immunofluorescent staining of extracellular fibrils and, to a lesser extent, intracellular punctate deposits (Fig. 6). These staining data are consistent with a fibroblast-like origin for the MDB^{CWD} ; however, other brain cells such as astrocytes can also express fibronectin (18). However, no staining was seen with antibodies against bovine GFAP, an activated astrocyte marker. In addition, little staining was seen with antibodies against bovine MAP2, a neuronal marker. The reactivity of these human and bovine antibodies to their corresponding mule deer antigens was confirmed with formalin-fixed and paraffin-embedded sections of mule deer brain tissue and similarly prepared $\text{MDB}^{\text{CWD}2}$ cell pellets

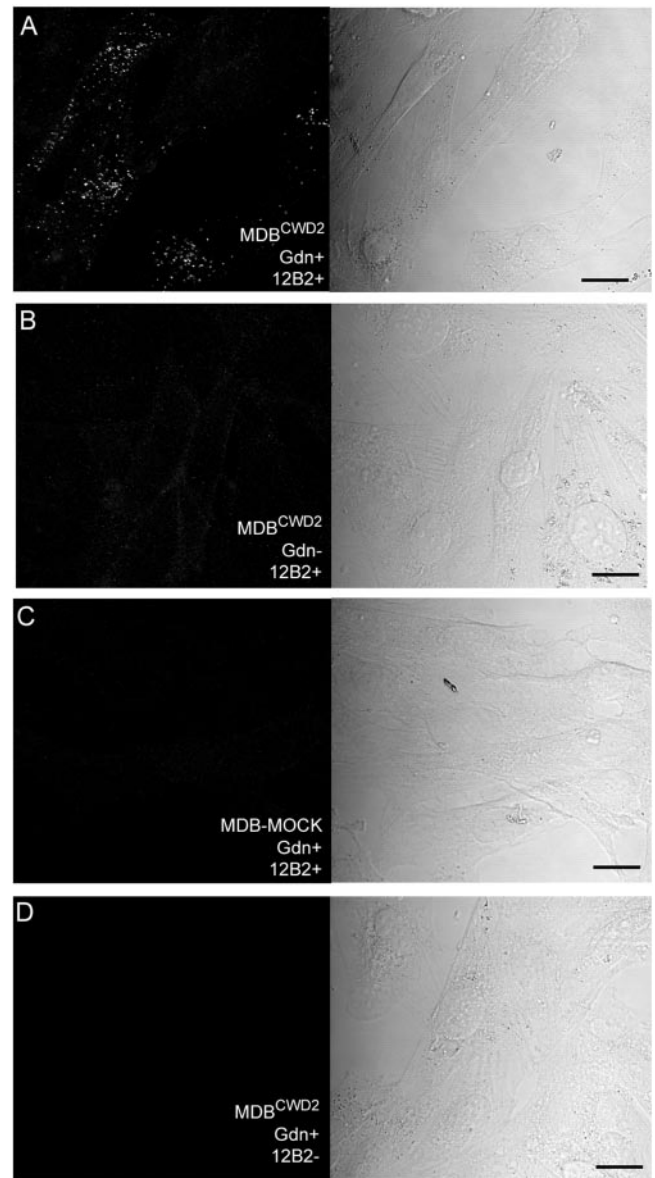


FIG. 5. Immunofluorescence staining of PrP^{CWD} in $\text{MDB}^{\text{CWD}2}$ cells. $\text{MDB}^{\text{CWD}2}$ cells were stained with anti-PrP antibody 12B2 after treatment with GdnSCN (Gdn+, panel A, left). The extensive punctate structures are typical of PrP-res staining. When GdnSCN treatment was omitted (Gdn-, panel B, left), only a few faint punctate structures were observed. Only very faint fluorescence was apparent in mock-infected cells (MDB-MOCK, panel C, left). The $\text{MDB}^{\text{CWD}2}$ cells stained only with secondary antibody (panel D, left) had no apparent fluorescence. All images are reconstructions of Z-series acquired by confocal microscopy with an interval of 0.54 μm . The panels on the right are corresponding differential interference contrast images to show the cell positions. Bar, 20 μm .

(data not shown). Therefore, these data support the conclusion that the $\text{MDB}^{\text{CWD}2}$ is a fibroblast-like cell line.

Inhibition of PrP^{CWD} accumulation in $\text{MDB}^{\text{CWD}2}$ cells. To investigate the utility of $\text{MDB}^{\text{CWD}2}$ cells for screening anti-CWD compounds, we tested the ability of two inhibitors of rodent PrP-res accumulation, sodium PPS (9) and In-TSP (W. S. Caughey, E. Olsen, D. A. Kocisko, B. Caughey, unpub-

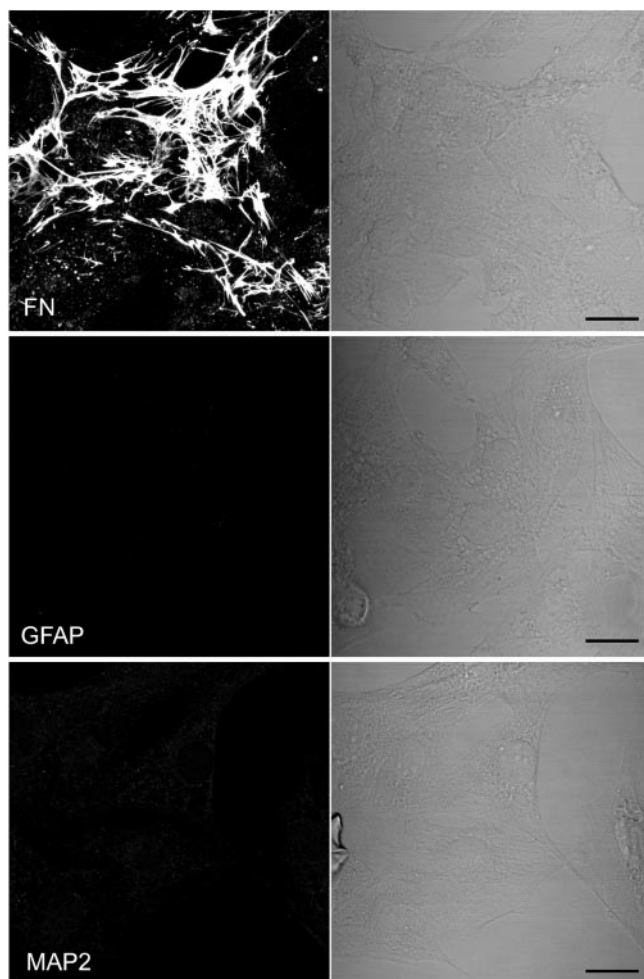


FIG. 6. Immunostaining of $\text{MDB}^{\text{CWD}2}$ cells. $\text{MDB}^{\text{CWD}2}$ cells were stained with antibodies against human fibronectin (FN), GFAP, and MAP2, which are markers for fibroblast cells, astrocytes, and neuronal cells, respectively. Reconstructed confocal microscopic images of Z-series acquired with an interval of $0.54 \mu\text{m}$ are shown in the left panel, and differential interference contrast images are shown in the right panel. Bar, $20 \mu\text{m}$.

lished data) to block PrP^{CWD} accumulation in these CWD-infected cells. PPS and In-TSP blocked PrP^{CWD} accumulation by $>90\%$ with IC_{50} s (concentrations giving half-maximal inhibition) of $\sim 10 \text{ ng/ml}$ ($\sim 3 \text{ nM}$, based on an average, but heterogeneous, molecular mass of $\sim 3,800 \text{ Da}$) and $<0.3 \mu\text{M}$, respectively (Fig. 7A and B). This IC_{50} for PPS is similar to the low nanomolar IC_{50} observed with RML-infected N2a cells (9). At effective inhibitory concentrations, these inhibitors appeared to be largely selective for PrP^{CWD} formation because they did not substantially alter the overall profile of cellular proteins (Fig. 7C) or PrP-sen levels detected by immunoblotting (Fig. 7D). When either $1 \mu\text{g/ml}$ PPS or $1 \mu\text{M}$ In-TSP was added directly to untreated $\text{MDB}^{\text{CWD}2}$ lysates immediately before the PK digestion step, the PrP-res detected was similar to that seen from untreated control lysates (data not shown); thus, these compounds did not artifactually alter the recovery or detection of PrP^{CWD} from cell lysates. No evidence of cytotoxicity as reflected by the rate of cell division and the gross

morphology of cells was seen at $\leq 3 \mu\text{M}$ for In-TSP. PPS started to show minor cytotoxicity at $1 \mu\text{g/ml}$, i.e., ~ 100 -fold higher than the IC_{50} for PrP^{CWD} inhibition. These results showed that PPS and In-TSP can potently block PrP^{CWD} accumulation in MDB^{CWD} cells at concentrations that are far below those required to affect cell growth or PrP-sen biosynthesis.

To test whether the PPS effect on PrP^{CWD} was reversible and also to attempt to cure the MDB^{CWD} cell line of the CWD infection, duplicate lines of $\text{MDB}^{\text{CWD}2}$ cells were serially passed 1:10 five times in the presence of $0.3 \mu\text{g/ml}$ PPS and then subsequently passed without PPS (Fig. 8). After the first pass in PPS, the immunoblot-detectable PrP^{CWD} was decreased to $<10\%$ of untreated duplicate parallel flasks, and after three passes, PrP^{CWD} was no longer detectable. The PPS-treated lines were tested for PrP^{CWD} at the first and ninth passes after removal of the PPS, and no signal was detected. Parallel untreated flasks of $\text{MDB}^{\text{CWD}2}$ cells showed no observable loss of PrP^{CWD} content throughout the series of passages.

DISCUSSION

Expansion of the known geographic distribution of CWD, whether due to the spread of the disease or increased surveillance, makes it important to develop screens for compounds that might prevent CWD spread among cervid populations and, potentially, the transmission from cervids to other species. As exemplified by the results of experiments with PPS and In-TSP shown in Fig. 7, the MDB^{CWD} cell line should be useful in the search for anti-CWD compounds. When administered prophylactically, pentosan polysulfate and certain porphyrins have been especially effective against intraperitoneal infections of rodent-adapted scrapie (30, 31). Those previous results and our observations that pentosan polysulfate and In-TSP are effective blockers of PrP^{CWD} accumulation in the MDB^{CWD} cell line provide evidence that these or related compounds might have activity against CWD in vivo. Thus, it is tempting to speculate that PPS or In-TSP may help prevent the spread of CWD on game farms and in the wild, where most transmissions would be expected to occur via peripheral routes of infection. In addition, our findings attest to the broad inhibitory activities of both sulfated glycans and porphyrins, which differ from some other inhibitors that have strain and/or species specificities (10, 22, 23).

The CWD infection in MDB^{CWD} cells appears to be persistent because PrP^{CWD} production has been stable and robust through 32 serial passes despite the fact that, in the first dilution cloning, 33% of the subclones were apparently negative for PrP^{CWD} . The reason for the generation of PrP^{CWD} -negative subclones from the original MDB^{CWD} culture is unclear, although similar observations of cell-to-cell differences in levels of PrP-res formation have been noted in other cell lines (21, 28, 41). It is possible that the cell line obtained in the first dilution cloning was not derived from a single-cell clone. This potential for lack of clonality should have been reduced in subsequent dilution cloning steps. Even if the initial MDB^{CWD} line was in fact clonal, it is possible that a certain percentage of daughter cells became less able to maintain the infection and produce PrP^{CWD} , as was apparent in the variable PrP^{CWD} band intensities from the secondary clones (Fig. 4). This might

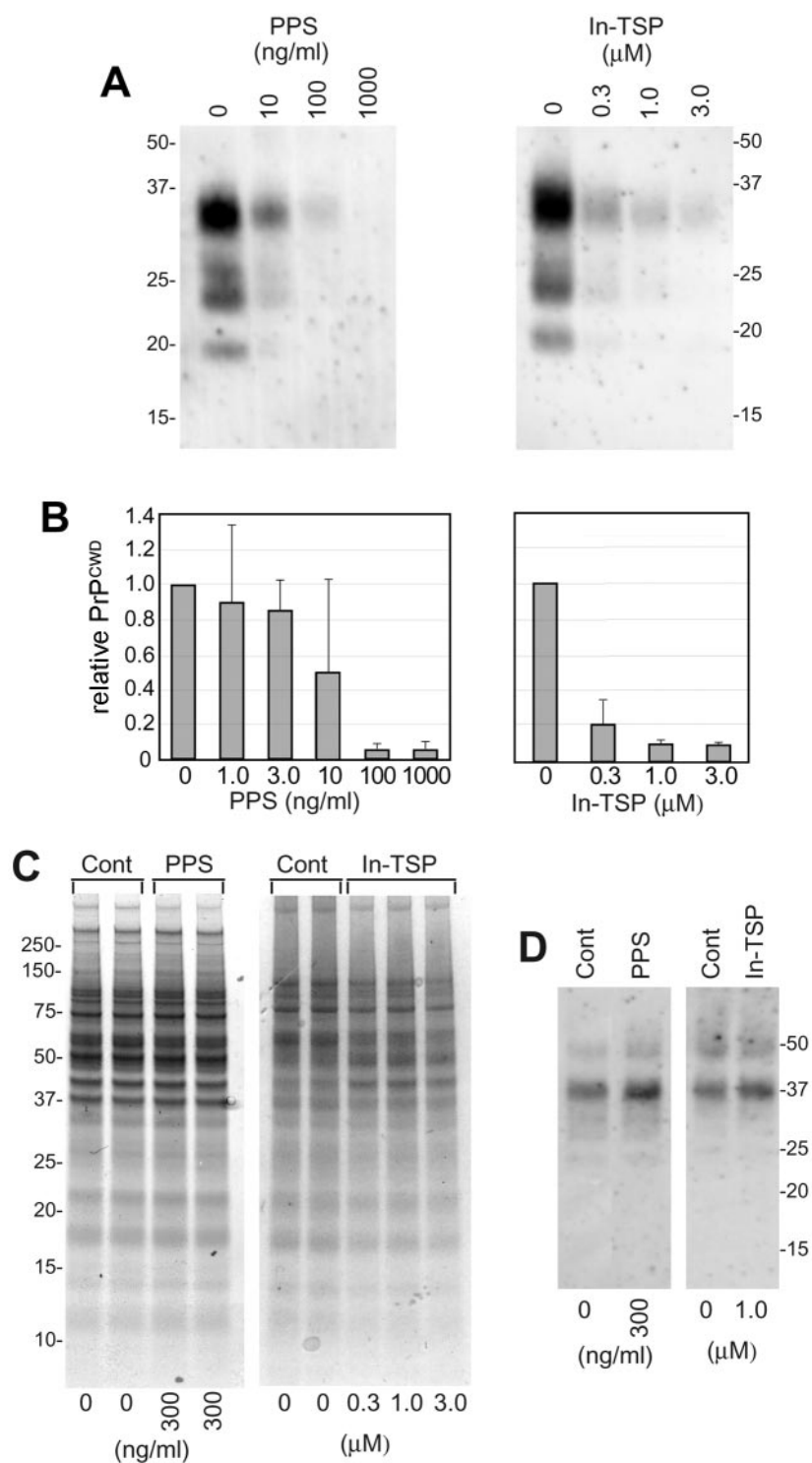


FIG. 7. Effects of PPS and In-TSP on PrP^{CWD} biosynthesis in MDB^{CWD2} cells. Upon plating at 1:10 dilution, cells were treated with the designated amounts of the compounds and grown until near confluence (~4 days). (A) Cell lysates were analyzed for PrP^{CWD} by immunoblotting using antibody 12B2. The migration of molecular mass standards in kilodaltons is shown on the sides. (B) Mean values and standard deviations of relative PrP^{CWD} band intensities (as proportions of untreated controls) from multiple experiments, like those shown in panel A. Three to six replicates of each concentration of inhibitor were tested. (C) GelCode blue-stained SDS-PAGE gels of equivalent aliquots of lysates (prior to PK treatment) from PPS-treated, In-TSP-treated, or untreated (Cont) cells. (D) Immunoblot of PrP immunoprecipitated from lysates (without PK treatment) of control (Cont), PPS-treated, and In-TSP-treated cells.

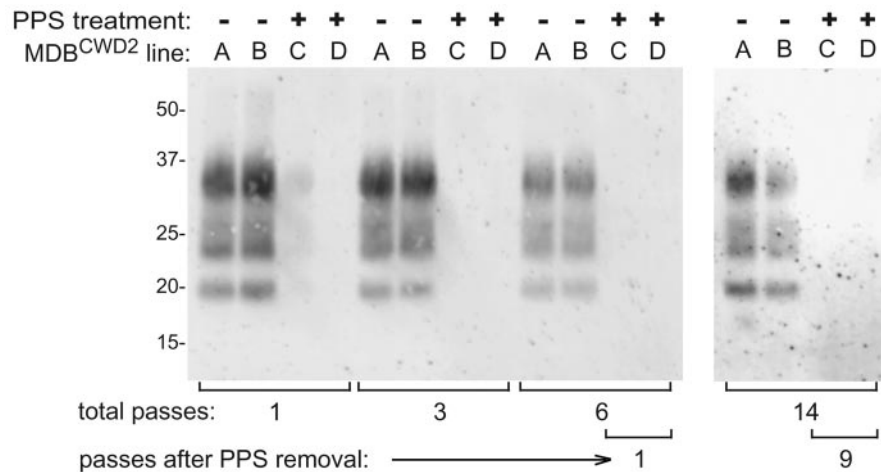


FIG. 8. Lack of recovery of PrP^{CWD} after PPS treatment of MDB^{CWD2} cells. Duplicate lines of MDB^{CWD2} cells were passed at ~1:10 dilution in medium for the designated number of passes (A, B) or in medium containing 0.3 µg/ml PPS for 5 passes and unsupplemented medium thereafter (C, D). One-half nearly confluent T-25 flask equivalents of PK-treated cell lysates were analyzed for PrP^{CWD} content by immunoblotting as described in Materials and Methods. +, PPS treated; -, not PPS treated.

be due to genetic instability (a common feature in transformed cell lines), to unequal distribution of PrP^{CWD} between daughter cells after division, to destabilizing effects of the dilution cloning itself (in which cells are forced to survive and proliferate at extremely low densities), or perhaps, to toxicity and death if cells accumulate too much PrP-res.

The probable fibroblast-like origin of MDB^{CWD} cells is not surprising because fibroblast-like cells have been shown to be capable of maintaining chronic scrapie infections (12, 14, 41). In brain-derived cultures, fibroblast-like cells are often derived from the meninges. This is interesting to consider in the context of iatrogenic transmissions of CJD that have occurred via dura mater transplants. Dura mater contains fibroblasts, and in this study, we have shown that fibroblast-like cells derived from brain tissue are susceptible to infection by CWD. If these cells are susceptible to TSE infection *in vivo*, they could represent a direct and integral source of CJD contamination of dura mater taken from CJD-infected humans.

The MDB^{CWD} cell line is the first to be persistently infected with CWD. The G⁹⁶M¹³²S¹³⁸S²²⁵Q²²⁶ PrP genotype of the mule deer donors of both the MDB cell line and the CWD infectivity is by far the most common in both mule deer and white-tailed deer (~95% in wild populations analyzed) (4, 20). Moreover, this allelic type is probably the most susceptible to natural CWD infection (M. Miller, unpublished observations). Thus, MDB^{CWD} cells appear to be an apt experimental model of CWD infection in *Odocoileus* spp. and should facilitate *in vitro* experimentation into the cell biology, molecular biology, biochemistry, and strain- and species-dependent characteristics of this TSE disease.

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Mefloquine, an Antimalaria Drug with Antiprion Activity In Vitro, Lacks Activity In Vivo

David A. Kocisko* and Byron Caughey

National Institute of Allergy & Infectious Diseases, National Institutes of Health, Hamilton, Montana 59840

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In view of the effectiveness of antimalaria drugs inhibiting abnormal protease-resistant prion protein (PrP-res) formation in scrapie agent-infected cells, we tested other antimalarial compounds for similar activity. Mefloquine (MF), a quinoline antimalaria drug, was the most active compound tested against RML and 22L mouse scrapie agent-infected cells, with 50% inhibitory concentrations of ~0.5 and ~1.2 μ M, respectively. However, MF administered to mice did not delay the onset of intraperitoneally inoculated scrapie agent, the result previously observed with quinacrine. While most anti-scrapie agent compounds inhibit PrP-res formation in vitro, many PrP-res inhibitors have no activity in vivo. This underscores the importance of testing promising candidates in vivo.

The transmissible spongiform encephalopathies (TSEs) or prion diseases show a common and unique posttranslational conversion of normal, host-encoded, protease-sensitive prion protein (PrP^{sen} or PrP^C) to an abnormal disease-associated isoform (PrP^{res} or PrP^{Sc}). The latter is an aggregation-prone and detergent-insoluble polymer resistant to proteolysis (5). Human TSEs include Gerstmann-Straussler-Scheinker disease, fatal familial insomnia, Creutzfeldt-Jakob disease (CJD), and kuru. The epidemic nature of prion diseases in domestic and wild animals could constitute serious health problems. Scrapie is a TSE of sheep which has been experimentally adapted to rodents, and bovine spongiform encephalopathy (BSE) is prominent in Europe and has also occurred in other continents, including North America. The appearance of a new form of CJD, presumably due to consumption of BSE-contaminated beef, created a troubling new scenario in the transmission of fatal prion diseases. As there is no deployable therapeutic TSE intervention immediately available, it is important to continue to pursue TSE drug development (reviewed in references 4, 12, and 17).

Compounds including polyene antibiotics, such as amphotericin B (18, 23); cyclic tetrapyrroles, such as porphyrins (7, 24); and polyanions, such as pentosan polysulfate (6, 9), inhibit PrP-res formation in infected cells and have also demonstrated antiscrapie activity in vivo. Many antimalarial compounds and related acridine and quinoline analogs have been shown to be effective inhibitors of PrP-res formation in infected mouse neuroblastoma (N2a) cells (11, 16, 19, 20). Thus, we were particularly interested in testing other antimalarial compounds, as many are FDA-approved drugs and some also cross the blood-brain barrier (BBB). Here we demonstrate mefloquine (MF) as an effective inhibitor of PrP-res in N2a cells infected with RML and 22L mouse strains of scrapie agent. We also tested MF, the most potent inhibitor found, against intraperitoneal (i.p.) scrapie infection in mice as a further evaluation of its potential as an anti-TSE drug.

Antimalarial compounds were tested for the ability to inhibit PrP-res formation in infected cells as described previously (14). MF was supplied by Roche, and other compounds tested were included in the Spectrum Collection from Microsource Discovery (Groton, CT). As shown in Table 1 with new and published data, many antimalarial molecules can inhibit RML PrP-res accumulation in N2a cells. The ability is especially pronounced for quinoline, 4-aminoquinoline, 8-aminoquinoline, and acridine analogs. Many more quinoline and acridine compounds have been reported as inhibitors than are listed here (16, 19, 20). MF was the most effective new inhibitor, so it was also tested against 22L-infected N2a cells. MF also inhibited 22L PrP-res, with a 50% inhibitory concentration (IC₅₀) of 1.2 μ M. Interestingly, antimalarial compounds not of the above-mentioned classes demonstrated no activity at concentrations lower than those toxic to the cells. Doxycycline, which has been reported to render preexisting PrP-res sensitive

TABLE 1. Inhibition of PrP-res in infected cells by various antimalarial compounds

Antimalarial compound	IC ₅₀ vs RML PrP-res (μ M) (reference) ^a	Compound class
Mefloquine	0.5	Quinoline
Quinine	6 (20)	Quinoline
Hydroquinine	12.5 (20)	Quinoline
Quinidine	3 (20)	Quinoline
Hydroquinidine	NR, toxic at 2.5 (20)	Quinoline
Cinchonine	6 (20)	Quinoline
Cinchonidine	18 (20)	Quinoline
Chloroquine	2.3 (11)	4-Aminoquinoline
Amodiaquine	0.5 (14)	4-Aminoquinoline
Hydroxychloroquine	1–10 (14)	4-Aminoquinoline
Primaquine	<10	8-Aminoquinoline
Quinacrine	0.4 (11)	Acridine
Doxycycline	NR, toxic at 5 ^b	Other
Pyrimethamine	NR, toxic at 10	Other
Atovaquone	NR, toxic at 10	Other
Artemisinin	NR, toxic at 10	Other
Dihydroartemisinin	NR, toxic at 1	Other

* Corresponding author. Mailing address: Rocky Mountain Laboratories, 903 S. 4th Street, Hamilton, MT 59840. Phone: (406) 375-9692. Fax: (406) 363-9286. E-mail: dkocisko@niaid.nih.gov.

^a NR, IC₅₀ not reached.

^b Against 22L-infected N2a cells.

TABLE 2. Test of MF for scrapie prophylaxis in Tg7 mice inoculated on day 0 with 50 μ l of 1% 263K brain homogenate^a

Treatment	Dosing regimen	Survival times (days)	Mean survival time \pm SD
None		73, 76, 77, 79, 79, 88, 88, 89	81.1 \pm 6.3
5 mg/kg MF ^b	Days -2, -1, and 0 and then 3/wk for 4 wk	74, 75, 80, 81, 88, 88, 89, 92	83.4 \pm 6.8

^a The Rocky Mountain Laboratories Animal Care and Use Committee approved this procedure.

^b In 10% dimethyl sulfoxide-phosphate-buffered saline; a single i.p. dose at 50 mg/kg was not tolerated.

to proteolysis at concentrations approaching 1 mM (13), had no PrP-res inhibitory activity at concentrations lower than that toxic to cells. These results emphasize that not all antimalarial compounds inhibit PrP-res accumulation and suggest additionally that the presence of a quinoline or acridine ring system is advantageous.

Because MF is an FDA-approved antimalaria drug that potently inhibits PrP-res formation in cells and crosses the BBB, it was an excellent TSE therapeutic candidate. MF was tested for scrapie prophylaxis in transgenic mice (Tg7) (25) that are very susceptible to hamster 263K scrapie agent. Mice were first given a loading dose of MF consisting of three daily i.p. injections of 5 mg of MF per kg of body weight. Immediately after the third MF dose, the mice were inoculated i.p. with 50 μ l of 1% 263K-infected brain homogenate (~1,000 50% infective doses). Based on pharmacokinetic studies of MF in mice (1), blood and brain levels should exceed 22L- or RML-PrP-res IC₅₀ values. Inoculation was on a Friday, and 5-mg/kg i.p. MF dosing continued on Mondays, Wednesdays, and Fridays for the next 4 weeks. As shown in Table 2, MF was not able to delay the onset of scrapie in mice. A similar prophylaxis test with different cyclic tetrapyrroles has shown a significant delay in scrapie onset (24), but amodiaquine in this type of test was also ineffective (15). It remains possible that prophylactic effects of MF or amodiaquine could be seen in different in vivo models having greater lymphoreticular involvement than 263K scrapie agent; however, effects on established central nervous system infections will be required to treat most CJD patients. Since treating such advanced TSE disease is likely to be even more challenging than prophylaxis, MF and amodiaquine were not considered further as potential therapeutic agents.

Quinacrine, another FDA-approved antimalaria drug that inhibits mouse PrP-res formation in cells about as potently as MF (11) and crosses the BBB, also was an excellent TSE therapeutic candidate (16). However, no antiscrapie activity has been observed in mice tested for prophylaxis by quinacrine oral gavage (8) and i.p. injections (2) and no therapeutic effects have been observed against existing mouse brain infections by infusion pumping of quinacrine into the brain (10). Additionally, quinacrine has been dosed experimentally to a limited number of human TSE patients, with no benefit to some and limited transient benefit to others (3, 21, 22). Liver dysfunction was also a common side effect of the quinacrine treatment. Surprisingly, it is now being considered for expanded clinical trials in the United Kingdom and United States.

Screening compounds for PrP-res inhibitory activity in infected cell cultures has successfully found classes of compounds with in vivo antiscrapie activity, such as the cyclic tetrapyrroles and sulfonated dyes. Antimalarials have been tested as TSE therapeutic candidates because of such screening. Most compounds with in vivo antiscrapie activity also

inhibit PrP-res formation in cells, regardless of how they were initially discovered. For instance, pentosan polysulfate demonstrated antiscrapie activity before it was found to inhibit PrP-res formation in cell culture (6, 9). Although in vitro tests are useful as initial compound screens, they cannot substitute for in vivo tests against actual TSE disease. Also, specific in vitro assays cannot be expected to test for all possible therapeutic mechanisms or provide information on optimum dosages for in vivo use. A compound that does not inhibit PrP-res in cells might have activity in vivo through a mechanism that does not involve the inhibition of PrP-res accumulation. In light of the fact that much is still unknown concerning the mechanisms of infection and disease processes of the TSEs, it would be prudent to demonstrate anti-TSE activity in vivo before a therapeutic candidate is advanced to clinical use.

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A Porphyrin Increases Survival Time of Mice after Intracerebral Prion Infection

David A. Kocisko,^{1*} Winslow S. Caughey,¹ Richard E. Race,¹ Grant Roper,²
Byron Caughey,¹ and John D. Morrey²

Laboratory of Persistent Viral Diseases, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, Montana,¹ and Institute for Antiviral Research, Animal, Dairy, and Veterinary Sciences Department, Utah State University, Logan, Utah²

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Prion diseases, including scrapie, are incurable neurodegenerative disorders. Some compounds can delay disease after a peripheral scrapie inoculation, but few are effective against advanced disease. Here, we tested multiple related porphyrins, but only Fe(III)meso-tetra(4-sulfonatophenyl)porphine injected into mouse brains after intracerebral scrapie inoculation substantially increased survival times.

The transmissible spongiform encephalopathies (TSEs or prion diseases) are neurodegenerative diseases that include Creutzfeldt-Jakob disease (CJD) of humans, bovine spongiform encephalopathy, chronic wasting disease of deer and elk, and scrapie of sheep. The infectious agent of TSEs is not fully characterized, but there is evidence that an abnormal, protease-resistant form of prion protein is involved (10). Over 160 cases of variant CJD, caused by the consumption of bovine spongiform encephalopathy-infected beef, have increased concern about the impact of TSEs on human health. While TSEs are incurable, various compounds dosed at or near the time of infection have delayed the onset of scrapie in animals after inoculation with high peripheral doses of infectant or even prevented disease after low peripheral doses (reviewed in references 1 and 4). Compounds that have delayed the onset of clinical scrapie after intracerebral (i.c.) inoculation include amphotericin B (7), pentosan polysulfate (PPS) (3), and, to a lesser extent, Congo red (6).

Most compounds active against scrapie, including cyclic tetra-

pyrroles, also inhibit protease-resistant prion protein formation in cell cultures (2), which may explain their *in vivo* activity. A metal-free phthalocyanine and two iron porphyrins, types of cyclic tetrapyrroles, have been shown to delay scrapie onset after peripheral but not i.c. inoculation (8, 9). In the search for more effective anti-TSE compounds, we evaluated two types of previously untested porphyrins with or without central metals (Fig. 1).

meso-tetra(4-sulfonatophenyl)porphine (TSP), iron(III)TSP (FeTSP), *meso*-tetra(4-*N,N,N*-trimethylanilinium)porphine (TAP), and iron(III)TAP (FeTAP) were tested for the ability to delay scrapie in transgenic mice (Tg7) that are very susceptible to hamster scrapie strain 263K (9, 11). (All animal use was approved by the appropriate institution's animal care and use committee.) All four porphyrins injected intraperitoneally (i.p.) prior to and for 4 or 5 weeks after i.p. scrapie inoculation significantly increased survival times (Table 1). FeTAP was most effective, increasing survival times more than fourfold. In a further test, FeTAP administered i.p. beginning 50 days after

TABLE 1. Porphyrins as prophylactic compounds against 263K scrapie infection

Compound	i.p. dose [mg/kg (mmol/kg)] ^a	Dosing regimen	i.p. scrapie inoculation (day 0) ^b	Survival times (days) ^c	Mean survival time ± SD
None			50 μl 1% BH	78, 82, 91, 91, 92, 92, 92, 100	90.0 ± 6.4
FeTSP	12.5 (0.012)	3 doses/wk for 6 wks starting 2 wks prior to inoculation	50 μl 1% BH	124, 143, 145, 147, 163, 171, 196, 203	161.5 ± 27.3 ^d
TSP	25 (0.025)	Dosing on days -2, -1, and 0, then 3 doses/wk for 5 wks	50 μl 1% BH	119, 122, 122, 126, 129, 136, 141, 161	132.0 ± 13.9 ^d
FeTAP	12.5 (0.012)	3 doses/wk for 6 wks starting 2 wks prior to inoculation	50 μl 1% BH	295, 299, 376, 388, 581, 686	437.5 ± 160.0 ^d
TAP	6.25 (0.006) ^e	3 doses/wk for 6 wks starting 2 wks prior to inoculation	50 μl 1% BH	100, 127, 142, 156, 182, 183, 205, 233	166.0 ± 43.3 ^d

^a In phosphate-buffered saline.

^b BH, 263K-infected brain homogenate in phosphate-buffered saline.

^c Tg7 mice dying from non-scrapie causes were removed from the data set.

^d *P* < 0.0001 versus control group by unpaired *t* test.

^e The dose of 12.5 mg/kg was toxic.

* Corresponding author. Mailing address: Rocky Mountain Laboratories, 903 S. 4th Street, Hamilton, MT 59840. Phone: (406) 375-9692. Fax: (406) 363-9286. E-mail: DKocisko@niaid.nih.gov.

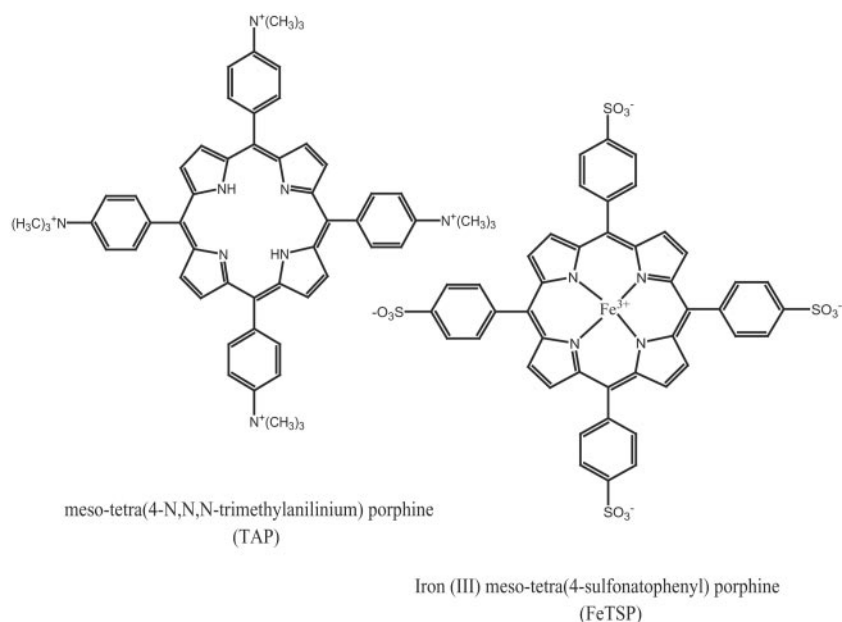


FIG. 1. Structures of the two types of porphyrins tested. Metal-free TAP is shown on the left. The central metal ion of these porphyrins is coordinated with the nitrogen atoms as is shown for FeTSP on the right.

i.p. scrapie challenge and continuing three times per week until near death was ineffective (average survival time \pm standard deviation of 85.0 ± 13.2 days versus 83.1 ± 7.5 days for the control). This is not surprising as TAP and TSP compounds may have little blood-brain barrier (BBB) permeability. Since these four porphyrins demonstrated prophylactic activity after i.p. scrapie inoculation in a test where infectant and compound can interact without crossing the BBB, they were further tested against scrapie via i.c. injections to bypass the BBB.

In one type of antisrapie assay, the test compound and infected brain homogenate are mixed prior to i.c. inoculation. Some compounds in such tests have produced increased survival times, presumably due to either direct inactivation of the infectant or the presence of the compound in the brain at the

time of infection (5). As FeTAP was the most effective prophylactic compound, FeTAP and other metal TAPs were tested in this manner. The toxicity of i.c.-administered TAP compounds varied greatly, and $50 \mu\text{l}$ of 0.5 mM TAP, ZnTAP, CrTAP, InTAP, or CdTAP was not tolerated (data not shown). The results from FeTAP and other tolerated TSP and TAP compounds are shown in Table 2. A dilution series of untreated infected brain homogenate was also included to allow estimation of the apparent reduction in scrapie titer. NiTAP and FeTAP, the most active compounds in this "inactivation" test, produced survival times that correlated with a reduction of between 3 and 4 logs of infectivity. When the metal was changed to Cu(II), the activity was greatly reduced, indicating the importance of the metal ion.

While this inactivation test can help rank compounds' abilities to slow the effects of scrapie inocula, it does not measure activity against late-stage TSE infection. To test therapeutic potential, a number of the more effective TAP and TSP scrapie inactivation compounds were dosed once a week for 5 weeks starting ~ 2 weeks after i.c. scrapie inoculation (Table 3). Compounds were injected i.c. to overcome suspected low BBB permeability. PPS, which has antisrapie activity when it is continuously infused into an infected brain (3), was injected directly to the brain as a positive control (Table 3). Other than a small but statistically significant increase in survival time with FeTAP, only FeTSP was effective as a therapeutic treatment, with activity comparable to that of a 10-fold-lower dose of PPS. The reason that FeTAP was the most active prophylactic compound but had little activity as a treatment after i.c. scrapie inoculation is not known. FeTSP was then further tested using six weekly i.c. doses of $50 \mu\text{l}$ of 0.5 , 0.16 , or 0.05 mM FeTSP (25 , 8 , or 2.5 nanomoles/mouse) (Table 3). The average survival time increased between the 8 - and 25 -nanomole doses but changed little between the 8 - and 2.5 -nanomole doses. ZnTSP

TABLE 2. Infectivity of scrapie-infected brain homogenate incubated with TAP or TSP compounds

Inoculum ($50 \mu\text{l}$) ^a	Survival times ^b (days)	Mean survival time \pm SD
1% BH	50, 50, 50, 51, 51, 52, 52, 56	51.5 ± 2.0
0.1% BH	50, 51, 52, 56, 56, 56, 56	53.9 ± 2.7
0.01% BH	56, 56, 58, 58, 61, 61, 62, 62	59.3 ± 2.5
0.001% BH	61, 61, 62, 62, 67, 68, 70	64.4 ± 3.8
0.0001% BH ^c	69, 74, 87, 89, 97, 98	85.7 ± 11.9
0.5 mM CuTAP + 1% BH	52, 52, 53, 56, 56, 56, 56, 62	55.4 ± 3.2
0.5 mM NiTAP + 1% BH	65, 70, 70, 70, 71, 71, 73, 77	70.9 ± 3.4^d
0.5 mM FeTAP + 1% BH	62, 67, 68, 68, 71, 73, 76, 79	70.5 ± 5.4^d
0.5 mM PdTAP + 1% BH	58, 61, 65, 66, 69, 76	65.8 ± 6.3^d
0.5 mM TSP + 1% BH	55, 55, 56, 56, 56, 56, 57, 57	56.0 ± 0.8^d
0.5 mM CuTSP + 1% BH	52, 52, 54, 54, 54, 54, 57, 59	54.5 ± 2.4
0.5 mM FeTSP + 1% BH	56, 56, 57, 58, 58, 60, 63, 65	59.1 ± 3.3^d

^a BH, 263K-infected brain homogenate in phosphate-buffered saline. BH was incubated for 1 hour at 37°C with different metal-substituted TAP or TSP compounds prior to i.c. inoculation into Tg7 mice.

^b Mice dying from nonscrapie causes were removed from the data set.

^c Not done at the same time as that of other controls, but data are typical.

^d $P < 0.0001$ versus 1% BH group by unpaired t test.

TABLE 3. Effect of compounds injected into the brain of Tg7 mice^a

Treatment (50 μ l in PBS)	Days of dose (postinoculation)	Survival times ^b (days)	Mean survival time \pm SD	Control mean survival time \pm SD
0.5 mM FeTAP	14, 16, 18, 21 ^c	45, 51, 51, 54, 54, 60, 60, 60	54.4 \pm 5.4 ^d	46.1 \pm 1.2
0.5 mM FeTAP	14, 21, 28, 35, 42	46, 47, 47, 47, 47, 48, 56, 57	49.4 \pm 4.4	48.3 \pm 3.0
0.5 mM NiTAP	13, 20, 27, 34, 41	44, 44, 49, 49, 49, 61, 61	50.1 \pm 7.2	48.7 \pm 6.8
0.25 mM PdTAP	13, 20, 27, 34, 41	43, 49, 51, 56	49.8 \pm 5.4	48.7 \pm 6.8
0.1 mM ZnTAP	13, 20, 27, 34, 41	49, 49, 50, 50, 51, 51, 53, 63	52.0 \pm 4.6	48.7 \pm 6.8
0.1 mM TAP	13, 20, 27, 34, 41	43, 44, 49, 49, 49, 51, 54, 54, 54	49.7 \pm 4.1	48.7 \pm 6.8
0.5 mM ZnTSP	13, 20, 27, 34, 41	47, 49, 49, 50, 51, 53, 53, 54, 57, 65	52.8 \pm 5.2	48.7 \pm 6.8
0.5 mM InTSP	13, 20, 27, 34, 41	44, 49, 51, 53, 54, 54, 56, 57, 65	53.7 \pm 5.8	48.7 \pm 6.8
0.5 mM FeTSP	13, 20, 27, 34, 41	57, 68, 70, 70, 72, 73, 83, 83, 85	73.4 \pm 9.0 ^e	48.7 \pm 6.8
0.5 mM FeTSP	14, 21, 28, 35, 42, 49	54, 68, 68, 74, 76, 76, 80	70.9 \pm 8.6 ^e	50.6 \pm 3.0
0.16 mM FeTSP	14, 21, 28, 35, 42, 49	54, 56, 56, 61, 64, 66, 66, 67	61.3 \pm 5.3 ^d	50.6 \pm 3.0
0.05 mM FeTSP	14, 21, 28, 35, 42, 49	52, 54, 57, 60, 62, 62, 64, 67	59.8 \pm 5.1 ^d	50.6 \pm 3.0
~0.05 mM PPS ^f	14, 21, 28, 35, 42	67, 68, 70, 72, 73, 73, 77, 78	72.3 \pm 3.9 ^e	48.3 \pm 3.0

^a Compounds were dosed after i.c. inoculation with 50 μ l of 1% 263K-infected brain homogenate. A control group of eight Tg7 mice dosed i.c. with 50 μ l of phosphate-buffered saline (PBS) at the same interval as that of treated mice was included with each experiment.

^b Mice dying from nonscrapie causes were removed from the data set.

^c Dosing was halted due to observed toxicity.

^d $P < 0.001$ versus corresponding control group by unpaired t test.

^e $P < 0.0001$ versus corresponding control group by unpaired t test.

^f This concentration was based on the average molecular weight of ~5,000.

and InTSP, injected at the same dose and frequency as that of FeTSP, gave no benefit, further demonstrating the importance of the central metal ion. It is also curious that NiTAP, which was quite effective in the inactivation test, was ineffective when dosed i.c. weekly starting 2 weeks after i.c. scrapie inoculation. Thus, differences in the central metal may affect not only porphyrin stereochemistries and reactivities but also, as shown here, antiscrapie potential. Understanding the reason for the differences in activity due to metal substitutions may be instructive in designing therapies for TSEs.

Based on its antiscrapie activity in mice, PPS is currently being infused into the brains of CJD patients as an experimental treatment (first patient described in reference 12). As there is no known effective CJD therapy, experimental treatment will likely start as soon as a diagnosis is made and will continue as long as possible. It is not known whether neurodegeneration can be stopped or reversed, but an important first goal is to slow disease progression. The discovery reported here that FeTSP has activity similar to that of PPS suggests that the use of cyclic tetrapyrroles as a CJD treatment is worth pursuing. With that goal in mind, testing of FeTSP by continuous brain infusion in mice to increase efficacy is ongoing. Until this brain infusion test is completed, it is impossible to know just how effective FeTSP treatment might be. Depending on these results and additional toxicology testing, a more informed decision on human clinical trials can be made. Finally, the demonstrated benefit of FeTSP against i.c.-inoculated scrapie suggests that other cyclic tetrapyrroles with even greater activity may yet be discovered.

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Potent Antiscrapie Activities of Degenerate Phosphorothioate Oligonucleotides

David A. Kocisko,^{1†} Andrew Vaillant,^{2†} Kil Sun Lee,¹ Kevin M. Arnold,¹ Nadine Bertholet,² Richard E. Race,¹ Emily A. Olsen,¹ Jean-Marc Juteau,^{2*} and Byron Caughey^{1*}

Laboratory of Persistent Viral Diseases, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, Montana,¹ and REPLICor Inc., Laval, Quebec, Canada²

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Although transmissible spongiform encephalopathies (TSEs) are incurable, a key therapeutic approach is prevention of conversion of the normal, protease-sensitive form of prion protein (PrP-sen) to the disease-specific protease-resistant form of prion protein (PrP-res). Here degenerate phosphorothioate oligonucleotides (PS-ONs) are introduced as low-nM PrP-res conversion inhibitors with strong antiscrapie activities in vivo. Comparisons of various PS-ON analogs indicated that hydrophobicity and size were important, while base composition was only minimally influential. PS-ONs bound avidly to PrP-sen but could be displaced by sulfated glycan PrP-res inhibitors, indicating the presence of overlapping binding sites. Labeled PS-ONs also bound to PrP-sen on live cells and were internalized. This binding likely accounts for the antiscrapie activity. Prophylactic PS-ON treatments more than tripled scrapie survival periods in mice. Survival times also increased when PS-ONs were mixed with scrapie brain inoculum. With these antiscrapie activities and their much lower anticoagulant activities than that of pentosan polysulfate, degenerate PS-ONs are attractive new compounds for the treatment of TSEs.

The transmissible spongiform encephalopathies (TSEs) or prion protein (PrP)-related diseases are infectious neurodegenerative diseases of mammals that include bovine spongiform encephalopathy, chronic wasting disease of deer and elk, scrapie in sheep, and Creutzfeldt-Jakob disease (CJD) in humans. TSEs are fatal after incubation periods that vary from months to years. The infectious agent of TSEs has not been conclusively identified, but abundant evidence implicates the abnormal, disease-specific protease-resistant conformation of prion protein (PrP-res) as a critical component (7, 35). In infected animals and cells, PrP-res is formed from the normal, protease-sensitive form of prion protein (PrP-sen), which is produced at the highest levels in the central nervous system.

Attempts to treat TSEs have often been based on compounds that prevent the formation of PrP-res in infected cell cultures (5). Many inhibitors of PrP-res in cell cultures have been identified (22), but relatively few have been tested against TSEs in vivo. Of the latter, many are effective prophylactically but have little or no benefit after TSE infection is established (5, 15). Thus, it remains important to identify new classes of drugs that are practical for prophylactic use and/or that are effective therapeutically.

Polyanionic sulfated glycans such as pentosan polysulfate (PPS) and dextran sulfate 500 (DS500) are among the most effective known anti-TSE compounds in vitro (4, 8, 17) and in vivo (3, 13, 14, 16, 24). PPS (molecular weight, ~5,000) and

DS500 (molecular weight, ~500,000) are polymers of xylose and glucose, respectively, and contain two and three sulfate units per sugar, respectively. While the antiscrapie activity of DS500 is significant, PPS appears to be more effective and less toxic to rodents (24). PPS is one of the few compounds known to lengthen the TSE incubation periods in animals that have been inoculated with scrapie directly into the brain (14). However, because PPS does not effectively cross the blood-brain barrier, it must be injected into the brain to be beneficial once the infection has reached the central nervous system. Orally dosed PPS (Elmiron) is a Food and Drug Administration-approved treatment for interstitial cystitis, and PPS is now being evaluated as a treatment for CJD in humans by the use of direct dosing into the brain (42).

Nucleic acids are a distinct class of polyanions that interact with PrP molecules. DNA binds to recombinant PrP molecules and, depending on the relative concentrations of peptide and nucleic acid, can promote or inhibit PrP-sen aggregation in cell-free reactions (9, 11, 18, 32, 33). Interestingly, the addition of vertebrate RNA but not DNA to cell-free conversion reactions of PrP-sen to PrP-res enhances PrP-res formation, but the mechanism of this effect is not known (12). Also, prophylactic treatments of mice with a specific immunomodulatory CpG deoxynucleotide (cpg1826) can prolong scrapie survival times by a mechanism that was hypothesized to involve stimulation of innate immunity (38). While natural nucleic acids ($\leq 10 \mu\text{g/ml}$) have not been found to affect PrP-res formation in scrapie-infected neuroblastoma cells (8), we show here that degenerate single-stranded phosphorothioated analogs of natural nucleic acids (the structures are provided in Fig. 1) bind to PrP-sen and potently inhibit PrP-res accumulation. Both the molecular sizes and the hydrophobicities of phosphorothioate oligonucleotides (PS-ONs) were important, implying that these

* Corresponding author. Mailing address for Jean-Marc Juteau: REPLICor Inc., Laval, Quebec, Canada H7V 5B7. Phone: (450) 688-6068. Fax: (450) 688-3158. E-mail: jmjuteau@replicor.com. Mailing address for Byron Caughey: NIH, Rocky Mountain Laboratories, 903 S. 4th Street, Hamilton, MT 59840. Phone: (406) 363-9264. Fax: (406) 363-9286. E-mail: bcaughey@niaid.nih.gov.

† These authors contributed equally to this work.

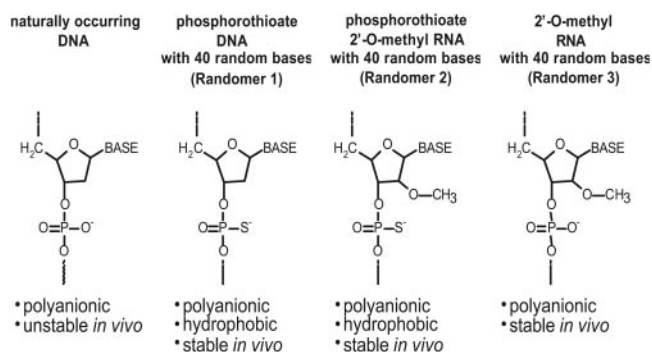


FIG. 1. Structures of nucleic acids tested. The phosphorothioate modification, which reduces enzymatic degradation and increases the hydrophobicities of ONs, and 2'-O-methylation, which only stabilizes ONs, were used to alter the chemical properties of ONs in this study.

inhibitors interact with a discrete amphipathic site on PrP-sen that influences conversion. PS-ONs dramatically prolong the lives of scrapie-infected rodents if they are administered prophylactically and are capable of effectively neutralizing scrapie titers in infected brain inocula. Thus, degenerate PS-ONs represent an attractive class of anti-TSE drugs that may also help to define the mechanism for PrP-res formation.

MATERIALS AND METHODS

Synthesis of ONs. All oligonucleotides (ONs) were designed and characterized at REPLICor (Montreal, Quebec, Canada) and were prepared by the University of Calgary DNA services laboratory by standard solid-phase synthesis methods. Combinations of phosphorothioation and/or 2'-O-methylation were combined to prepare ONs (Fig. 1). Good manufacturing practice (GMP)-grade Randomer 1, used for *in vivo* prophylaxis studies with mice, was prepared by Grinidus America Inc. under contract with REPLICor. Fluorescent ONs were synthesized with a single label on the 3' end of the ONs by using commercially available 3'-(6-fluorescein) or 3'-(6-rhodamine) CPG supports (Glen Research). Rhodamine-tagged Randomers (rh-Randomers) had different specific fluorescent intensities (presumably due to the intramolecular quenching caused by the presence of the 2'-O-methyl modification), with rh-Randomers 2 and 3 having intensities that were 40% and 24% of that of rh-Randomer 1, respectively. The synthesis of completely degenerate ONs was accomplished by using equal molar amounts of adenosine, cytidine, guanosine, or thymidine amidites in each coupling reaction during the solid-phase synthesis, which produced a pool of equivalently sized ONs that collectively have no sequence-specific antisense or aptameric activity. Approximately equivalent incorporation of individual nucleotides was found by high-pressure liquid chromatography quantification of the proportion of each nucleotide present following the oxidation and degradation of an ON into its constituent nucleotides by using S1 nuclease or snake venom phosphodiesterase (1, 37) (data not shown).

PrP-res dot blot assay. A dot blot assay was used as described previously to test the inhibition of RML and 22L mouse scrapie PrP-res (22) or sheep scrapie PrP-res (23) in infected cells. Briefly, infected mouse neuroblastoma (N2a) cells were plated at a low density and grown to confluence in the presence of potential inhibitors. At confluence, the cells were carefully examined by light microscopy for any morphological changes or other evidence of toxicity. Following this, the cells were lysed and treated with proteinase K before they were applied to a polyvinylidene difluoride membrane by use of a 96-well dot blot apparatus. The proteins on the polyvinylidene difluoride membrane were then denatured with 3 M guanidine thiocyanate to expose epitopes, and then the membrane was blocked with 5% (wt/vol) skim milk to prevent nonspecific antibody interactions. The membranes were probed with monoclonal antibody 6B10 (22), followed by an alkaline phosphatase-conjugated goat antimouse secondary antibody. Immunoreactivity was detected with enhanced chemifluorescence, and the amount of PrP-res was quantified by using ImageQuant software. The concentrations giving half-maximal inhibition (IC_{50} s) were determined by graphing PrP-res inhibition curves by using the points from at least three independent determinations.

PrP-sen *in vitro* binding assay. The binding affinities of mouse and hamster PrP-sens to Randomers 1, 2, and 3 were monitored by using recombinant mouse PrP (23-231) (mouse rPrP-sen) (20) and hamster PrP (23-231) (hamster rPrP-sen) (41). These proteins were serially diluted in assay buffer (10 mM Tris, pH 7.2, 80 mM NaCl, 1 mM EDTA, 10 mM β -mercaptoethanol, 0.1% Tween 20) and allowed to interact with 3 nM fluorescein isothiocyanate-labeled Randomer for 30 s. Protein binding was monitored by fluorescence polarization at 535 nm with a Tecan Ultra plate reader. The equilibrium dissociation constant (K_D) was determined from the concentration of protein which resulted in 50% of the maximal polarization observed (saturated protein interaction). For the competition assays, each of the three different fluorescein isothiocyanate-labeled modified Randomers was loaded to saturation with recombinant PrP-sen from mouse or hamster (0.5 μ g protein for Randomer 1 and 2 and 2 μ g protein for Randomer 3). Serial dilutions of unlabeled Randomers or other polyanions were then used to challenge the Randomer-PrP-sen interaction. Competition was monitored by determination of the reduction in fluorescence polarization. The reported averages and standard deviations of the K_D values and K_i values (the concentration achieving 50% competition of bound, labeled Randomer) were from at least three independent measurements.

Transient transfection. 22L-infected N2a or SN56 cells were plated in glass-bottom culture dishes (MaTek) at 10% confluence. On the following day the cells were transfected with Effectene transfection reagent (QIAGEN) with plasmids expressing either green fluorescent protein (GFP)-labeled PrP (GFP-PrP) (27) or GFP-labeled glycosylphosphatidylinositol (GFP-GPI) (a gift from Benjamin J. Nichols and J. Lippincott-Schwartz, MRC Laboratory of Molecular Biology, United Kingdom, and Cell Biology and Metabolism Branch, NICHD, NIH) under control of the cytomegalovirus promoter. The transfection was performed according to the manufacturer's instructions with 0.3 μ g of DNA and 6 μ l of Effectene reagent per plate. Following 14 to 16 h of incubation with the transfection reagent, the cells were washed twice and incubated in fresh culture medium.

Uptake of Randomers into N2a cells. Nontransfected N2a or SN56 cells were incubated with 100 nM rh-Randomer 1, 2, or 3 for various times. Incubation of the transfected cells with the compounds did not begin until the transfection reagent was completely removed by washing the cells with fresh culture medium. At the desired length of incubation, the cells were washed three times and fixed with 2.5% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min at room temperature. Images of the cells were acquired with a Bio-Rad MRC 1024 laser scanning confocal system coupled to a Zeiss microscope with a water immersion objective ($\times 40$, 1.2 numerical aperture). Image processing and analysis were performed with Confocal Assistant, Adobe Photoshop, and Image J software.

Bioassay for disinfection of scrapie infectivity. The amount of infectivity in dilutions of hamster 263K scrapie-infected brain homogenate was bioassayed in transgenic mice that overexpress hamster PrP (Tg7). Untreated 10% (wt/vol) homogenates of 263K hamster scrapie-infected brains were sonicated for 1 min and then diluted with PBS to 1, 0.1, 0.01, or 0.001% (wt/vol) and incubated at 37°C for 1 h. A total of 50 μ l of each of these diluted homogenates was then injected intracerebrally (i.c.) into Tg7 mice. Separate 10% 263K-infected brain homogenate solutions were diluted to 1% with PBS and 1 mM Randomer solution to the desired final concentrations. These mixtures of brain homogenate and Randomer were also incubated at 37°C for 1 h. As with the control homogenates, 50 μ l of each of these was injected i.c. into Tg7 mice. The mean survival times of different groups of animals were compared by one-way analysis of variance and a Tukey multiple-comparison posttest with GraphPad Prism 4 software. Prism reports *P* values for multiple-comparison posttests in discrete ranges rather than an exact value.

Rocky Mountain Laboratories is an AALAC-accredited facility, and all animal procedures were approved by the institution's Animal Care and Use Committee. Scrapie was identified as the cause of death by the clinical signs observed and detection of PrP-res in the brain. Data for animals that died from causes other than scrapie have been excluded.

Prophylaxis of scrapie progression *in vivo*. Tg7 mice were first dosed with GMP-grade Randomer 1 at 10 mg/kg of body weight in 5% dextrose subcutaneously (s.c.) or intraperitoneally (i.p.) daily for 3 days. Also, on the third day immediately after the third dose of Randomer 1, the animals were inoculated i.p. with 50 μ l of 1% 263K hamster scrapie-infected brain homogenate. Afterwards, the animals were dosed on Mondays, Wednesdays, and Fridays for either the next 4 or the next 10 weeks with the amount of Randomer 1 mentioned above. Groups of Tg7 mice were also inoculated i.p. with 50 μ l of 1% 263K hamster scrapie-infected brain homogenate and dosed with 5% dextrose either s.c. or i.p. as a control. The mean survival times of the different groups of animals were statistically analyzed by an unpaired *t* test with GraphPad Prism 4 software.

TABLE 1. The cell culture anti-PrP-res activities of PS-ONs are largely dependent on size and chemistry

Compound ^a	Avg \pm SD IC ₅₀ for 22L scrapie		Avg \pm SD IC ₅₀ (nM) for sheep scrapie
	nM	ng/ml	
6-base Randomer 1	55,000 \pm 11,000	110,000 \pm 22,000	>25,000 ^b
10-base Randomer 1	3,100 \pm 400	10,000 \pm 1,000	17,000 \pm 4,000
11-base Randomer 1	3,800 \pm 700	14,000 \pm 3,000	8,400 \pm 4,800
12-base Randomer 1	920 \pm 80	3,600 \pm 300	4,300 \pm 400
13-base Randomer 1	1,200 \pm 500	5,100 \pm 2,100	2,200 \pm 1,000
14-base Randomer 1	630 \pm 180	2,900 \pm 800	3,600 \pm 700
15-base Randomer 1	280 \pm 90	1,400 \pm 400	3,300 \pm 200
16-base Randomer 1	290 \pm 50	1,500 \pm 300	2,900 \pm 600
17-base Randomer 1	78 \pm 3	440 \pm 20	730 \pm 80
18-base Randomer 1	70 \pm 30	420 \pm 180	780 \pm 30
19-base Randomer 1	65 \pm 5	410 \pm 30	620 \pm 150
20-base Randomer 1	58 \pm 11	380 \pm 70	410 \pm 80
25-base Randomer 1	24 \pm 3	200 \pm 30	150 \pm 100
28-base Randomer 1	25 \pm 3	230 \pm 30	48 \pm 8
30-base Randomer 1	27 \pm 5	270 \pm 50	58 \pm 18
Randomer 1	21 \pm 10	270 \pm 130	51 \pm 14
50-base Randomer 1	11 \pm 5	180 \pm 80	28 \pm 2
80-base Randomer 1	18 \pm 7	480 \pm 190	31 \pm 2
120-base Randomer 1	9 \pm 1	360 \pm 40	33 \pm 2
40-base DNA	15,000 \pm 3,000		61,000 \pm 22,000
Randomer 2	20 \pm 8		35 \pm 6
Randomer 3	90,000 \pm 11,000		~100,000 ^c
Poly(A) Randomer 1	31 \pm 13		28 \pm 2
Poly(T) Randomer 1	23 \pm 2		21 \pm 5
Poly(C) Randomer 1	20 \pm 4		31 \pm 9
Poly(G) Randomer 1	67 \pm 9		NT ^d
Poly(AC) Randomer 1	23 \pm 5		47 \pm 2
Poly(TC) Randomer 1	9 \pm 1		8 \pm 1
Poly(AG) Randomer 1	12 \pm 5		59 \pm 22
Poly(TG) Randomer 1	25 \pm 4		12 \pm 4

^a The sequence is degenerate unless specified otherwise; Randomers 1, 2, and 3 without a specified number of bases have 40 bases.

^b Inhibition of ~30% at 25 μ M.

^c Inhibition of ~45% at 100 μ M.

^d NT, not tested.

Effects of PPS and Randomers 1 and 2 on aPPT. On three different days, Randomers 1 and 2 and PPS were dissolved in normal saline at equimolar concentrations and were added to freshly drawn human blood with a 1/10 volume dilution. The activated partial thromboplastin times (aPPTs) were then determined by using a clinically accepted assay at a local clinical laboratory. Normalized aPPT ratios were determined by normalizing individual aPPT times to the result obtained with normal saline for each daily measurement and represent the fold increase over the values obtained with normal saline for that particular day. The average and standard deviations from three separate trials were plotted for analysis.

RESULTS

Inhibition of PrP-res accumulation by degenerate PS-ONs.

As a number of polyanions are effective antiscrapie compounds, differently modified ONs were investigated for the ability to inhibit PrP-res accumulation. By using the well-controlled "building block" approach available for ON synthesis, we prepared 40-base fully degenerate ONs which were phosphorothioated (Randomer 1), phosphorothioated and 2'-O methylated (Randomer 2), or only 2'-O methylated (Randomer 3) (Fig. 1). At each coupling step in the synthesis, equimolar mixtures of nucleotides were included, generating a fully random mixture of sequences. These degenerate ON preparations were used to avoid any potential antisense or sequence-specific aptameric activity. The different backbone chemistries were chosen to allow the comparison of the antiscrapie activities of ONs that are resistant to enzymatic degradation (25, 39) with a minimal hydrophobic character (Randomer 3) or an enhanced hydrophobic character (Randomers 1 and 2) (1).

The ability of Randomers 1, 2, and 3 to prevent 22L, RML, or sheep PrP-res accumulation in cell culture models was tested (Table 1 and Fig. 2A). Both Randomers 1 and 2 had IC₅₀s of 20 to 51 nM, while Randomer 3 was >1,000-fold less effective. An unmodified degenerate DNA composed of 40 random bases was also much less effective. The inhibition of PrP-res accumulation by Randomer 1 was not due to effects on the biosynthesis of PrP-sen, as the steady-state levels of PrP-sen in uninfected N2a cells were not altered by its presence (Fig. 2B). No cytotoxicity was observed by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell viability assay with mouse neuroblastoma (N2a) or rabbit epithelial (Rov9) cells grown in \leq 100 μ M Randomer 1, 2, or 3 (data not shown). Moreover, these Randomers did not artifactually interfere with the detection of PrP-res when they were added directly to

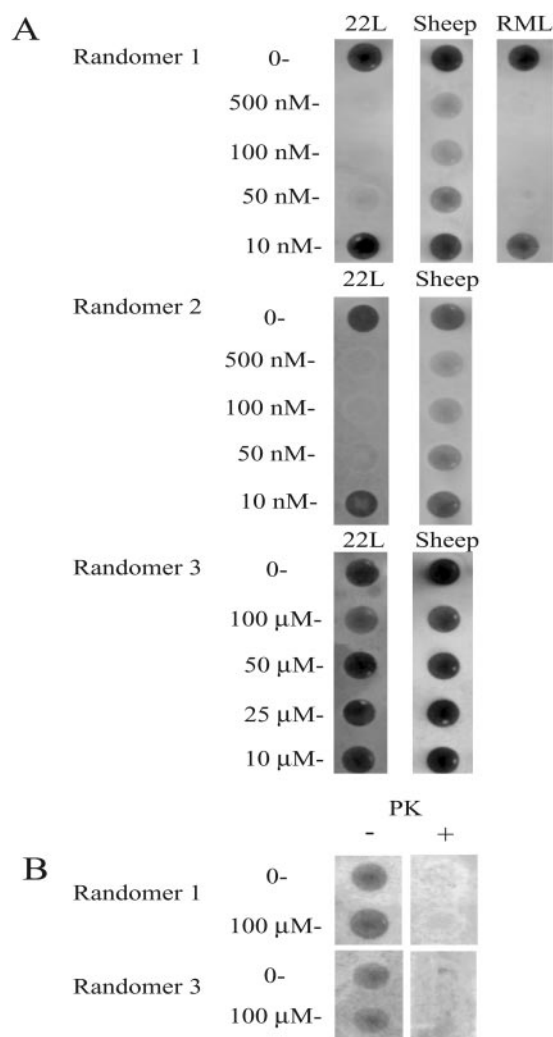


FIG. 2. Randomers 1 and 2 but not Randomer 3 were potent inhibitors of 22L PrP-res and sheep scrapie PrP-res in cells. (A) The three sets of panels are dot blots of the indicated type of PrP-res from cells grown from low density to confluence in the presence of the indicated amounts of Randomer in cell medium. (B) Mouse PrP-sen from uninfected N2a cells grown to confluence with the indicated amount of Randomer in the cell medium. Proteinase K (PK) treatment eliminates all signals, as the PrP-sen is completely degraded.

TABLE 2. Affinity of PS-ON binding to rPrP-sen, determined by fluorescence polarization in vitro, is size and chemistry dependent

Compound ^a	Avg \pm SD K_D for mouse rPrP-sen		Avg \pm SD K_D (nM) for hamster rPrP-sen
	nM	ng/ml	
6-base Randomer 1	2,400 \pm 300	4,800 \pm 600	2,800 \pm 100
10-base Randomer 1	420 \pm 30	1,400 \pm 100	430 \pm 30
20-base Randomer 1	70 \pm 16	460 \pm 110	87 \pm 31
Randomer 1	16 \pm 10	210 \pm 130	12 \pm 6
80-base Randomer 1	63 \pm 8	1,700 \pm 200	56 \pm 30
Randomer 2	13 \pm 4	170 \pm 50	25 \pm 1
Randomer 3	250 \pm 60	3,300 \pm 800	210 \pm 60

^a The sequences of all compounds are degenerate; Randomers 1, 2, and 3 without a specified number of bases have 40 bases.

the scrapie-infected N2a cell lysates at 100 μ M prior to the dot blot assay (data not shown). The lack of anti-PrP-res activity of Randomer 3 in these assays suggested that the polyanionic nature of these molecules was insufficient for inhibition and that the added hydrophobicity of the phosphorothioate modification was important.

Effect of base composition on anti-PrP-res activities of PS-ONS. Although the degenerate nature of these ONs strongly implied that the anti-PrP-res activities did not require a specific ON sequence, there was a possibility that the activities were due to a small proportion of ONs enriched in a particular base. To address this question, the anti-PrP-res activity of Randomer 1 was compared with those of other phosphorothioated 40 base homo- and heteropolymeric ONs of defined compositions (Table 1). The various hetero- and homopolymer ONs showed activities comparable to that of Randomer 1.

These results indicated that the antiscrapie activities of PS-ONS are minimally dependent on base composition.

Size dependence of anti-PrP-res activities of PS-ONS. To test the effect of ON length on anti-PrP-res activities, a series of Randomer 1 analogs from 6 to 120 bases in length were tested for their anti-PrP-res activities against 22L and sheep scrapie (Table 1). Size-dependent activity was apparent against both scrapie strains, with longer ONs having more potent activities. Nearly maximal anti-PrP-res activities were reached with ONs of \sim 25 to 28 bases. This was especially apparent when the IC₅₀s against 22L scrapie were compared on the basis of mass per volume rather than molarity (Table 1), in order to compensate for the differences in molecular mass. The activities of ONs 25 bases in length and shorter were generally greater against 22L scrapie than against sheep scrapie.

Interactions between ONs and PrP-sen. To test the possibility that PS-ONS might also interact directly with PrP molecules as part of their inhibitory mechanism, the binding of various PS-ONS and related molecules to recombinant mouse and hamster PrP-sen (rPrP-sen) molecules were examined by using a cell-free, fluorescence polarization-based assay. In agreement with the in vitro anti-PrP-res activities of PS-ONS, fluorescently labeled Randomers 1 and 2 showed at least eight-fold stronger binding to both mouse and hamster rPrP-sen than fluorescent Randomer 3 (Table 2). The size dependence of fluorescently labeled PS-ON binding to mouse and hamster rPrP-sens was also examined by using analogs of Randomer 1, with larger ONs resulting in stronger binding (Table 2). The optimum size for binding was between 20 and 40 bases whether the binding was compared by molarity or mass per volume, consistent with the size-dependent anti-PrP-res activities of the PS-ONS in vitro.

TABLE 3. K_i values (50% competition) with fluorescence-labeled Randomer 1, 2, or 3 bound to mouse or hamster rec-PrP^a

Competitor	Avg \pm SD K_i (nM)					
	Randomer 1-FL		Randomer 2-FL		Randomer 3-FL	
	Mouse	Hamster	Mouse	Hamster	Mouse	Hamster
Randomer 1	16 \pm 4	31 \pm 10	29 \pm 3	48 \pm 16	16 \pm 5	58 \pm 20
Randomer 2	20 \pm 1	37 \pm 18	29 \pm 2	53 \pm 18	26 \pm 12	52 \pm 14
Randomer 3	130 \pm 30	220 \pm 70	170 \pm 50	200 \pm 60	62 \pm 4	140 \pm 30
Dextran, 5,000 MW	NC	NC	NC	NC	NC	NC
Dextran, 12,000 MW	NC	NC	NC	NC	NC	NC
Dextran sulfate, 5,000 MW	MC	MC	MC	MC	44 \pm 3	150 \pm 70
Dextran sulfate, 8,000 MW	MC	MC	MC	MC	31 \pm 3	100 \pm 40
Dextran sulfate, 10,000 MW	MC	120 \pm 70	MC	83 \pm 15	14 \pm 3	48 \pm 16
Dextran sulfate, 500,000 MW	7.7 \pm 4.4	3.5 \pm 2.3	0.7 \pm 0.1	2.5 \pm 0.8	1.1 \pm 0.8	1.7 \pm 0.3
Heparan sulfate (Sigma H7640), 10,000 to 14,000 MW	NC	NC	NC	NC	MC	MC
Heparan sulfate, fast-moving fraction (Sigma H9902), \sim 14,000 MW	NC	MC	MC	MC	MC	MC
Heparan sulfate proteoglycan (Sigma H4777), $>$ 200,000 MW	NC	NC	NC	NC	NC	NC
Heparin (Sigma H3149), \sim 17,000 MW	3,300 \pm 700	2,600 \pm 400	1,900 \pm 500	MC	42 \pm 18	120 \pm 70
Pentosan polysulfate, \sim 5,000 MW	1,600 \pm 300	2,100 \pm 600	1,700 \pm 400	2,400 \pm 700	100 \pm 20	360 \pm 20
Chondroitin sulfate A (Sigma C9819)	NC	NC	NC	NC	NC	NC
Chondroitin 6-sulfate (Sigma C4384), \sim 60,000 MW	NC	NC	NC	NC	NC	NC

^a Abbreviations: FL, fluorescence label; MW, molecular weight; NC, no competition at up to 100 μ g/ml competitor; MC, minimal competition (some competition was observed, but $>$ 100 μ g/ml competitor was required to achieve the IC₅₀).

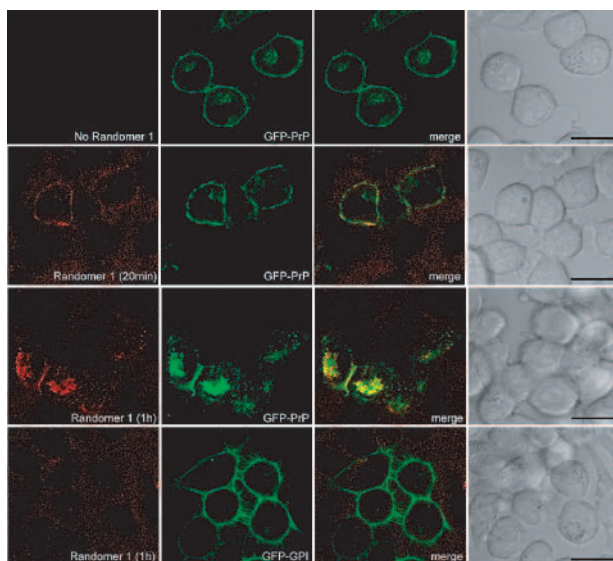


FIG. 3. Enhanced binding and uptake of rh-Randomer 1 in N2a cells expressing GFP-PrP. N2a cells transiently expressing GFP-PrP (green) or GFP-GPI, a green GPI-linked GFP control, were incubated for the designated times with 100 nM rh-Randomer 1 (red). The yellow fluorescence in the merged images indicates the colocalization of rh-Randomer 1 and GFP-PrP. The corresponding differential interference contrast images (grey scale) showing all of the cells in each field are shown in the right column. Bars, 20 μ m.

Considering the common polyanionic character of the PS-ONs and known sulfated glycan inhibitors of PrP-res, we compared the relative abilities of unlabeled PS-ONs and a variety of sulfated polysaccharides to displace fluorescent Randomers that were bound to mouse and hamster rPrP-sen (Table 3). The abilities of Randomer 1 and Randomer 2 to displace other bound Randomers were equivalent with both mouse and hamster rPrP-sens. As expected, based on their relative K_D values in Table 2, Randomers 1 and 2 were more effective than Randomer 3 at displacing other Randomers. Dextran sulfates showed a size-dependent ability to displace all three Randomers, with larger polymers being more efficient and Randomer 3 being the most easily displaced. Of the other sulfated saccharides used in competition with bound Randomers, only heparin and pentosan polysulfate displayed a substantial ability to displace Randomers from mouse and hamster rPrP-sens, and both of these polymers displaced Randomer 3 more easily than Randomers 1 and 2. PPS, heparin, and DS500 differ substantially in their molecular masses; and when they are considered in terms of mass per volume rather than molarity, their average K_i values (8, 56, and 4 μ g/ml, respectively, with mouse r-PrPsen) were more similar. Collectively, these data provide evidence that the inhibitory Randomers and sulfated glycans compete for the same or overlapping binding sites on PrP-sen.

Cellular binding and uptake of Randomers. To visualize the interactions of the Randomers with intact cells, rhodamine (red)-tagged Randomers were added to N2a cells expressing a GFP-PrP chimera and were observed by confocal microscopy (Fig. 3). This GFP-PrP chimera, like normal PrP-sen, was anchored to the cell membrane by a GPI moiety. Without rh-Randomer treatment, GFP-PrP fluorescence was seen in a

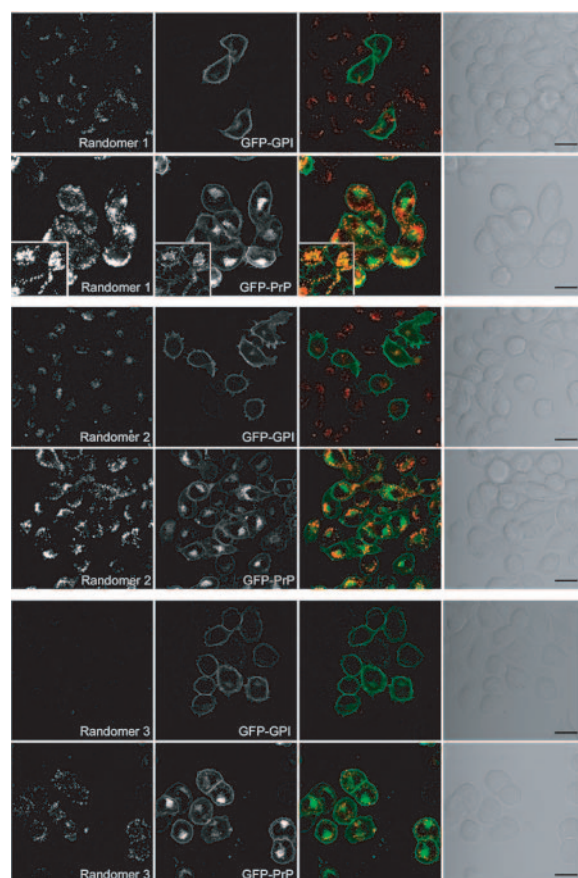


FIG. 4. Comparison of GFP-PrP-dependent uptake of rh-Randomers 1, 2, and 3 in N2a cells. N2a cells transiently expressing GFP-GPI (rows 1, 3, and 5) or GFP-PrP (rows 2, 4, and 6) were incubated for 1 day with 100 nM rh-Randomer 1 (rows 1 and 2), 100 nM rh-Randomer 2 (rows 3 and 4), or 400 nM rh-Randomer 3 (rows 5 and 6). The first column shows rh-Randomer, and the second column shows GFP-GPI or GFP-PrP. Merged images (the rh-Randomer is red, and GFP-GPI or GFP-PrP is green) of both fluorescences is shown in the third column, and the corresponding differential interference contrast images are in the fourth column. The inset in row 2 shows a population of cells with colocalization of GFP-PrP and rh-Randomer 1. Bars, 20 μ m.

mostly diffuse pattern on the cell surface and in a more punctate intracellular distribution. In cells treated with rh-Randomer 1 for 20 min, punctate rh-Randomer fluorescence on the cell surface colocalized with a pattern of cell surface GFP-PrP fluorescence that was more punctate than that in the untreated cells. This suggested that rh-Randomer 1 bound to GFP-PrP and caused it to cluster. After 1 h, much of both the rh-Randomer 1 and GFP-PrP fluorescence had moved from the cell surface to intracellular sites where colocalization was often, but not always, apparent. Individual cells with high levels of expression of GFP-PrP had enhanced binding and internalization of rh-Randomer 1 compared to those of nontransfected cells (visible in differential interference contrast images) or cells expressing GFP alone attached to the GPI anchor (Fig. 3). This indicated that the PrP portion of the GFP-PrP chimera enhanced rh-Randomer 1 binding and internalization relative to the baseline levels that may be mediated by the endogenous

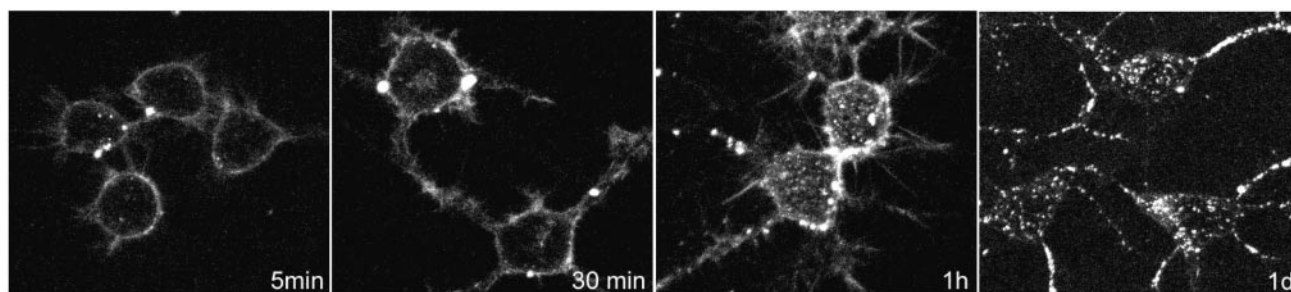


FIG. 5. Uptake of rh-Randomer 1 in SN56 cells. SN56 cells were incubated with 100 nM of rh-Randomer 1, and images were acquired by confocal microscopy after the designated times. Binding of rh-Randomer 1 on the cell surface was observed prior to the internalization. d, day.

unlabeled PrP molecules. After 1 day, much less colocalization of the internalized rh-Randomer 1 and GFP-PrP was observed in most cells (Fig. 4), providing evidence that after internalization, these two molecules separated. This was also observed with Randomer 2 (Fig. 4). The 4- and 1.6-fold lower specific fluorescence intensity of rh-Randomer 3 relative to those of Randomers 1 and 2, respectively (see Materials and Methods), made quantitative comparisons between Randomers difficult. Nonetheless, internalization of rh-Randomer 3 appeared to be markedly less efficient than that of the other rh-Randomers, even when compensations such as the use of a fourfold higher concentration of rh-Randomer 3 (Fig. 4) or a threefold increase in the laser power (data not shown) were made. The apparently reduced internalization of rh-Randomer 3 correlated with its lower affinity for recombinant PrP-sen (Table 2) and reduced activity as a PrP-res inhibitor (Table 1).

The uptake of rh-Randomer 1 was also evaluated in SN56 cells, another murine septum-derived neuronal cell line that is readily infected with scrapie (30), to determine if the rh-Randomer uptake was specific to N2a cells. In SN56 cells, rh-Randomer 1 was detected on the cell surface within 5 min and punctate intracellular staining was detected within 30 min (Fig. 5). Thus, the cell surface binding and internalization of rh-Randomer 1 occurred in SN56 cells as well as N2a cells. As was observed with the N2a cells, a high degree of colocalization between rh-Randomer 1 and GFP-PrP was observed at the cell surface. However, after 2 days there was a dramatic decrease in the GFP-PrP signal at the cell surface and little colocalization was observed between the intracellular signal of GFP-PrP and rh-Randomer 1 (Fig. 6). Again, it appeared that the Randomers interacted with PrP molecules preferentially on the cell surface and separated after internalization.

Lack of effect of PrP-res on the cellular uptake of Randomers. To assess whether PrP-res and scrapie infection alters the observed cellular interactions of Randomers, the levels of uptake of rh-Randomers 1 and 2 were compared in N2a cells that were either scrapie infected or cured of their infection by the use of pentosan polysulfate. In both of these cell cultures, punctate intracellular fluorescence of both the rh-Randomers was observed, and the fluorescence gradually increased in intensity through at least 24 h (Fig. 7). Internalized rh-Randomers were distributed throughout the cell bodies, but in most cells, rh-Randomers were concentrated in the perinuclear region. No effect of scrapie infection on the uptake and intracellular transport of these Randomers was observed, suggesting

that the primary interactions between the Randomers and these cells were not mediated by PrP-res.

In vivo antiscrapie activities of Randomers. GMP-grade Randomer 1 was tested against scrapie infections of Tg7 mice (34, 36), which overexpress hamster PrP. To test for prophylactic efficacy, 10 mg/kg Randomer 1 was dosed i.p. or s.c. to Tg7 mice daily for 3 days prior to an i.p. inoculation of 263K hamster scrapie brain homogenate (10^4 i.p. lethal doses) on the third day. The Randomer 1 dosing continued for three times per week for 4 weeks in one group of mice and for 10 weeks in another. Randomer 1 had strong prophylactic antiscrapie activity, with the s.c. and i.p. dosing regimens more than doubling and tripling the survival times, respectively (Table 4). Animals that died at days 58, 75, and 79 had shown no clinical signs of scrapie and did not have PrP-res in the brain. The Tg7 mouse that died at day 58 had been dosed 27 times, and the other animals had each been dosed 32 times. It is not known if this frequent dosing regimen contributed to their deaths.

GMP-grade Randomer 1 was also tested for its ability to prolong the survival time simply by being premixed with the scrapie brain inoculum prior to i.c. inoculation (10^6 i.c. lethal doses). In the first experiment, in which 0, 100 nM, or 10 μ M Randomer 1 was mixed with 1% scrapie brain homogenate, a significant 9-day increase in the survival time was observed

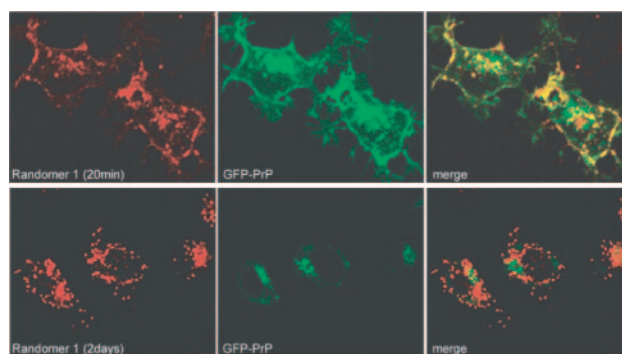


FIG. 6. Colocalization of rh-Randomer 1 with GFP-PrP at the cell surface in SN56 cells. SN56 cells expressing GFP-PrP (green) were incubated with rh-Randomer 1 (red) for 20 min or 2 days. After 20 min of incubation, an extensive colocalization between rh-Randomer 1 and GFP-PrP was observed. After 2 days of incubation, the GFP-PrP signal at the plasma membrane was greatly decreased and only little colocalization between the intracellular signal of GFP-PrP and rh-Randomer 1 was observed.

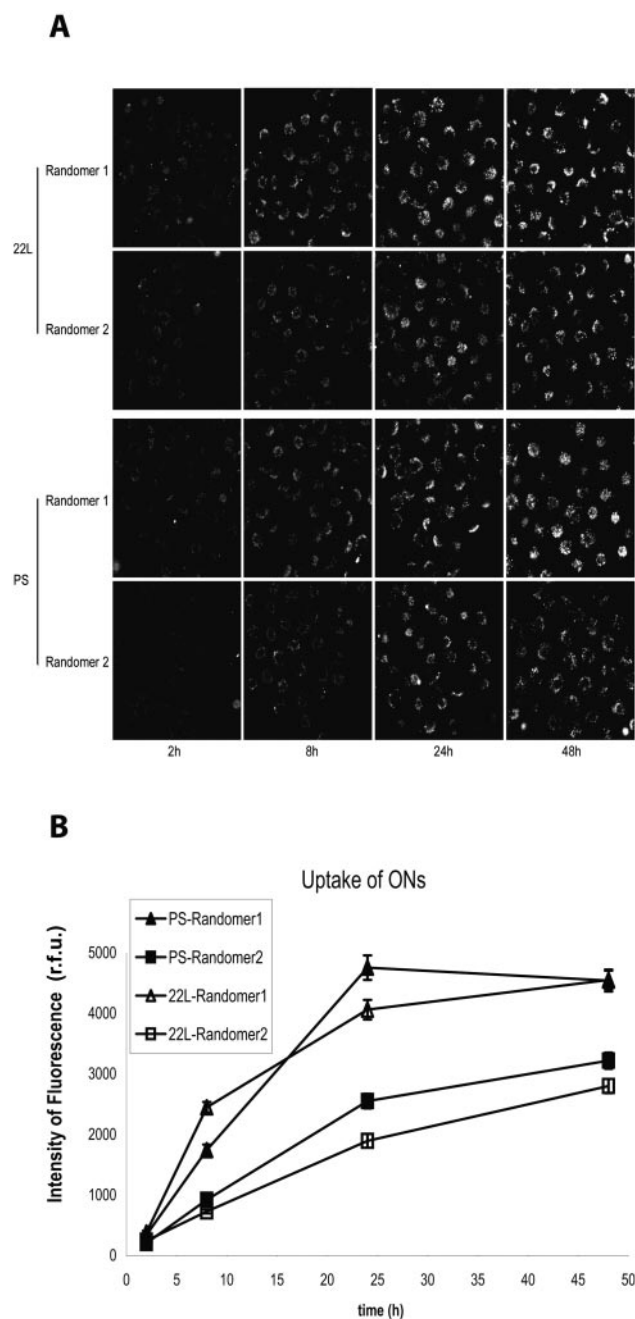


FIG. 7. Uptake of Randomers 1 and 2 in scrapie-infected and cured N2a cells. (A) Images of 22L-infected (22L) or pentosan polysulfate-cured (PS) N2a cells were acquired after 2, 8, 24, or 48 h of incubation with 100 nM rh-Randomer 1 or 2 by confocal microscopy. (B) To quantitate the uptake of Randomers, the fluorescence intensity of individual cells was measured by using Image J software, and the mean value of intracellular signal was graphed. Bars represent standard error. r.f.u., relative fluorescent units.

with 10 μ M Randomer 1 (Table 5). A second experiment added a 100 μ M Randomer 1 treatment as well as serial dilutions of untreated homogenate (1%, 0.1%, 0.01%, etc.) to correlate the delay of the survival time with the reduction in the titer of scrapie infectivity. Randomer 1 at 1 mM in diluted brain homogenate was not tolerated by Tg7 mice after rapid

i.c. administration. Treatments of 1% scrapie brain homogenate with Randomer 1 at 10 μ M and 100 μ M gave survival times equivalent to those of 0.01% and 0.001% homogenates, respectively and, thus, reduced the effective scrapie infectivity levels by approximately 100- and 1,000-fold, respectively (Table 5). In contrast, Randomer 3 had no effect. A third experiment with Randomer 2 showed that it had activity similar to that of Randomer 1. Finally, a 40-base poly(C) analog of Randomer 1, which contains no CpG motifs, also had activity comparable to those of Randomer 1 and Randomer 2 in this in vivo assay. Overall, these experiments showed that when they were added to a source of infection, Randomer 1, Randomer 2, and a poly(C) analog of Randomer 1 can each substantially reduce the apparent infectivity levels (as indicated by incubation period) even when the sample is inoculated directly into the brain.

Anticoagulant activities of Randomers 1 and 2. Sulfated glycans are known to interact with the coagulation cascade in blood. It is therefore possible that the dose-limiting factor for any of these compounds is their impact on blood coagulation. In light of the fact that side effects such as hematomas potentially related to the anticoagulation properties of PPS have complicated animal experiments (14) involving PPS administration into the brain, we examined the relative anticoagulant activities of PPS and Randomers 1 and 2. By using the aPTTs in human blood (normalized to the aPTTs in the presence of vehicle alone) as an indirect measure of the effect on blood coagulation, treatment with Randomers 1 and 2 resulted in a significantly lower increase in the normalized aPTT compared to that obtained with PPS at equivalent molar doses (Fig. 8). In general, in a clinical setting it is safe to maintain the aPTT within 1.5 times the baseline value. When clinical therapeutic anticoagulation is desired, the aPTT is usually maintained between 1.5 and 2 times the baseline values. These results suggest that although Randomer 1, Randomer 2, and PPS have comparable IC_{50} s against PrP-res formation (e.g., 51 nM, 35 nM, and \sim 100 nM [23], respectively, in sheep scrapie-infected Rov9 cells), the Randomers should have a much milder anticoagulant activity at equivalent molar doses compared to that of PPS. Because Randomers 1 (13 kDa) and 2 (14 kDa) have more than twice the 5-kDa average molecular mass of PPS, they would have even lower relative anticoagulant activities when their activities are compared on the basis of mass per volume rather than on a molar basis.

DISCUSSION

Given that no practical and effective anti-TSE prophylaxes or therapies have been established, it is critical to identify new therapeutic approaches. The present data reveal that degenerate PS-ONs are a new class of PrP-res inhibitors that have potent antiscrapie activities in vivo and in vitro. These observations have both mechanistic and practical implications for potential TSE therapies.

Antiscrapie mechanism of action of PS-ONs. From a mechanistic point of view, it is difficult to fully define the antiscrapie mechanism of action of the PS-ONs or any other anti-TSE agent without knowing the molecular, cellular, and organismal mechanisms of PrP-res formation. Nonetheless, it is likely that PS-ONs act by binding directly to PrP molecules. The preferential binding

TABLE 4. Randomer 1 (10 mg/kg) as a scrapie prophylactic in Tg7 mice inoculated i.p. with 50 μ l of 1% 263K brain homogenate

Treatment after inoculation	Survival times (days)	Avg \pm SD survival time (days)	Significance between groups (unpaired <i>t</i> test)
4 wk s.c. 5% dextrose	77, 83, 83, 85, 87, 90, 91, 94, 98	87.6 \pm 6.4	
4 wk s.c. Randomer 1	181, 201, 238, 254	218.5 \pm 33.4	<i>P</i> < 0.0001 vs s.c. control
10 wk s.c. Randomer 1	175, 186, 226 ^a	195.7 \pm 26.8	<i>P</i> < 0.0001 vs s.c. control
4 wk i.p. 5% dextrose	75, 76, 76, 76, 77, 77, 79, 82, 83, 86, 103	80.9 \pm 8.1	NS, ^c <i>P</i> > 0.05 vs s.c. control
4 wk i.p. Randomer 1	276, 281, 300, 462	329.8 \pm 88.8	<i>P</i> < 0.0001 vs i.p. control
10 wk i.p. Randomer 1	260, 326 ^b	293	<i>P</i> < 0.0001 vs i.p. control

^a One animal in the group died of a nonscrapie cause.

^b Two animals in the group died of a nonscrapie cause.

^c NS, not significant.

of PS-ONS to PrP-sen rather than PrP-res is suggested by several experiments. When the binding of rh-Randomer 1 to hamster PrP-res was measured by a centrifugation assay, the preliminary apparent K_D value was found to be at least 5 μ M (data not shown), i.e., >400-fold higher than the corresponding value for Randomer 1 binding to recombinant hamster PrP-sen shown in Table 2. Moreover, the similar internalization of rh-Randomers in scrapie-infected and PPS-cured N2a cells (Fig. 7) also suggests that PS-ON interactions with PrP-res are minimal and that the antiscrapie activities of PS-ONS are mediated primarily by binding to PrP-sen. By binding selectively to PrP-sen, PS-ONS might prevent interactions between PrP-sen and PrP-res that are critical in the conversion of PrP-sen to PrP-res. Nucleic acids are known

to alter the conformation and aggregation state of PrP-sen in cell-free reactions (9, 11, 18, 32, 33), which suggests the possibility that PS-ONS cause similar but even more stable changes in the PrP-sen conformation, preventing its PrP-res-induced conversion.

By testing different lengths and chemical modifications of ONs, their antiscrapie activities were found to be dependent on two properties: their length and the presence of a phosphorothioate backbone. This dependence on a phosphorothioate backbone was not simply due to stabilization of ONs, as a stable ON lacking a phosphorothioate backbone (Randomer 3) weakly interacted with PrP-sen and had negligible antiscrapie activity both in vitro and in vivo. The fact that no particular PS-ON sequence was required was indicated not

TABLE 5. Effects of Randomers in scrapie brain homogenate on survival times following i.c. inoculation into Tg7 mice

Expt. no. and inoculum (50 μ l)	Survival times (days)	Avg \pm SD survival time (days)	Significance between groups (Tukey test)
Expt. 1			
1% BH ^a	48, 50, 50, 52, 48, 44, 47, 48	48.4 \pm 2.4	
1% BH + 100 nM Randomer 1	49, 50, 52, 51	50.5 \pm 1.3	NS ^b ; <i>P</i> > 0.05 vs 1% BH
1% BH + 10 μ M Randomer 1	58, 58, 57, 58, 57	57.6 \pm 0.5	<i>P</i> < 0.001 vs 1% BH
Expt. 2			
1% BH	47, 46, 48, 45, 45, 45	46.0 \pm 1.3	
0.1% BH	51, 51, 53, 54, 52, 55	52.7 \pm 1.6	<i>P</i> < 0.001 vs 1% BH
0.01% BH	54, 59, 60, 59, 57	57.8 \pm 2.4	<i>P</i> < 0.01 vs 0.1% BH
0.001% BH	66, 65, 58, 62, 62, 61	62.3 \pm 2.9	<i>P</i> < 0.01 vs 0.01% BH
1% BH + 10 μ M Randomer 1	58, 57, 58, 57, 58, 58, 57	57.6 \pm 0.5	<i>P</i> < 0.001 vs 1% BH; NS, <i>P</i> > 0.05 vs 0.01% BH
1% BH + 100 μ M Randomer 1	62, 62, 61, 62, 64, 61, 67	62.7 \pm 2.1	<i>P</i> < 0.001 vs 1% BH; NS, <i>P</i> > 0.05 vs 0.001% BH
1% BH + 100 μ M Randomer 3	48, 48, 45, 46, 45	46.4 \pm 1.5	NS, <i>P</i> > 0.05 vs 1% BH
Expt. 3			
0.01% BH	53, 54, 55, 57, 58, 58	55.8 \pm 2.1	
0.001% BH	61, 63, 65, 69, 71, 75	67.3 \pm 5.3	<i>P</i> < 0.001 vs 0.01% BH
1% BH + 10 μ M Randomer 2	56, 56, 59, 62, 62, 56, 56, 54	57.6 \pm 3.0	NS, <i>P</i> > 0.05 vs 0.01% BH
1% BH + 50 μ M Randomer 2	70, 60, 67, 67, 71, 70, 77, 76	69.8 \pm 5.4	<i>P</i> < 0.001 vs 0.01% BH; NS, <i>P</i> > 0.05 vs 0.001% BH
1% BH + 100 μ M Randomer 2	72, 70, 70, 70, 67, 70, 72	70.1 \pm 1.7	<i>P</i> < 0.001 vs 0.01% BH; NS, <i>P</i> > 0.05 vs 0.001% BH
1% BH + 10 μ M 40-base Randomer 1 analog, poly(C)	52, 52, 52, 53, 54, 55, 55	53.3 \pm 1.4	NS, <i>P</i> > 0.05 vs 0.01% BH
1% BH + 50 μ M 40-base Randomer 1 analog, poly(C)	54, 55, 55, 56, 56, 56, 56, 57	55.7 \pm 0.9	NS, <i>P</i> > 0.05 vs 0.01% BH
1% BH + 100 μ M 40-base Randomer 1 analog, poly(C)	55, 56, 57, 58, 58, 59, 60	57.8 \pm 1.7	NS, <i>P</i> > 0.05 vs 0.01% BH

^a BH, brain homogenate.

^b NS, not significant.

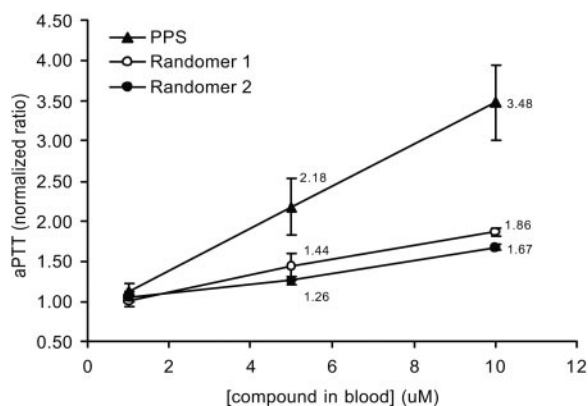


FIG. 8. aPPTs of Randomers 1 and 2 and PPS. Normalized aPTT ratio represents the fold increase in aPTT compared to that obtained with normal saline.

only by the fully degenerate nature of Randomers but also by the comparable PrP-res inhibitory activities of equivalently sized homo- and heteropolymers in vitro. This argues that the antiscrip activities of PS-ONs are derived mainly from their physiochemical properties rather than the sequence of the nucleotides. However, this does not rule out the possibility that more potent antiscrip PS-ONs might be obtained from a uniform population of a specific sequence.

For the ONs tested here, the IC_{50} s for PrP-res inhibition in vitro and the K_D values for binding to rPrP-sen were well correlated for both the size dependence (the optimum reached between 20 and 40 bases) and the requirement for a phosphorothioate backbone. The discovery of a size optimum for PrP-sen binding and activity is inconsistent with a simple charge interaction and suggests that the target for PS-ON interaction is also sterically defined. As the phosphorothioate backbone increases the hydrophobicity of oligonucleotides (1), the data presented here suggest that hydrophobic interactions and not simply the charge displayed by polyanions are important in PS-ON antiscrip activity and, furthermore, that the PS-ON binding site on PrP-sen is amphipathic. Since the data presented here show that PS-ONs and sulfated glycans bind to similar regions of PrP-sen, it seems likely that the activities of sulfated glycans and other polyanions also depend on amphipathic interactions. This would be consistent with the ability of sulfated glycans to displace bound PS-ONs from PrP-sen in correlation with their relative inhibitory activities. These observations may help to explain why not all polyanions have the ability to inhibit PrP-res formation. For instance, striking differences in antiscrip activities have been demonstrated between various sulfated glycans, even between those with similar sulfate densities (8). Sulfated glycans, like oligonucleotides, can have various degrees of amphipathic character that can depend on both the density and the distribution of sulfates. Thus, both PS-ONs and sulfated glycans probably work to prevent PrP conversion by similar mechanisms, namely, by binding to a complementary amphipathic site on PrP-sen.

The nature of the interaction between PS-ONs and PrP-sen suggests several possible mechanisms underlying the antiscrip activities of PS-ONs. PS-ONs may block or compete with interactions between PrP molecules and endogenous cel-

lular glycosaminoglycans or proteoglycans that appear to be critical in sustaining PrP-res production in infected cells (2, 43), a mechanism that has already been suggested for PPS (6, 8, 17, 43). PS-ONs also induce the internalization of PrP-sen, an effect that appears to be similar to that induced by PPS (40). This internalization might move PrP-sen to an intracellular compartment where the interaction with PrP-res and/or conversion does not occur. Finally, while the possibility of allosteric inhibition of PrP-sen conversion by PS-ONs (and sulfated glycans) cannot be excluded, it is possible that the amphipathic, sterically defined domain targeted by these molecules may be directly involved in the conformational changes required for conversion to PrP-res. Because PS-ONs frequently interact with amphipathic helices (A. Vaillant, unpublished results), it is tempting to speculate that they bind to helices with a partial hydrophobic character, such as helix 2 in the C-terminal folded domain of PrP-sen.

The fact that PS-ONs lengthened the survival times when they were added directly to the i.c. scrapie inoculum (Table 5) could be explained most simply by a direct interaction between the PS-ONs and PrP-res that interfered with the infection of relevant cells in the host. However, the apparent preferential interaction of PS-ONs with PrP-sen over PrP-res suggests that other, alternative mechanisms should also be considered. For instance, the presence of PS-ONs could affect the convertibility of PrP-sen in the vicinity of the inoculum or modify the host's clearance of the inoculum without directly interacting with PrP-res. In any case, the mechanism of action of this effect of PS-ONs remains unclear.

Potential for PS-ON treatment of TSEs. The in vivo antiscrip activities of degenerate PS-ONs were indicated in two types of experiments. When i.p. Randomer 1 treatments were initiated before a high-dose (10,000 LD₅₀s) i.p. scrapie inoculation, the survival times more than tripled (Table 4). When they were mixed directly with an intracerebral scrapie inoculum, Randomers 1 and 2 reduced the effective infectivity by ~1,000-fold (Table 5). The lack of obvious toxicity in mice after long-term parenteral dosing suggests that higher and more frequent dosing of PS-ONs by peripheral routes might be tolerated to improve prophylactic efficacy. As with PPS, PS-ONs do not appreciably cross the blood-brain barrier, so direct administration into the brain will likely be required to achieve therapeutic benefits once infections have reached the central nervous system. Rapid administration of 1 mM PS-ONs directly into the brain of a mouse was not tolerated, but gradual administration by an infusion pump might greatly reduce the toxicities of higher doses. In any case, although variations in experimental animal models and protocols complicate direct comparisons to published studies, Randomer 1 appears to be as effective prophylactically as any known anti-TSE compound.

The in vivo antiscrip activity of a CpG containing PS-ON (cpg1826) has been attributed to the stimulation of innate immunity through TLR-9-mediated mechanisms (38). The initial observation that CpG PS-ONs were effective against prion disease was surprising, as these PS-ONs resulted in the proliferation of the very cells involved in prion neuroinvasion (19). More striking was the observation that cpg1826 treatment strongly reduced the humoral response and immunoglobulin G (IgG) class switching (19), which can be used to argue that another mechanism of action, independent of the stimulation

of innate immunity, is responsible for the antiscrapie activity of cpg1826. Our data suggest that the *in vivo* antiscrapie activities of PS-ONS in the presence or the absence of CpG motifs may occur by preventing PrP conversion by direct interaction with PrP^{sen}. In our *in vivo* studies, a 40-base poly(C) PS-ON, which contains no CpG motifs, had activity comparable to that of Randomer 1, strongly suggesting that TLR-9-mediated activity was not the source of the antiscrapie activity of this PS-ON. Although non-CpG PS-ONS such as guanosine-enriched PS-ONS stimulate the proliferation of cytotoxic T cells (29) and macrophages (26) in a TLR-9-independent fashion, the actual ability of non-CpG PS-ONS to stimulate innate immunity is unclear. Liang et al. (28) demonstrated that degenerate PS-ONS (analogous to Randomer 1) as well as homopolymeric PS-ONS [poly(A), poly(T), poly(G), or poly(C)] had little or no ability to induce the proliferation of human B cells in comparison to that of a CpG-containing PS-ON. Moreover, in the same study, it was also demonstrated that degenerate, poly(C), and poly(T) PS-ONS were much weaker in inducing the production of IgA, IgG, and IgM by B cells. Since cpg1826 is basically a 20-base phosphorothioated ON, it should also directly interact with PrP^{sen} in a manner similar to the interactions described here for degenerate PS-ONS and PS-ON homopolymers. Our data argue that this direct PrP^{sen} interaction contributes to the antiscrapie efficacy of cpg1826 *in vivo*. Finally, repeated daily dosing with 60 μ g (~1.5 to 2 mg/kg in mice) of CpG PS-ONS resulted in specific TLR-9-mediated alteration of lymphoid organ morphology, including the induction of liver necrosis and hemorrhagic ascites (19). None of these toxic side effects were observed with a much more aggressive dosing regimen of Randomer 1 in animals that had received numerous repeated 10-mg/kg doses, suggesting that TLR-9-mediated toxicity is absent from Randomer 1. In any case, the *in vivo* effect of Randomer 1 in this study (a >248% increase in the survival time) is greater than that previously reported for any ON, including the \geq 82% increase in survival time reported for cpg1826 (38).

The reduced anticoagulant activities of PS-ONS compared to that of PPS may also give them a practical advantage in terms of potential side effects. This is an important consideration, because intracerebroventricular administration of PPS to animals can lead to hematomas (14), a complication likely related to the anticoagulant properties of PPS. In addition, because the Randomers are fully degenerate, there is virtually no chance for molecules of any particular sequence or group of closely related sequences to be concentrated enough to exert any meaningful aptameric or antisense effects. Furthermore, aside from the acute toxicity after rapid administration of 1 mM Randomer 1 into the brain, no *in vivo* toxicity was observed with any of the effective *in vivo* doses described here. In fact, PS-ONS (as antisense agents) have been shown to be generally well tolerated when they are administered parenterally to humans in several clinical trials (10, 21, 31). Thus, degenerate PS-ONS represent an attractive new type of anti-TSE compound that should be considered for clinical trials of treatments for CJD.

Prophylactic PS-ON treatments may have utility for reducing the risks from TSE exposure under a variety of circumstances. Prophylaxis might become warranted in at-risk animal populations after outbreaks of bovine spongiform encephalo-

pathy, chronic wasting disease, or scrapie to limit the spread of these infections. In humans, TSE prophylaxis might be considered with certain medical procedures or travel to areas where TSE is endemic. It might also be practical to add prophylactic compounds such as a PS-ON to blood products prior to transfusion to reduce the risk of TSE transmission. Nonetheless, drugs that are effective against established TSE infections will also be needed. Further experimentation will be required to assess the efficacies of PS-ONS in therapeutic circumstances and against TSE infections other than scrapie.

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[14] Searching for Anti-Prion Compounds: Cell-Based High-Throughput *In Vitro* Assays and Animal Testing Strategies

By DAVID A. KOCISKO and BYRON CAUGHEY

Abstract

The transmissible spongiform encephalopathies (TSEs) or prion diseases are infectious neurodegenerative diseases of mammals. Protease-resistant prion protein (PrP-res) is only associated with TSEs and thus has been a target for therapeutic intervention. The most effective compounds known against scrapie *in vivo* are inhibitors of PrP-res in infected cells. Mouse neuroblastoma (N2a) cells have been chronically infected with several strains of mouse scrapie including RML and 22L. Also, rabbit epithelial cells that produce sheep prion protein in the presence of doxycycline (Rov9) have been infected with sheep scrapie. Here a high-throughput 96-well plate PrP-res inhibition assay is described for each of these scrapie-infected cell lines. With this dot-blot assay, thousands of compounds can easily be screened for inhibition of PrP-res formation. This assay is designed to find new PrP-res inhibitors, which may make good candidates for *in vivo* anti-scrapie testing. However, an *in vitro* assay can only suggest that a given compound might have *in vivo* anti-scrapie activity, which is typically measured as increased survival times. Methods for *in vivo* testing of compounds for anti-scrapie activity in transgenic mice, a much more lengthy and expensive process, are also discussed.

Introduction

The transmissible spongiform encephalopathies (TSEs) or prion diseases are closely related incurable infectious neurodegenerative diseases of humans and other mammals. The incubation periods of these diseases range from months to decades. Creutzfeldt-Jakob disease (CJD) is a human TSE with an incidence of about 1 case per million people per year. Bovine spongiform encephalopathy (BSE) is a well-known TSE that has caused many billions of dollars of economic damage worldwide. BSE is also most likely responsible for approximately 180 cases of human variant CJD transmitted by consumption of contaminated beef. Strict measures to stop the spread of BSE and protect the food supply have resulted in a greatly reduced incidence in cattle and seem to have reduced the

incidence of variant CJD as well (Andrews *et al.*, 2003; Smith and Bradley, 2003).

Prion protein (PrP) is a 33–35-kDa membrane-associated glycoprotein of unknown function. The only form of prion protein found in healthy mammals is detergent soluble and sensitive to protease-degradation (PrP^C or PrP-sen). A TSE-associated form of prion protein (PrP^{Sc} or PrP-res) is highly aggregated and resistant to protease degradation (Caughey and Lansbury, 2003). PrP-res and PrP-sen have the same amino acid sequence (Stahl *et al.*, 1993), and PrP-res is formed from PrP-sen by a posttranslational conformational modification (Borchelt *et al.*, 1990; Caughey and Raymond, 1991). PrP-res is the major component of purified infectivity and is postulated to be the infectious particle of the TSEs (Prusiner, 1998).

PrP-res has consequently been a target for therapeutic intervention of the TSEs (Aguzzi *et al.*, 2001; Brown, 2002; Cashman and Caughey, 2004; Dormont, 2003). The role of PrP in TSE pathology is not well understood mechanistically, but animals lacking PrP are not susceptible to TSE infection (Bueler *et al.*, 1993). Compounds that have demonstrated anti-scrapie activity *in vivo*, which is typically measured as increased survival times, are usually also inhibitors of PrP-res in cell culture. Pentosan polysulfate, perhaps the most active anti-scrapie compound *in vivo* (Diringer and Ehlers, 1991; Doh-ura *et al.*, 2004; Ladogana *et al.*, 1992), strongly inhibits PrP-res formation in cells (Caughey and Raymond, 1993). Amphotericin B (Adjou *et al.*, 1995; Mange *et al.*, 2000; Pocchiari *et al.*, 1987) and a number of porphyrins (Caughey *et al.*, 1998; Priola *et al.*, 2000) with anti-scrapie activity also inhibit the formation of PrP-res in cell culture. Regardless of the mechanism by which these compounds work *in vivo*, inhibition of PrP-res in cell culture is one feature these anti-scrapie compounds share. Thus, new compounds that effectively inhibit PrP-res in cell culture are good candidates for the expensive and time-consuming process of testing against scrapie *in vivo*. High-throughput screening of compound libraries for PrP-res inhibitors is an efficient way to find these new candidates. In this chapter, high-throughput testing of compounds for PrP-res inhibitory activity using TSE-infected cells and a dot-blot apparatus is discussed. Demonstrating anti-TSE activity requires *in vivo* experimentation, and several different approaches to this testing in transgenic mice are also discussed.

Cell Lines Chronically Infected with TSEs

Cell lines chronically infected with TSEs have been useful tools for studying cellular processes of PrP-res (reviewed by Solassol *et al.*, 2003). However, relatively few chronically infected cell lines have been developed despite the efforts of many research groups; among these are RML mouse

scrapie strain (RML) (Race *et al.*, 1988), 22L mouse scrapie strain (22L) (Nishida *et al.*, 2000), and Fukuoka mouse-adapted CJD strain (Ishikawa *et al.*, 2004) infected mouse neuroblastoma cells (N2a). 22L also infects two different mouse fibroblast lines, NIH/3T3 and ψ 2C2 (Vorberg *et al.*, 2004). In addition, mouse neuronal gonadotropin-releasing hormone cells have been infected with RML (Sandberg *et al.*, 2004). A rabbit epithelial cell line that produces sheep PrP in the presence of doxycycline (Rov9) has been chronically infected with sheep scrapie (Vilette *et al.*, 2001). Recently, we have developed a mule deer brain cell line persistently infected with chronic wasting disease (MDB-CWD) (Raymond *et al.*, 2005). Although scrapie-infected hamster cells (Taraboulos *et al.*, 1990) and CJD-infected human cells (Ladogana *et al.*, 1995) have been reported, they seem to have been lost. Hence, Rov9 and MDB-CWD are the only non-mouse TSE-infected cell lines that are currently available.

Compounds can be tested for the ability to inhibit PrP-res accumulation in chronically TSE-infected cell lines. An assay based on cells grown in 96-well plates with dot-blot PrP-res detection can greatly increase the throughput of such testing. A requirement for this increase in throughput is that the cell line must produce enough PrP-res from one well of a 96-well plate to be readily detected on a dot blot. Mouse N2a cells infected with RML and 22L (Kocisko *et al.*, 2003), and Rov9 cells infected with sheep scrapie produce enough PrP-res to be used with dot-blot detection and 96-well plate testing (Kocisko *et al.*, 2005). In the next sections, the use of these cells in a high-throughput assay will be discussed.

N2a Cell-Based High-Throughput PrP-res Inhibition Assay

The following description of the assay is written in the context of testing a commercially available compound library such as the Spectrum Collection (Microsource Discovery). In this case, the compounds were received as 10 mM DMSO solutions in 96-well format, which was convenient for this assay.

Before the addition of compounds, approximately 20,000 RML or 22L-infected N2a cells are added to each well of a Costar 3595 flat-bottom 96-well plate with a low evaporation lid (Corning) in 100 μ l of OPTIMEM cell medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen). The OPTIMEM and the FBS lots used are pretested for the ability to sustain RML scrapie infection in mouse N2a cells for five passes as measured by analysis of PrP-res signal on immunoblot. For unknown reasons RML scrapie infections can be rapidly lost with growth in a majority of recent individual lots of OPTIMEM and rare lots of Invitrogen certified FBS. 22L-infected cells were developed by the curing of RML-infected N2a

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cells by seven passages including treatment with 1 $\mu\text{g/ml}$ pentosan polysulfate. The cured cells were then reinfected with 22L using the method of Nishida *et al.* (2000). The N2a cells reinfected with 22L scrapie have continuously expressed PrP-res for more than 80 passages. The cells are allowed to settle for at least 90 min in a 5% CO_2 incubator at 37° before compounds are added.

The 10-mM solutions of compounds in DMSO are diluted several times with PBS before addition to the cell medium. Typically, compounds are screened at 1 or 10 μM . From the final dilution into PBS, 5 μl is added to the 100- μl cell medium. For example, if compounds are being screened at 1 μM , then 5 μl of 21 μM compound solution is added to the 100 μl cell medium. If aqueous-soluble compounds are being tested, up to 20 μl of physiologically compatible aqueous solutions containing no DMSO or other solvent have been added to the cell medium without decreasing PrP-res production. Final DMSO concentrations in the cell media as the cells grow to confluence are never higher than 0.5% (v/v). DMSO concentrations higher than 0.5% (v/v) have caused morphological changes in the cells. After compound is added, the cells are allowed to grow for 4–6 days at 37° in a 5% CO_2 incubator before being lysed at confluence.

Immediately before cell lysis, the cells of each well are inspected for toxic effects, bacterial contamination, and density by light microscopy. Any differences in the cells compared with controls are noted. Cytotoxicity detected initially by light microscopy is confirmed with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) cell viability assays (May *et al.*, 2003). However, under the conditions of growth from low density to confluence in the presence of test compounds, cytotoxicity is usually obvious by light microscopy. So far, the MTT assay results have always agreed with what was noted as cytotoxicity by light microscopy.

After removal of the cell media, 50 μl of lysis buffer is added to each well. Lysis buffer contains 150 mM NaCl, 5 mM EDTA, 0.5% (w/v) triton X-100, 0.5% (w/v) sodium deoxycholate, and 5 mM tris-HCl, pH 7.4, at 4°. At this point the plates containing cell lysates can be frozen at -20° for up to 2 weeks, thawed, and the processing continued without any loss of signal. The frozen cell lysates may be stable longer than 2 weeks, but this has not been tested. Several minutes after adding lysis buffer, 25 μl of 0.1 mg/ml proteinase K (PK) (Calbiochem) in TBS is added to each well and incubated at 37° for 50 min. The treatment with PK eliminates PrP-sen and most other proteins in the lysate but only has a limited effect on PrP-res, which can then be more easily detected; 225 μl of 1 mM Pefabloc (Boehringer Mannheim) is then added to each well to inhibit PK before dot-blot analysis.

Rov9 Cell-Based High-Throughput PrP-res Inhibition Assay

The creation and characterization of the sheep scrapie-infected Rov9 cells used in this 96-well plate assay has been reported by Vilette *et al.* (2001). Rov9 cells must be grown in the presence of 1 $\mu\text{g}/\text{ml}$ ($\sim 1 \mu\text{M}$) doxycycline to maintain expression of ovine PrP. Rov9 cells are grown at 37° in 5% CO₂ and are passaged at a 1:4 dilution weekly. We have adapted Rov9 cells from MEM supplemented with 10% FBS to OPTIMEM (Invitrogen) supplemented with 10% FBS (Invitrogen), because cells chronically infected with TSEs often maintain infection better when grown with pretested lots of OPTIMEM. This adaptation was completed over the course of three passages by increasing to 50, 75, and finally 100% OPTIMEM and resulted in an increase in PrP-res production by the cells (Kocisko *et al.*, 2005). Rov9 cells are plated in 96-well plate wells in 100 μl medium, as was the case with N2a cells. After at least 90 min, appropriate dilutions of potential inhibitors in DMSO or PBS solutions are added and the cells allowed to grow to confluence during the next 7 days. DMSO in the medium at up to 0.5% (v/v) does not affect Rov9 cell growth or morphology. At confluence, any morphological changes or toxicities seen by light microscopy caused by test compounds are noted as described for N2a cells. An MTT cell viability assay (May *et al.*, 2003) is also useful with inhibitors of PrP-res in the Rov9 cells to corroborate any toxicity noted by light microscopy. The cell medium is then removed by aspiration, and 50 μl of lysing buffer is added; 25 μl of 0.2 U/ μl benzonase (Sigma) is added 5 min after lysis, and the lysates are then incubated for 30 min at 37°. The benzonase treatment eliminates clumps of nucleic acids to produce more homogeneous signals in the subsequent dot blots. This treatment is critical with the Rov9 cells and is optional with the N2a cells; 25 μl of 100 $\mu\text{g}/\text{ml}$ PK is added after benzonase treatment to give a final concentration of 25 $\mu\text{g}/\text{ml}$, and the plates are incubated at 37° for 50 min. Immediately after protease treatment, 200 μl of 1 mM Pefabloc is added to each well to inhibit further proteolysis.

Dot-Blot Procedure and Immunodetection of PrP-res on Membranes

The dot-blot procedure and immunodetection of PrP-res are identical for RML- and 22L-infected N2a cells and sheep-scrapie infected Rov9 cells. Each opening of the dot-blot apparatus (Minifold I dot-blot system, Schleicher and Schuell) is rinsed with 500 μl of TBS. The suction is adjusted so that 500 μl of liquid will go through the apparatus in about 30 sec. Variation in suction strength can lead to distortion of the signal. The PK-treated cell lysates are then put onto a PVDF membrane (Immobilon-P, 0.45- μm pore size, Millipore) through the dot-blot apparatus along with a

second rinse of 500 μ l TBS. The membrane is removed, treated with 3 M guanidine thiocyanate for 10 min, and blocked in 5% (wt/v) milk in TBS-T (TBS with 0.5% [v/v] Tween 20 added). The 3 M guanidine thiocyanate denatures PrP-res and makes it more accessible to an antibody. The membrane is then incubated with an anti-PrP monoclonal antibody, in our case 6B10 (Kocisko *et al.*, 2003), which was effective against mouse and sheep PrP-res with low background. 6H4 antibody (Prionics) is effective and presumably others will work as well. The membrane is then incubated with an alkaline phosphatase-conjugated goat anti-mouse secondary antibody in 5% milk, and then after TBST-T rinsing, an enhanced chemifluorescence agent (Zymed) is applied. PrP-res is quantified by scanning the membrane with a Storm Scanner (Molecular Dynamics) and using ImageQuant software.

The amount of input PrP-res is virtually undetectable from RML- and 22L-infected N2a cells, and the quantified PrP-res data can be used at this point. However, because the Rov9 cells are initially plated at ~25% confluent density before addition of potential inhibitors, the amount of input PrP-res in the seeded cells needs to be subtracted from all wells for more accurate results. To measure the amount of preexisting PrP-res in seeded Rov9 cells, a cytotoxic compound such as 20 μ M thiothixene is added to at least three wells per 96-well plate to prevent new PrP-res formation while cells in other wells are growing to confluency. The addition of the cytotoxic compound does not affect detection of PrP-res in the input (seeded) cells. New PrP-res accumulation during growth to confluency is calculated as the difference between the total PrP-res signal intensity and the average signal intensity from the wells containing the cytotoxic compound.

Inhibitors Found with This Assay

This assay has been useful to screen several libraries of compounds for PrP-res inhibitory activity. Many new inhibitors have been discovered through screening compounds (Kocisko *et al.*, 2003). This assay is also useful for testing smaller numbers of compounds at a range of concentrations to determine IC₅₀ values. An arbitrary IC₅₀ value of 1 μ M or less has been used as a standard for advancing a compound to animal scrapie testing, but this is only a guide, because porphyrins with *in vivo* activity have IC₅₀ values between 1 and 10 μ M (Caughey *et al.*, 1998; Priola *et al.*, 2000). Because RML and 22L mouse strains are available as chronic infections in N2a cells, any differences in compounds' inhibitory activity between these strains can be readily detected. Many compounds have been found that are better inhibitors of RML than 22L PrP-res (Kocisko *et al.*, 2005).

The availability of sheep scrapie-infected Rov9 cells allows the comparison of a compound's PrP-res inhibitory activity to be extended to other species. Many compounds that are good inhibitors of RML or 22L PrP-res are not inhibitors of sheep PrP-res in the Rov9 cells (Kocisko *et al.*, 2005), and it is not clear whether this is due primarily to differences in PrP-res or cell type. Nonetheless, these examples show that PrP-res inhibitors can have striking species-, strain-, and/or cell-type specificities that should be considered as a potential confounding aspect in anti-TSE applications.

Screening Throughput

For chronically infected cell lines amenable to a 96-well plate assay with dot-blot detection, testing compounds for PrP-res inhibitory activity is much more rapid than using a Western blot-based assay. A person assaying compounds by this method should be able to screen hundreds of compounds per week, but this number depends on how batches of test compounds are received. As noted previously, receiving a library of compounds pre-solubilized in 96-well format saves considerable setup time. Quantifying PrP-res from two plates in a day at the same time is easy. Experienced personnel can increase output to four per day by processing plates in parallel batches. Culturing multiple flasks of cells that are passed on different days of the week can help increase testing output. Finally, knowing that plates of cell lysates can be frozen to process later allows more scheduling freedom. This assay may be amenable to robotics, but this has not been attempted.

The Use of Transgenic Mice for *In Vivo* Anti-Scrapie Testing

These *in vitro* assays select promising candidates for *in vivo* anti-TSE activity on the basis of inhibition of PrP-res formation in chronically infected cell culture. Unfortunately, there is no substitute for animal testing to prove that a compound actually has *in vivo* anti-TSE activity. The TSEs are known for long incubation periods, so testing compounds for anti-TSE activity *in vivo* is a lengthy and expensive process. However, transgenic mice have been developed with greatly reduced incubation periods. One such line, Tg7 (Priola *et al.*, 2000; Race *et al.*, 2000), overexpresses hamster PrP and is highly susceptible to hamster 263K scrapie (263K) infection. High doses of 263K given intracerebrally (IC) into Tg7 mice cause disease in about 44 days, whereas high doses given intraperitoneally (IP) cause disease in 80–90 days. Another transgenic mouse line, Tga20 (Fischer *et al.*, 1996), overexpresses mouse PrP and its incubation period from RML is roughly the same as the incubation period of Tg7 mice from 263K.

Compounds can be tested for either prophylaxis or postexposure activity, depending on when dosing begins relative to scrapie inoculation. A prophylaxis test has the greater chance of success, because compound is present before inoculation. Also, a prophylaxis test against an IP inoculation allows a compound to intercept infectivity before it gets established in the brain. Once infection is established in the brain, the blood-brain barrier penetration of the compound is an issue. In general, designing *in vivo* anti-scrapie experiments involves arbitrary decisions such as when compound dosing is started relative to inoculation and how long it lasts. There are many other valid experimental designs besides the schemes outlined in the following, which have been used with Tg7 mice and 263K infection (Kocisko *et al.*, 2004).

Another variable in animal testing is the amount of infectivity to deliver. Regardless of the route of inoculation, using high amounts of infectivity has the advantage of shorter and less variable incubation periods. This must be balanced with the possibility that high amounts of inoculated infectivity may make therapy or prophylaxis more difficult. Naturally occurring infections are likely to involve much lower levels of infectivity than can be dosed in a laboratory setting. A compromise approach is to use intermediate doses of infectivity that result in reasonable incubation periods. In the case of 263K dosed IC into Tg7 mice, 50 μ l of 0.001% brain homogenate results in incubation periods of approximately 70 days, and this has been used in some tests (Kocisko *et al.*, 2004).

To test for treatment of an established scrapie infection in the brain, compound administration is started 2 weeks after IC scrapie inoculation and continues for 5–6 weeks. A 2-week delay after IC inoculation before starting treatment allows the disease time to progress before the compound is administered. To test for prophylaxis, compound is administered for a total of 6 weeks starting 2 weeks before and continuing for 4 weeks after IP scrapie inoculation. *In vivo* compound levels should be approaching a steady state in the mouse at the time of inoculation, enabling it to block peripheral scrapie infectivity from being established in the brain. The treatment after inoculation conceivably allows time for the animal to eliminate infectious material while the compound prevents further formation of PrP-res.

Compounds are administered either as an IP injection or in the drinking water. For IP injections, compounds are dissolved or suspended in an appropriate buffer and the dose volume is 10 ml/kg. Generally, the highest known tolerated dose of a compound in mice is given to maximize the chance of seeing an effect in all types of testing. Injections are given three times per week on Monday, Wednesday, and Friday. This dosing schedule is largely for convenience of laboratory personnel; many other dosing

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regimens are possible. If it is available, a compounds' pharmacokinetics can be helpful in planning a dosing regimen. Compound administration in drinking water is less labor intensive for sufficiently stable and soluble molecules that have known oral bioavailability. Solutions of compounds in drinking water are made to yield the desired dose on the basis of the average daily consumption of water by mice, 15 ml/100 g body weight. Compound dissolved in the drinking water is the mouse's only source of water during the dosing period. All 263K scrapie brain homogenates made up for inoculation are in PBS supplemented with 2% fetal bovine serum. Tg7 mice are euthanized when clinical signs of scrapie such as ruffed fur, lethargy, ataxia, and weight loss are present. Animals that die from inoculation, dosing, anesthetizing procedures, and any other non-scrapie causes are excluded from the data. In the course of experiments involving mice, there will be occasional deaths for reasons other than scrapie. Watching mice regularly for clinical signs of scrapie and testing brain homogenates for PrP-res by protease treatment and Western blot can eliminate scrapie as a cause of death.

Another way that compounds can be tested for *in vivo* activity is to mix them with infectious brain homogenates before inoculation. After 1-h incubation at 37°, 50 μ l of the homogenate/compound mixture is inoculated IC to see whether infectivity in the sample has been reduced. The IC inoculation route is used, because it has the fastest incubation period and no other compound administrations are done. This method has the advantages of needing only one injection and using very little compound. Compounds have been dosed directly into the ventricle of the brain by catheter and osmotic pump to test for scrapie treatment activity (Doh-ura *et al.*, 2004), but this is a labor-intensive procedure. A "mixing" experiment as described here can test many compounds for activity with much less labor. However, a compound directly injected into the brain by a needle or osmotic pump can result in problematic toxicity. Even a compound that seems relatively nontoxic dosed IP may be toxic directly injected into the brain because the blood-brain barrier has been bypassed.

Conclusion

The high-throughput dot-blot assay is a rapid and easy way to measure the amount of PrP-res produced by chronically infected cells as they grow from low density to confluence over the course of 4–6 days. A single addition of potential inhibitors to wells of a 96-well plate, added soon after plating cells, allows for inhibition of PrP-res formation to be assayed. The output data from the assay is that a given concentration of compound added to cell medium allows accumulation of a certain amount of PrP-res

in that time. As mentioned previously, the most effective known anti-scrapie compounds *in vivo* inhibit PrP-res formation in cell culture. Exactly how these compounds fight scrapie *in vivo* is not clear, and the precise role of PrP-res in disease pathology is not understood. However, on a purely practical level, screening compounds for the ability to inhibit PrP-res in cells is a rational way to seek new compounds that might be active *in vivo*. This assay on its own is not designed to discriminate between the different mechanisms that can be envisioned for inhibiting PrP-res production. For example, the assay cannot distinguish between PrP-res accumulation because of a compound binding to PrP-sen or PrP-res or inhibiting some cellular process required for PrP-res accumulation. Regardless of how inhibitors work in cell culture, testing in animals must be done to show *in vivo* anti-scrapie activity.

Cell lines infected with additional strains and species of TSEs will hopefully be available soon. As previously noted, compounds that inhibit one strain or species of PrP-res cannot be assumed to be inhibitors of all. Different activities against various mouse scrapie strains *in vivo* by the same compound have already been demonstrated (Ishikawa *et al.*, 2004). Certainly the best cell-based test for compounds effective against human TSEs will be cells infected with human TSEs, but these are currently not available.

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[15] A *Drosophila* Model of Alzheimer's Disease

By DAMIAN C. CROWTHER, RICHARD PAGE,
DHIANJALI CHANDRANATNA, and DAVID A. LOMAS

Abstract

The development of a model of Alzheimer's disease in *Drosophila* allows us to identify and dissect pathological pathways using the most powerful genetic tools available to biology. By reconstructing essential steps in Alzheimer's pathology, such as amyloid β peptide and tau over-expression, we can observe clear and rapid phenotypes that are surrogate markers for human disease. The characterization of progressive phenotypes

Prions and Transmissible Spongiform Encephalopathy (TSE) Chemotherapeutics: A Common Mechanism for Anti-TSE Compounds?

B. CAUGHEY,*† W. S. CAUGHEY,†
D. A. KOCISKO,† K. S. LEE,† J. R. SILVEIRA,† AND
J. D. MORREY‡

*National Institute of Allergy and Infectious Diseases,
National Institutes of Health, Rocky Mountain Laboratories,
Hamilton, Montana, and Institute for Antiviral Research,
Animal, Dairy and Veterinary Sciences Department, Utah
State University, Logan, Utah*

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ABSTRACT

No validated treatments exist for transmissible spongiform encephalopathies (TSEs or prion diseases) in humans or livestock. The search for TSE therapeutics is complicated by persistent uncertainties about the nature of mammalian prions and their pathogenic mechanisms. In pursuit of anti-TSE drugs, we and others have focused primarily on blocking conversion of normal prion protein, PrP^C, to the TSE-associated isoform, PrP^{Sc}. Recently developed high-throughput screens have hastened the identification of new inhibitors with strong *in vivo* anti-TSE activities such as porphyrins, phthalocyanines, and phosphorothioated oligonucleotides. New routes of administration have enhanced beneficial effects against established brain infections. Several different classes of TSE inhibitors share structural similarities, compete for the same site(s) on PrP^C, and induce the clustering and internalization of PrP^C from the cell surface. These activities may represent a common mechanism of action for these anti-TSE compounds.

Introduction

The transmissible spongiform encephalopathies (TSEs) or prion diseases are infectious neurodegenerative syndromes of mammals that include bovine spongiform encephalopathy (BSE), chronic wasting disease (CWD) of deer and elk, scrapie in sheep, and Creutzfeldt–Jakob disease (CJD) in humans. TSEs have incubation periods of months to years but after the appearance of clinical signs are rapidly progressive, untreatable, and invariably fatal. Attempts to develop therapeutic strategies for these diseases are

Byron Caughey is Chief of the TSE/Prion Biochemistry Section of LPVD, Rocky Mountain Laboratories (RML).

Winslow S. Caughey is Professor and Chair Emeritus in the Department of Biochemistry and Molecular Biology at Colorado State University and volunteer at RML.

David A. Kocisko is a Staff Scientist in LPVD, RML.

Kil Sun Lee and Jay R. Silveira are postdoctoral fellows in the TSE/Prion Biochemistry Section, RML.

John D. Morrey is a Professor at the Institute for Antiviral Research at Utah State University.

hobbled by gaping holes in the understanding of the transmissible agent (or prion) and the pathologic consequences of its propagation in the host. Nonetheless, recent studies have placed tighter limits on the nature of TSE infectivity, suggested salient features of TSE neurotoxicity, and revealed new anti-TSE compounds and treatment regimens that prolong the lives of infected individuals.

The Nature of TSE Infectivity: Protein-Only Prions?

The full molecular nature of TSE infectivity and its propagation mechanism remain unclear. One critical component appears to be an abnormal form of prion protein called PrP^{Sc}. PrP^{Sc} is defined loosely by its apparent association with TSE infectivity but, otherwise, has variable properties and is poorly understood structurally.¹ Usually, if not always, PrP^{Sc} is multimeric and has greater β sheet secondary structure and protease resistance than normal PrP (PrP^C). Relative protease resistance is often used practically to discriminate PrP^{Sc} from PrP^C and gives rise to the operationally defined alternative term, PrP-res. PrP^{Sc} is made post-translationally from the normal protease-sensitive prion protein. The mechanism of this conversion is not well understood but involves the ability of multimeric PrP^{Sc} to bind PrP^C and induce a conformational change as PrP^C is recruited into the growing PrP^{Sc} multimer.

The prion hypothesis posits that PrP^{Sc} is the only necessary component of TSE infectivity.² Efforts to test this hypothesis have led to recent reports of the *in vitro* generation of TSE prions.^{3,4} Synthetic truncated prion protein (PrP) fibril preparations were shown to accelerate disease when inoculated into transgenic mice that vastly overexpress the same truncated PrP construct.⁴ However, these fibrils were not infectious for normal mice and thus were $\geq 10^8$ -fold less infectious than bona fide PrP^{Sc}. Although it was concluded that prions had been synthesized from recombinant PrP^C alone, the lack of controls leaves open the possibility that the recipient transgenic mice were spontaneously making prions.

In contrast, others have shown compelling evidence for continuous serial amplification of robust TSE infectivity in cell-free reactions containing crude brain homogenate.³ This landmark result virtually eliminates the possibility that replication of an agent-specific nucleic acid genome is required. However, these studies also do not prove the “prion protein-only” model for TSE infectivity because many other host-encoded molecules besides PrP were present in the reaction.

The Most Infectious Prion Protein Particles

A fundamental question with many neurodegenerative protein misfolding diseases is whether large fibrillar

* Corresponding author. Mailing address: Rocky Mountain Labs, 903 S. 4th St., Hamilton, MT 59840. Phone: (406) 363 9264. Fax: (406) 363 9286. E-mail: bcaughey@nih.gov.

† National Institute of Allergy and Infectious Diseases.

‡ Utah State University.

deposits or smaller subfibrillar oligomers are the prime causes of disease.¹ To address this question with respect to TSE diseases and characterize the basic infectious unit of TSE infectivity, we have fractionated infectious PrP-containing aggregates by flow field-flow fractionation and compared their infectivity per unit protein (i.e., specific infectivity).⁵ Nonfibrillar particles between about 300–600 kDa (mass equivalent to ~14–28 PrP molecules) had much higher specific infectivity than larger fibrils or smaller oligomers (≤ 5 -mers) of PrP. These most infectious particles were ~25 nm in diameter, consistent with particles detected previously in filtration⁶ and field flow fractionation⁷ experiments. In our analyses, the infectivity levels were nearly proportional to the concentration of *particles* rather than protein, suggesting that as long as PrP^{Sc} oligomers are above a minimal size, they are similarly infectious *in vivo*.⁵ Accordingly, per unit mass, smaller particles are more infectious than larger ones. Although the predominant protein constituent of the “most infectious” particles was PrP, it remains possible that other molecular constituents are important.

Thus, our results also fall short of providing firm support for a protein-only nature of mammalian prions. On the contrary, it seems just as plausible to argue that host-derived molecules besides PrP might be required for robust TSE infectivity. For example, there is growing evidence that sulfated glycosaminoglycans (GAGs),^{8–10} nucleic acids, or both could be essential, at least as cofactors in pathological PrP conversion.^{11–13} Indeed, as discussed below, compounds such as these, or analogues thereof, can interact with PrP, alter its conformation, and have potent anti-TSE activities. Nonetheless, these findings support the emerging view that with many protein aggregation diseases, smaller nonfibrillar oligomers are more pathological than large fibrils or clusters of fibrils (plaques).

Neuropathologic Mechanisms

Although the enigmatic PrP^{Sc} multimer seems almost certain to be a major component of the transmissible agent, it is not necessarily the main neurotoxin of TSE diseases. Alternative forms of PrP have also been observed that may play primary roles in neuropathogenesis (reviewed in ref 1). Furthermore, there is evidence that the neuropathology of TSE infections is greatly enhanced by the presence of PrP^C^{14,15} and, more specifically, PrP^C that is anchored to cellular membranes by its glycosphosphatidylinositol (GPI) anchor.¹⁶ In scrapie-infected transgenic mice expressing only anchorless PrP^C, PrP^{Sc} (PrP-res) and TSE infectivity are propagated, but the resulting neuropathological and clinical effects are dramatically reduced.¹⁶ Thus, it is likely that in addition to being the substrate for PrP^{Sc} formation, GPI-anchored PrP^C somehow transduces or potentiates the neurotoxicity of TSE infections.

Prophylactic and Therapeutic Strategies

Despite fundamental uncertainties regarding the infectious agent, its replication mechanism, and neuropatho-

logical manifestations, a number of anti-TSE interventions have been pursued. An important but elusive goal is to be able to treat the disease after the appearance of clinical signs. This will most likely involve some combination of inhibiting PrP^{Sc} formation, destabilizing existing PrP^{Sc}, blocking neurotoxic effects of the infection, and promoting the recovery of lost functions in the central nervous system (CNS). Another worthwhile goal is to reduce the risk of infection in the first place by neutralizing sources of infection, blocking infections via the most common peripheral routes, or blocking neuroinvasion from the periphery. Although immunotherapies are being pursued with some tantalizing results,^{17,18} we have focused primarily on chemotherapeutic approaches. Although no clinically proven anti-TSE drug has been developed, significant progress has been made, especially in identifying compounds with prophylactic activity.

In Vitro Screens for Anti-PrP^{Sc} Compounds

Most TSE drug discovery efforts to date have attacked PrP^{Sc} accumulation.¹⁷ Our usual approach has been first to screen for inhibitors using TSE-infected cell cultures and then to test the most promising inhibitors against scrapie infections in rodents. Higher throughput screens have enabled the testing of thousands of compounds against multiple strains of murine and sheep scrapie in cell cultures.^{19,20} Recent development of the first deer cell line chronically infected with CWD has enabled us to begin screening compounds for activity against this cervid TSE disease as well.²¹ Unfortunately, no cell lines are available that are infected with BSE or human CJD, despite the great significance of these TSEs to public health and agriculture. The importance of testing compounds against multiple TSEs in multiple cell types is indicated by the striking species and strain specificities of PrP^{Sc} inhibitors that have been observed already.^{19,20}

Testing in Animals

A much slower process in TSE drug development is the testing of compounds against infections in animals. Despite possible problems with strain and species dependence of anti-TSE compounds, most *in vivo* testing has been done in rodents, which allow for much faster and less expensive screening than is possible in the natural, large-animal host species. Drug treatments initiated after high-dose intracerebral inoculations test for potential therapeutic activities in hosts with established CNS infections, the most difficult challenge in TSE therapeutics. Often it is also of interest to test for prophylactic protection against lower dose inoculations by peripheral routes (e.g., intraperitoneal).

Anti-TSE Compounds

A growing list of compounds has been reported to have anti-TSE activity *in vitro* and *in vivo* (Table 1). Of those that are known to inhibit PrP^{Sc} accumulation in TSE-infected cell cultures, many, but not all, also have pro-

Table 1. Compounds with in Vivo Anti-TSE Activity

class or compound	examples	inhibit PrP ^{Sc} in infected cell culture	activity prior to or soon after ip TSE inoculation	activity post-ic TSE inoculation or clinically	refs
sulfonated dyes	Congo red, suramin	+	+	+	40,55,56
sulfated glycans	pentosan polysulfate, dextran sulfate	+	+	+	52,57,58,59
polyoxometalates	HPA23	+	+	-	59,60
cyclic tetrapyrroles	porphyrins, phthalocyanines	+	+	+	23,24,25,34
polyene antibiotics	amphotericin B, MS8209	+	+	+	28,29,61
quinolines	mefloquine, quinine, quinidine	+	-	±	31,33,62
metal chelators	penicillamine	+	+	?	63
DMSO		+	±	±	24,64
flupirtine		+	?	+	65
tetracyclines	doxycycline	-	±	-	66,67
peanut oil		?	+	?	68
prednisone		?	+	?	69
phosphorothioate oligonucleotide		+	+	?	26,27

phylactic anti-scrapie activity against peripheral (e.g., intraperitoneal) infections in vivo. The most effective examples, such as, pentosan polysulfate,²² certain cyclic tetrapyrroles (cTPs),^{23–25} and phosphorothioated oligonucleotides (PS-ONs)^{26,27} can more than triple survival times of rodents inoculated intraperitoneally with high scrapie titers (e.g., 10³–10⁴ lethal doses) and completely protect animals receiving lower titers. In contrast, few compounds are known to have any beneficial effects if treatment is initiated after infection of the CNS. Many of the test compounds that are effective prophylactically have problems with blood–brain barrier penetration due to high molecular weight, charge, or both. Exceptions include the polyene antibiotics,^{28,29} which have significant toxicity problems. Much attention has been given to the anti-malarial drug quinacrine, which has anti-scrapie activity in cell culture,³⁰ crosses the blood–brain barrier, and is being administered to numerous CJD patients. However, there is no clear evidence that quinacrine is effective in vivo. We have found that the same is true of mefloquine (another anti-malarial drug),³¹ curcumin (unpublished results), and a number of other CNS-permeable compounds that potently inhibit PrP^{Sc} formation in cell culture.³² In the absence of evidence of anti-TSE efficacy in vivo, it is hard to understand the rationale for continued clinical trials of quinacrine against CJD.

Delivery of Anti-TSE Compounds into the Brain

To bypass the blood–brain barrier, Doh-Ura and colleagues have used osmotic pumps to deliver PrP^{Sc} inhibitors such as pentosan polysulfate directly to the brains of rodents via intraventricular cannulas.³³ As a result, significant extensions of scrapie incubation period were observed even with treatments directed against established CNS infections. Based on those results, similar intraventricular administrations of pentosan polysulfate have been initiated in human CJD patients, but the effects of such treatments are not clear.

cTPs, that is, porphyrins and phthalocyanines (Figure 1), are among the most promising of the anti-TSE compounds. Compounds of this class are PrP-res inhibitors in cultured cells infected with sheep scrapie, mouse scrapie, and mule deer chronic wasting disease.^{20,21,23} As noted above, cTPs can have strong prophylactic anti-scrapie activity rivaling that of pentosan polysulfate.^{24,25} Although some porphyrins are thought to cross the blood–brain barrier to some extent, this may not be true of our cTPs that are the most effective when used prophylactically or in cell cultures.

To test the efficacy of these compounds against CNS infections, we have directly injected cTPs into the brain as a crude substitute for Doh-Ura's sophisticated intraventricular osmotic pumping technique.³⁴ When weekly injections of the anionic Fe(III) *meso*-tetra(4-sulfonatophenyl)porphine (Fe-TSP) were initiated 2 weeks after a high dose (10⁶ lethal doses) intracerebral scrapie inoculation, the survival times increased by an average of 51%. Interestingly, indium- and zinc-bound TSP and various metal complexes of a cationic porphyrin *meso*-tetra(4-*N,N,N*-trimethylanilinium)porphine (TMP) had no statistically significant effects in the same experiment. In another experiment, porphyrins were mixed directly with the scrapie brain inoculum just prior to intracerebral injection to test for an ability to mask or decontaminate infectivity. Interestingly, Fe-TSP was less active in this protocol than Fe-TMP, which increased survival times as if the inoculum were diluted by 10³–10⁴.

Structure—Activity Relationships of cTPs

Compounds from each class of cTP in Figure 1 have shown anti-TSE activity in cell-free PrP conversion reactions, cell cultures, and animals.^{20,21,23–25,34} Many different types of structures were active, whereas others with seemingly similar structures were much less active. The results obtained thus far suggest that for anti-TSE activity, numerous permutations of cTP structure can often be

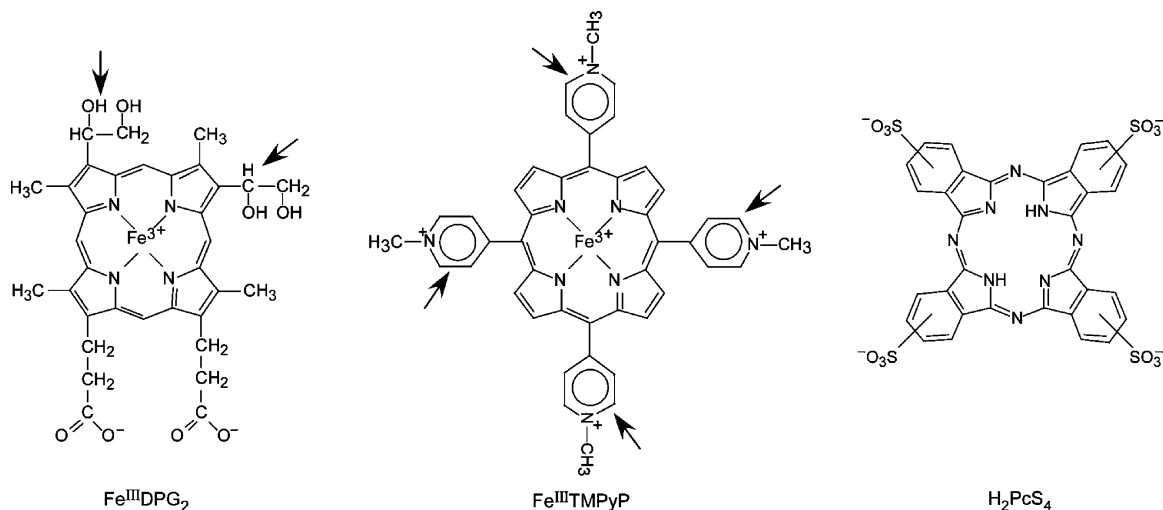


FIGURE 1. Representative cyclic tetrapyrrole (cTP) structures with anti-TSE activity. The cTPs most extensively studied have structures related to these. On the left, iron(III) deuteroporphyrin IX 2,4-bis(ethylene glycol), designated $\text{Fe}^{\text{III}}\text{DPG}_2$, represents one of many deuteroporphyrin IX derivatives with different substituents at the 2 and 4 ring positions (indicated by arrows). In the center, iron(III) *meso*-tetra(4-*N*-methylpyridyl)porphine ($\text{Fe}^{\text{III}}\text{TMPyP}$) represents synthetic porphyrins that possess aryl substituents (denoted by arrows) on the linking *meso* carbons but no peripheral ring substituents on pyrrole moieties. Aryl substituent variations include cationic 4-*N,N,N*-trimethylanilinium and anionic phenyl-4-sulfonates (not shown). On the right, phthalocyanines with one to four sulfonic acid peripheral substituents are represented by phthalocyanine tetrasulfonate (H_2PcS_4). The structure shown does not designate specific binding sites for each sulfonate group in that the preparations we have used were mixtures of isomers.

tolerated, but their influence can depend on other structural elements and the type of anti-TSE assay employed. Such differences include peripheral ring substituents and centrally bound metals.

One property that appears to correlate with anti-TSE activity is the ability to assemble into supramolecular aggregates. Aggregation of many phthalocyanines and porphyrins to dimers, trimers, and higher-order oligomers in aqueous media is well-known. The extent of such self-aggregation is influenced by cTP structure and concentration, as well as the solution conditions.^{35,36} Certain cTPs can also occupy sites on proteins, nucleic acids, and other polymers as both monomers and π -stacked aggregates.^{35,37} In solution, aggregate formation could affect cTP tissue bioavailability, whereas assembly on the surface of a biopolymer such as PrP^C or PrP^{Sc} could block PrP conversion, propagation of infectivity, or both.

Comparison of anti-TSE activity with self-aggregation propensity for various metal PcS₄'s (Figure 1) supports a relationship between the two properties. Specifically, the Al^{III} derivatives exhibited much lower anti-TSE activities in vitro than did metal-free PcS₄ or several other metal PcS₄'s.²³ At the same time, the Al^{III} derivative has a lower tendency to aggregate in aqueous media than the others.³⁶ Further studies are needed to test the role of supramolecular assembly in cTP anti-TSE activities. Fortunately, a variety of techniques can be used to monitor the nature of cTP interactions with themselves and with proteins.^{35,36} Furthermore, the use of cTPs in several other medical areas has provided useful information on the biodistribution, toxicity, retention, and methods of administration of cTPs. Particularly notable are the frequently low toxicities of cTPs.^{37–39}

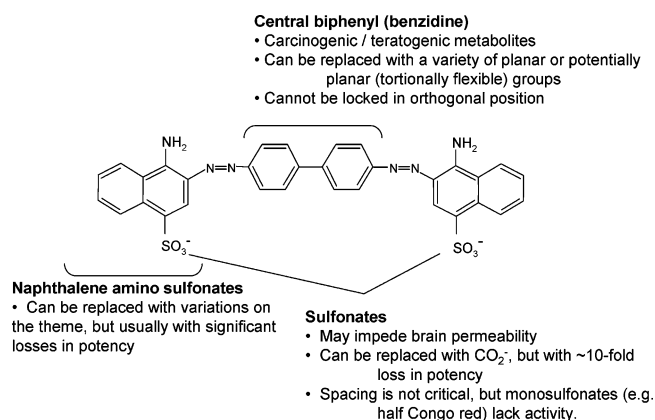


FIGURE 2. Structure–activity relationships of Congo red and analogues.

Structure–Activity Relationships with Other Anti-TSE Compounds

Like the cTPs, several other types of inhibitors of PrP^{Sc} accumulation that we have identified are planar, highly conjugated, multi-ringed molecules that are likely to have the ability to form π -stacked aggregates or similar interactions with planar nonionic surfaces on PrP molecules. Those with the best activity in vivo also tend to have one or more charged or polar moieties on the edges of the planar ring system. For example, the prototypic PrP^{Sc} inhibitor Congo red^{40–42} is a sulfonated dye (Figure 2) that is thought to form stacked aggregates within proteins such as RNA polymerase⁴³ and immunoglobulins⁴⁴ (Figure 3C).

Also notable are the observations that oligonucleotides, which contain polyanionic backbones and π -stacked bases, bind to PrP^C and induce conformational changes.^{11,12,45} More to the point are observations of PrP^C binding, PrP^{Sc} inhibition, and anti-TSE activity by phos-

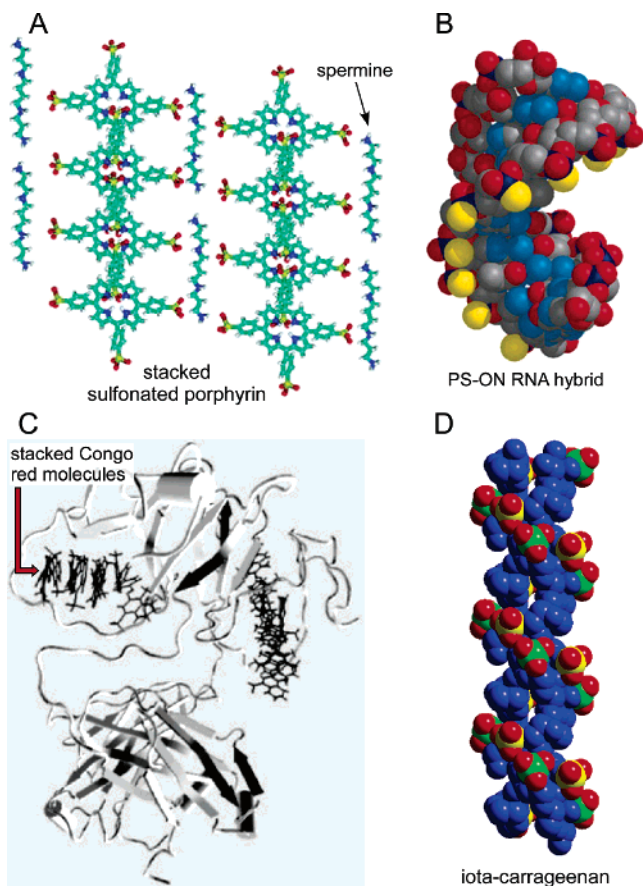


FIGURE 3. Structural similarities among different classes of anti-TSE compounds. Like the phosphorothioated oligonucleotides (PS-ONs) and sulfated glycans, planar π -stacked supramolecular aggregates of sulfonated cTPs and dyes can be extended structures with periodic negative charges and hydrophobic surfaces. Panel A shows a molecular model of tetrakis(4-sulfonatophenyl)porphyrin molecules stacked in the “J” grouping in association with the polyamine, spermine. Reproduced with permission from ref 70. Copyright 2005 Royal Society of Chemistry. Panel B shows molecular graphics of a 10-base phosphorothioate oligonucleotide hybridized with a complementary 10-base RNA. Reproduced with permission from ref 71. Copyright 2003 Biophysical Society. Panel C shows a molecular dynamics simulation of four Congo red molecules stacked in a pocket of immunoglobulin L chain λ . Reproduced with permission from ref 44. Copyright 2005 Wiley Interscience. Panel D shows an X-ray diffraction-based double-helical structure of iota-carrageenan⁴⁶ (courtesy of S. Janaswamy & R. Chandrasekaran, Purdue University).

phorothioated oligonucleotides (PS-ONs).^{26,27} The importance of the extended oligomeric character of PS-ONs was indicated by the strong dependence of activity on polymer length.²⁷ PS-ON inhibition was also dependent upon the phosphorothioate modification of the oligonucleotide backbone, which adds hydrophobicity to the polymer, but was mostly independent of base composition. Even sulfated glycan inhibitors such as pentosan polysulfate, a polysaccharide containing ~12–18 pentose disulfate sulfate units, and iota-carrageenan, a double helical sulfated glycosaminoglycan,⁴⁶ have structural analogies to both PS-ONs and stacked oligomers of sulfonated dyes and anionic cTPs, namely, repeated negative charges and hydrophobic domains (Figure 3).

A Common Inhibitor Binding Site on PrP

These analogies raise the possibility that the anionic cTPs, sulfonated dyes, PS-ONs, and sulfated glycans exert their inhibition by binding to PrP molecules at the same or overlapping sites. Indeed, competitive binding studies have shown that sulfated glycans compete with Congo red⁴⁷ and PS-ONs²⁷ for binding to PrP^C. It is tempting to speculate that the dimensions of this common inhibitor binding site on PrP^C corresponds approximately to a PS-ON 25-mer because inhibitory activity is reduced substantially with shorter PS-ON polymers.²⁷ In that case, multiple cTPs, sulfonated dyes, and other planar aromatic molecules might stack together to mimic polymeric PS-ONs or sulfated glycans (Figure 4). The display of multiple alternating anionic and nonpolar surfaces by such oligomeric inhibitors suggests that the binding site on PrP^C should include repeated cognate cationic and nonpolar surfaces. Such surfaces might be provided by the five octapeptide repeats and additional pseudorepeats in the flexible amino-terminal domain. Each repeat contains a cationic histidine residue and an aromatic tryptophan (or tyrosine) residue. The histidines might pair with anionic substituents on the edges of the inhibitors, while the tryptophan side chains could interact with nonpolar surfaces and even intercalate between planar aromatic regions of inhibitor molecules (Figure 4). Analyses of the sulfated glycan binding site on PrP^C by several groups have produced evidence for the involvement of residues in three different segments of the amino acid sequence: the highly cationic amino-terminal residues, the octapeptide repeats, and a more carboxy-terminal site containing residues 110–128, with differing views as to which residues are most important.^{48–50} We expect that the residues involved in binding different classes of anionic PrP^{Sc} inhibitors might vary somewhat, depending on the size and specific nature of the particular inhibitor. For instance, long sulfated glycans or PS-ONs might be able to bind to residues in all three segments of PrP^C, while the smaller planar aromatic inhibitors might have a preference for interacting with the tryptophan side chains of octapeptide repeats. In addition, planar aromatic inhibitors with anionic substituents might also be able to π -stack against themselves while forming ion pairs with adjacent PrP^C molecules as depicted in the figure at the amino-termini of the PrP^C molecules.

Whatever the precise PrP binding mechanism(s), one net effect of these inhibitors in several cases is the aggregation of PrP^C in cells. For instance, it is known that pentosan polysulfate,⁴⁹ sulfonated dyes,⁵¹ and the PS-ONs²⁷ cause PrP^C to cluster on the surface of cells and then become internalized. Furthermore, we have found that Congo red and cTPs (R. Kodali and B. Caughey, unpublished data) can cause aggregation of recombinant PrP^C. Hence, in the model depicted in Figure 4, we show PrP^C molecules being pulled together by the inhibitors. In each case, it seems plausible for these inhibitors to serve as a bridge between PrP^C molecules. With this in mind, it is noteworthy that activity is eliminated by cutting

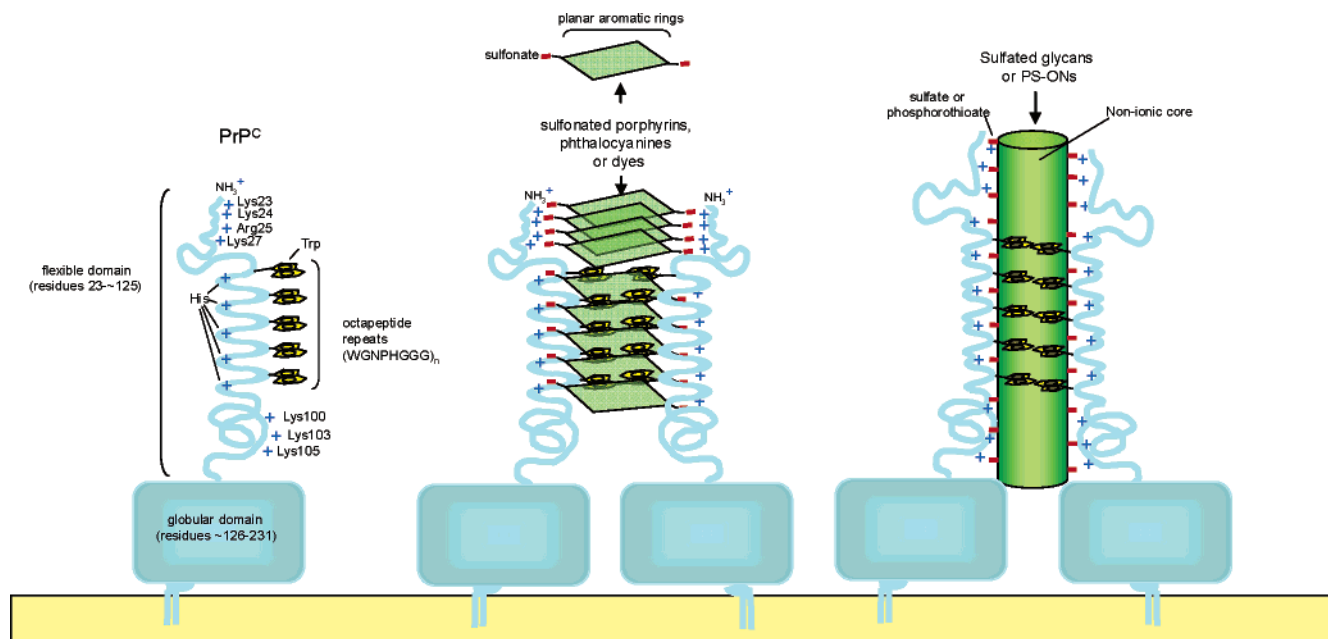


FIGURE 4. Model of possible interactions between PrP^C and various PrP^{Sc} inhibitors that cause PrP^C aggregation. The left panel shows diagrammatic PrP^C structure emphasizing the planar aromatic tryptophan side chains in the octapeptide repeats and cationic residues in regions that have been implicated in sulfated glycan binding as described in the main text. In the middle panel, planar aromatic sulfonated inhibitors such as the sulfonated porphyrins, phthalocyanines, and azo dye molecules (e.g. Congo red) are shown to be stacked directly against one another while ion-pairing with cationic residues at the amino-terminus, and co-stacked with tryptophan (Trp) side chains in the octapeptide region while ion-pairing to histidine (His) residues. In the right panel, extended polyanionic inhibitors such as sulfated glycans and phosphorothioated oligonucleotides are also shown to bind via similar ion pairs and hydrophobic interactions with aromatic side chains in the octapeptide repeats. These interactions could result in dimerization (as shown) or higher order clustering of PrP^C molecules as has been observed on the cell surface with several of these types of inhibitors.

Congo red in half⁴¹ (see Figure 2) or removing a third ring system in some planar aromatic polyphenols.¹⁹ Such molecules may lack sufficient planar aromatic area to be able to bind two PrP^C molecules at once. Although for simplicity we show the dimerization of PrP^C, the formation of higher order PrP^C aggregates might well be induced in a similar fashion by the inhibitor molecules or their supramolecular aggregates. Alternatively, it remains possible that aggregation of PrP^C is not mediated directly by the inhibitor molecules as depicted in the model but by induction of aggregation-prone conformations in PrP^C. At the cellular level, the PrP^C aggregation caused by these classes of inhibitors may lead to sequestration of PrP^C in a state or subcellular location that is incompatible with conversion to PrP^{Sc}.

Implications for Physiological Mechanisms of PrP Function and Conversion

The fact that several different structural classes of PrP^{Sc} inhibitors share certain properties, PrP binding sites, and abilities to cause PrP aggregation and internalization begs the question of how these phenomena might relate to the normal function of PrP^C and the mechanism of conversion to PrP^{Sc}. More specifically, it seems likely that these inhibitors bind to a site normally reserved for physiological ligands that are important in the conversion to PrP^{Sc}. Prime candidates for such ligands are sulfated glycosaminoglycans such as heparan sulfate, which bind to PrP^C,^{47,52} associate with PrP^{Sc} deposits in vivo,⁵³ and support PrP

conversion.^{8,9} Consistent with this view is the observation that many of the PrP^{Sc} inhibitors discussed above can be viewed as glycosaminoglycan analogues or mimics. If PrP molecules interact with polyanions, then it is also reasonable to expect that the polycationic inhibitors (e.g., branched polyamines⁵⁴ and cationic cTPs^{23,34}) could mask cellular polyanionic molecules such as GAGs that must bind to induce and stabilize the conversion of PrP^C. Polycations might also interact directly with PrP, possibly via bridging cations. In addition, crucial interactions with other cellular ligands and surfaces might be directly or indirectly affected by inhibitor binding. While such effects may block PrP^{Sc} formation, they might also have negative consequences relating to functions of PrP^C. Hopefully, further studies of the normal and disease-associated interactions and functions of PrP isoforms will suggest new and improved therapeutic strategies for the TSE diseases.

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Enhanced Antiscrapie Effect Using Combination Drug Treatment

David A. Kocisko,^{1*} Byron Caughey,¹ John D. Morrey,² and Richard E. Race¹

Laboratory of Persistent Viral Diseases, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, Montana,¹ and Institute for Antiviral Research, Animal, Dairy, and Veterinary Sciences Department, Utah State University, Logan, Utah²

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Combination treatment with pentosan polysulfate and Fe(III)meso-tetra(4-sulfonatophenyl)porphine in mice beginning 14 or 28 days after scrapie inoculation significantly increased survival times. This increase may be synergistic, implying that the compounds act cooperatively in vivo. Combination therapy may therefore be more effective for treatment of transmissible spongiform encephalopathies and other protein-misfolding diseases.

The transmissible spongiform encephalopathies (TSEs), or prion diseases, include Creutzfeldt-Jakob disease (CJD) in humans, bovine spongiform encephalopathy, chronic wasting disease of deer and elk, and scrapie of sheep and goats. The appearance of variant CJD, linked to consumption of bovine spongiform encephalopathy-infected cattle, has increased awareness of TSEs. These diseases are characterized by the accumulation of an abnormal protease-resistant form of prion protein (PrP-res), derived from normal prion protein (PrP-sen) (2). Considerable evidence indicates that PrP-res is either the infectious TSE agent or a critical component (8).

Some compounds have been able to delay scrapie onset in rodents when administered at or near the time of peripheral infection, but few have helped after intracerebral (i.c.) inoculation. Two compounds effective after i.c. scrapie inoculation include pentosan polysulfate (PPS) (5) and Fe(III)meso-tetra(4-sulfonatophenyl)porphine (FeTSP) (7), which, due to poor blood-brain barrier penetration, must be administered directly to the brain. PPS, a semisynthetic carbohydrate polymer approved as an oral therapy for interstitial cystitis (Elmiron), is being infused into the brains of CJD patients as an experimental therapy (11). FeTSP, a porphyrin, recently demonstrated antiscrapie activity when administered via i.c. injections to mice with established brain infections (7). Here, we report significant antiscrapie activity by using the combined formulation of PPS and FeTSP.

Increased survival time after scrapie inoculation is a common measure of antiscrapie activity. Here, transgenic mice overexpressing hamster prion protein (Tg7) were used because of their relatively short scrapie incubation period (9). All mice were inoculated i.c. with 50 μ l of 1% (wt/vol) brain homogenate from 263K scrapie-infected hamster brains. The first of five weekly i.c. drug injections was initiated 14, 28, or 35 days later. Tg7 mice in this study were euthanized when they showed obvious scrapie clinical symptoms, which in this strain is usually within 1 day of death (5). Animal procedures were approved by the guidelines of the Rocky Mountain Laboratory Animal Care and Use Committee. FeTSP and Fe(III)meso-

tetra(4-*N,N,N*-trimethylanilinium)porphine (FeTAP) were purchased from Porphyrin Products (Logan, UT), and PPS was a gift from Biopharm Australia (Bondi Beach, Australia). Statistical calculations were made using GraphPad Prism 4 software.

Scrapie-infected mice injected i.c. separately with either PPS or FeTSP beginning 14 days after inoculation had an average increased survival time of 26.5 or 16.9 days, respectively (Fig. 1A). Treatment with a combination of PPS and FeTSP by the same dosing regimen increased survival time by an average of 52.4 days (Fig. 1A). This delay was 9 days or 21% more than the sum of the delays induced by the drugs individually (26.5 days + 16.9 days = 43.4 days). Using two-way analysis of variance (ANOVA) (10), the combined use of PPS and FeTSP produced a statistically significant positive interaction effect ($P = 0.0004$). In contrast to combined FeTSP and PPS treatment, FeTAP, an iron-substituted porphyrin without antiscrapie activity under these circumstances, did not result in an increased antiscrapie effect when combined with PPS (Fig. 1B). Consequently, although FeTSP and PPS treatment resulted in an enhanced antiscrapie effect, this is not a characteristic of all porphyrins.

Testing of PPS, FeTSP, and their combination was also started at 28 or 35 days after inoculation or at the onset of clinical symptoms (~40 to 50 days). Treatment starting at 28 days postinoculation was less effective than at 14 days. FeTSP increased survival time by an average of 3.4 days, marginally significant by an unpaired *t* test ($P = 0.057$), but PPS treatment extended life span by an average of 12.4 days (Fig. 2A). The combination extended life span by an average of 29.0 days, which is 13.2 days or 84% more than the sum of the single-compound treatment extensions. As with treatment starting at 14 days, two-way ANOVA showed a statistically significant positive interactive effect for the combined use of PPS and FeTSP ($P = 0.03$). Treatment starting at 35 days postinoculation demonstrated no significant benefit with either single-treatment group or the combination (Fig. 2B). To investigate PPS and FeTSP as a possible therapy for late-stage treatment, animals were treated with one dose of PPS and FeTSP intracerebrally and 10 mg PPS/kg of body weight intraperitoneally at the onset of clinical symptoms. Even with the additional intraperitoneal dose of PPS, no benefit was observed.

* Corresponding author. Mailing address: Rocky Mountain Laboratories, 903 S. 4th Street, Hamilton, MT 59840. Phone: (406) 375-9692. Fax: (406) 363-9286. E-mail: DKocisko@niaid.nih.gov.

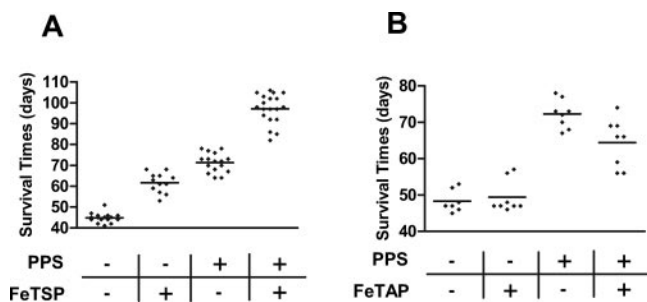


FIG. 1. Combined PPS and porphyrin treatments beginning at 14 days postinfection. Phosphate-buffered saline was the vehicle for all weekly 50- μ l i.c. injections. Injections were phosphate-buffered saline only, 0.5 mM porphyrin only, 0.05 mM PPS only, or 0.5 mM porphyrin and 0.05 mM PPS in the same solution. The line in each scatter group indicates the mean value. (A) Survival times of FeTSP- and/or PPS-treated mice after i.c. scrapie inoculation, using the combined data from two independent but identically conducted tests that gave the same results. (B) Survival times of FeTAP- and/or PPS-treated mice after i.c. scrapie inoculation.

Determination of the antiscrapie mechanism of the FeTSP-and-PPS combination treatment in vivo is hindered by an incomplete understanding of TSE infection and disease mechanisms. However, two-way ANOVA of the results from combination treatment at 14 and 28 days postinoculation suggests synergy rather than a simple additive effect (10). One possible explanation is that the presence of PPS or FeTSP might increase the half-life of the other compound by inhibiting an enzyme important in that compound's metabolism. Alternatively, each may differentially bind PrP and/or other molecules which might slow PrP-res accumulation or its pathological consequences. PPS and FeTSP individually inhibit the formation of PrP-res in chronically scrapie-infected cell cultures (3, 4); however, combinations of PPS and FeTSP were additive, and not synergistic, in this in vitro PrP-res inhibition model (Fig. 3). Also, PPS treatment alone has been shown to vastly reduce PrP-res in scrapie-infected mouse brains (5). This suggests that the in

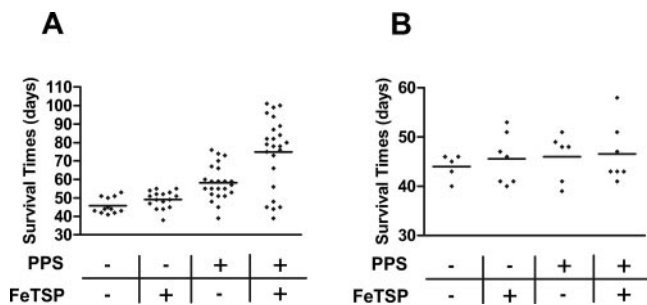


FIG. 2. Combined PPS and FeTSP treatments beginning at 28 or 35 days postinfection. Physiological saline was the vehicle for all weekly 50- μ l i.c. injections. Injections were saline only, 0.5 mM FeTSP only, 0.1 mM PPS only, or 0.5 mM FeTSP and 0.1 mM PPS in the same solution. The line in each scatter group indicates the mean value. (A) Survival times of mice treated with FeTSP and/or PPS starting 28 days after i.c. scrapie inoculation, using the combined data from two independent but identically conducted tests that gave similar results. (B) Survival times of mice treated with FeTSP and/or PPS starting 35 days after i.c. scrapie inoculation.

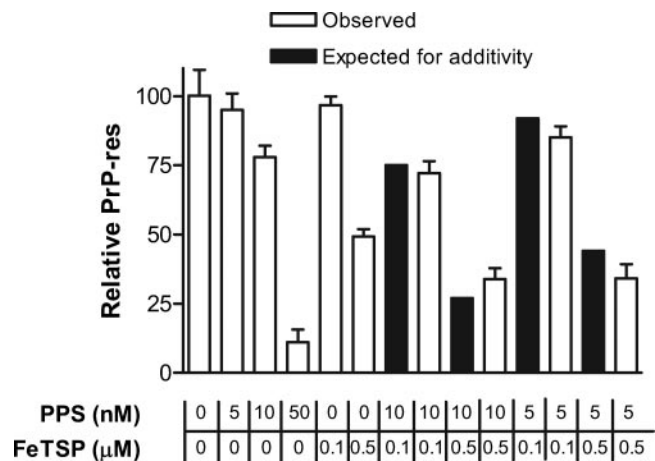


FIG. 3. Additive inhibitory effect with combinations of FeTSP and PPS in scrapie-infected mouse neuroblastoma cells. Cells chronically infected with the 22L scrapie strain were seeded at 5% confluent density and grown to confluence in the presence of the designated inhibitor concentrations. The cells were lysed and analyzed for accumulated PrP-res by dot blotting (6). The bars represent relative PrP-res contents (means \pm standard errors of the means; $n \geq 6$). "Expected for additivity" represents the sum of the mean PrP-res reductions from individual treatments. No combination of FeTSP and PPS showed a statistically significant positive interaction effect compared to separate treatments (P values were all >0.23) by two-way ANOVA (10).

vivo effects seen may involve more-complex biological interactions than the inhibition of PrP-res accumulation seen in cell culture or in vivo.

Regardless of the mechanism of action, on a practical level, the combination therapy was more effective than separate treatments. As PPS is being infused into the brains of CJD patients, the initial results reported here suggest that the addition of FeTSP to the treatment might be beneficial. Because the results from weekly i.c. dosing were so encouraging, further experiments are planned to continuously deliver PPS, FeTSP, and PPS/FeTSP to the brain by an infusion pump. It is hoped that brain infusion will be a more effective route of administration by providing a more constant concentration of drug over a longer period of time and that it will also allow a greater total dose of the combination to be safely administered. Finally, toxicology studies of PPS/FeTSP are needed, but a number of other porphyrins and porphyrin analogs have been approved for clinical use (1). Based on this finding, combination therapy for TSE treatment may lead to more-effective intervention for neurodegenerative diseases in general.

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Notes & Tips

Identification of prion inhibitors by a fluorescence-polarization-based competitive binding assay

David A. Kocisko^a, Nadine Bertholet^b, Roger A. Moore^a,
Byron Caughey^a, Andrew Vaillant^{b,*}

^a *Laboratory of Persistent Viral Diseases, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, MT, 59840 USA*

^b *REPLICor Inc., Laval, Que., Canada H7V 5B7*

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Transmissible spongiform encephalopathies (TSEs)¹ or prion diseases are associated with the misfolding of naturally occurring prion protein (PrP) into an abnormal isoform termed PrP^{Sc}. Scrapie-infected murine neuroblastoma cells are commonly used to identify compounds with potential anti-TSE activity [1] because almost all compounds with in vivo anti-TSE activity also inhibit PrP^{Sc} formation in these cells; however, many in vitro PrP^{Sc} inhibitors have not delayed TSEs in vivo [2,3]. Furthermore, cell-based assays are time consuming and costly which limits their utility for screening large numbers of compounds. Recently, antiprion screens using surface plasmon resonance [4], fluorescence correlation spectroscopy [5], and amyloid fibril formation [6] have been developed, which all show promise.

A novel in vitro antiprion screening method is presented here whose predictive ability to find anti-TSE compounds is validated by anti-TSE activity in rodent models. Phosphorothioate oligonucleotides (PS-ONs) bind strongly to natively folded recombinant PrP (rPrP) and are among the most potent anti-TSE compounds known [7]. PS-ONs longer than 30 bases are highly effective at preventing PrP^{Sc} formation in cell culture and this activity is dependent on the sequence-independent amphipathic properties of phosphorothioate oligonucleotides [7]. Known antiprion

compounds such as sulfated glycans bind at or near the PS-ON binding site on rPrP [7], suggesting that both types of molecules reversibly bind to rPrP at the same binding site. Since this regiospecific and quantifiable binding was correlated to the anti-TSE activity of the competitor sulfated glycans, we reasoned that this competitive binding could be used as an indicator of in vivo anti-TSE activity. Thus, a fluorescence polarization (FP; reviewed in [8])-based competitive binding assay was evaluated for its predictive accuracy with a larger set of compounds previously tested in rodents for anti-TSE activity [3,6,9–12].

Randomerl-FL¹ was synthesized with a single label using 3'-(6-fluorescein) CPG supports (Glen Research) and characterized as described [7]. Hamster rPrP (residues 23–231, the mature PrP sequence in vivo) was expressed in *Escherichia coli* without affinity tags and purified using a modification [13] of the method of Zahn et al. [14]. Desired concentrations of rPrP to be tested were diluted in FP assay buffer [7] in a black 96-well plate. The FP of Randomerl-FL was measured at excitation and emission wavelengths of 485/535 nm, respectively. Randomerl-FL was added to a final concentration of 3 or 10 nM and FP measured in a Tecan Ultra or Victor 3 microplate reader, respectively, with similar results. A saturating amount of rPrP (5 µg/mL; ~200 nM) and Randomerl-FL at 3 or 10 nM were incubated together for at least 30 s to ensure complete binding [7]. Test compounds in dimethyl sulfoxide were freshly diluted in assay buffer and then immediately added to the Randomerl-FL/rPrP solution to a final concentration of 10 µM. Other plate formats were suitable for this assay and a number of samples measured over the course of several hours had essentially constant millipolarization (mP) values (data not shown). Displacement of Rando-

* Corresponding author. Fax: +1 450 688 3138.

E-mail address: availlant@replicor.com (A. Vaillant).

¹ *Abbreviations used:* TSEs, transmissible spongiform encephalopathies; PrP, prion protein; PrP^{Sc}, abnormal form of prion protein; PS-ONs, phosphorothioate oligonucleotides; rPrP, recombinant prion protein; Randomerl-FL, fluorescein-labeled degenerate 40-base phosphorothioate oligonucleotide; FP, fluorescence polarization; mP, millipolarization units.

merl-FL from rPrP by the test molecules was monitored as a reduction in FP in mP. Typical FP mP baseline values of solutions containing only Randomerl-FL were 59 ± 3 . The rate of molecular tumbling decreases with binding events, thereby increasing the mP readout by increasing the polarization of light emitted from the excited fluorophore. An mP value of 261 ± 5 was typically obtained upon binding between rPrP and Randomerl-FL and the K_D for this binding was 16 nM [7]. As expected, increasing concentrations of unlabeled Randomerl competed for binding to rPrP, which lowered mP values and indicated displacement of bound Randomerl-FL. Compounds in this assay were revalidated with a dose–response curve which was then used to generate K_i values (50% competition of bound, Randomerl-FL). Each compound identified as a competitor was also further tested in the absence of rPrP to rule out direct interaction with free Randomerl-FL, which would be detected as an increase in FP readout. Table 1 lists 24 compounds previously determined to possess PrP^{Sc}-inhibitory activity in infected cells and/or anti-TSE activity in rodents. A “yes” for cell culture antiscurpie activity indicates that the compound has an IC_{50} (the concentration inhibiting 50% of PrP^{Sc} formation in cells) value $\leq 10 \mu\text{M}$ in scrapie-infected murine neuroblastoma

cells. A “yes” for in vivo antiscurpie activity means that the compound has at least demonstrated a statistically significant prophylactic effect in an animal model. Each compound was initially tested at $10 \mu\text{M}$ for the ability to displace rPrP from Randomerl-FL. Results from this screening were then compared to the cell-based PrP^{Sc} inhibition by those compounds at $\leq 10 \mu\text{M}$. By this simple and direct comparison the FP-based competition assay more accurately predicted the in vivo anti-TSE activity of these compounds than the cell-based PrP^{Sc} inhibition assay (73% vs 40% accuracy, Table 1). Unfortunately, some compounds cannot be tested in scrapie-infected cells due to cytotoxicity, as seen with the tetracyclines included in Table 1. Moreover, some PrP^{Sc} inhibitors identified in cell culture failed to displace Randomerl-FL from rPrP in the FP-based assay, thus highlighting the need for complimentary in vitro models.

The relationship between K_i (FP assay), cell culture IC_{50} , and prophylactic anti-TSE activity in vivo was examined for 23 compounds previously tested for in vivo antiscurpie activity in rodents (Supplementary Table 1). Compounds with FP competition K_i values of $< 6000 \text{ nM}$ typically had anti-TSE activity in vivo (6 of 7), while 12 of the remaining 16 compounds with K_i values $\geq 6000 \text{ nM}$ did not. A trend

Table 1

Ability of the FP-competition- or cell-based in vitro PrP^{Sc} inhibition assay to predict in vivo antiscurpie activity

Competitor added to Randomerl-FL bound to rPrP	Avg \pm SD FP (mP)	Competitor in FP assay	Anti-scurpie activity		Predictive of in vivo activity	
			Cell culture ^a	In vivo	FP	Cell culture
Unbound Randomerl-FL (No competitor)	59 ± 3	NA	NA	NA	NA	NA
Bound Randomerl-FL (No competitor)	261 ± 5	NA	NA	NA	NA	NA
500 nM Randomerl	86 ± 3	Yes	Yes	Yes	✓	✓
10 μM Randomerl	60 ± 6	Yes	Yes	Yes	✓	✓
10 μM Trifluoperazine	258 ± 2	No	Yes	No	✓	✗
10 μM Tetracycline	276 ± 1	No	No ^b	Yes	✗	NA
10 μM Tannic acid	251 ± 3	No	Yes	No	✓	✗
10 μM Doxycycline	257 ± 6	No	No ^b	Yes	✗	NA
10 μM Thiothixene	254 ± 8	No	Yes	No	✓	✗
10 μM Tetrandrine	252 ± 6	No	Yes	No	✓	✗
10 μM Thioridazine	253 ± 3	No	Yes	No	✓	✗
10 μM Congo red	260 ± 9	No	Yes	Yes	✗	✓
10 μM Amodiaquine	264 ± 11	No	Yes	No	✓	✗
10 μM Minocycline	276 ± 2	No	No ^b	Yes	✗	NA
10 μM Mefloquine	257 ± 4	No	Yes	No	✓	✗
10 μM Curcumin	NA ^c	NA	Yes	No	NA	✗
10 μM NiPCTS	50 ± 3	Yes	Yes	Yes	✓	✓
10 μM PCTS	56 ± 6	Yes	Yes	Yes	✓	✓
10 μM CuPCTS	82 ± 8	Yes	Yes	Yes	✓	✓
10 μM Deuterohemin Cl	123 ± 4	Yes	Yes	No	✗	✗
10 μM CuTSP	190 ± 7	Weak	Yes	No	✓	✗
10 μM ZnTSP	226 ± 6	Weak	Yes	No	✓	✗
10 μM Hemin	246 ± 3	No	Weak	Minimal ^d	✓	✓
10 μM AlPCTS	255 ± 10	No	No	Minimal ^d	✓	✓
10 μM InTSP	265 ± 1	No	Yes	No	✗	✓
10 μM TSP	280 ± 4	No	Yes	Yes	✓	✗
Accuracy of in vivo activity prediction					16/22 (73%)	8/20 (40%)

NA, not applicable; PCTS, phthalocyanine tetrasulfonate; TSP, meso-tetra(4-sulfonatophenyl) porphine.

^a PrP^{Sc} $IC_{50} \leq 10 \mu\text{M}$ in scrapie-infected murine neuroblastoma cells.^b Cytotoxic concentrations were $< 10 \mu\text{M}$.^c The inherent fluorescence of curcumin prevented its use.^d Slight, but statistically significant effect.

between cell culture IC_{50} values and in vivo anti-TSE prophylaxis is harder to define. Lower IC_{50} values did not necessarily correlate with increased activity in vivo; however, virtually all of the compounds with in vivo activity also inhibited PrP^{Sc} formation in scrapie-infected cell culture.

The FP-based competition assay presented here measures the ability of test compounds to displace Randomer1-FL bound to rPrP. As Randomer1 strongly binds to rPrP and ranks among the most effective prophylactic anti-TSE compounds in vivo, this PrP binding site has direct relevance for anti-TSE activity in vivo [7]. Compounds identified by this method are likely to bind to the same site on rPrP as Randomer1. The FP competition assay is therefore an indirect way to screen libraries for compounds that bind to rPrP specifically at the Randomer1 binding site. This distinguishes the FP-based competition assay from others measuring direct PrP–compound interactions, which may vary significantly in their specificity [4,5]. The ability to quantify binding affinity specifically to a therapeutically relevant region of rPrP may explain the predictive capabilities of the FP assay and allow further detailed structure–activity relationship studies. On a practical level, the assay is well-suited to high-throughput screening because the FP reaction comes to equilibrium within 30 s, is stable for several hours at room temperature, and is readily adaptable to multiple plate formats.

Virtually every compound that has demonstrated in vivo anti-TSE activity also inhibits PrP^{Sc} formation in infected neuroblastoma cells. However, these assays are labor intensive and require days for cell growth and PrP^{Sc} quantification. The fact that many inhibitors of PrP^{Sc} formation in cell culture do not work in vivo also suggests that there are aspects of in vivo PrP^{Sc} formation and compound bioavailability that are not recapitulated in cell cultures. Moreover, compound cytotoxicity prevents the cell-based approach from assessing all molecules in chemical libraries, which hinders the establishment of structure–activity relationships. The data presented here demonstrate that a FP-based competition assay as an initial screen prior to evaluation by other methods may be the most predictive test for in vivo activity. The convenience and predictive ability of this FP-based competition assay makes it a potential tool to analyze ever-expanding chemical libraries.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.ab.2006.11.007](https://doi.org/10.1016/j.ab.2006.11.007).

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Cyclic Tetrapyrrole Sulfonation, Metals, and Oligomerization in Antiprion Activity[∇]

Winslow S. Caughey, Suzette A. Priola, David A. Kocisko,[†] Lynne D. Raymond, Anne Ward, and Byron Caughey*

Laboratory of Persistent Viral Diseases, Rocky Mountain Laboratories, NIAID, NIH, Hamilton, Montana 59840

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Cyclic tetrapyrroles are among the most potent compounds with activity against transmissible spongiform encephalopathies (TSEs; or prion diseases). Here the effects of differential sulfonation and metal binding to cyclic tetrapyrroles were investigated. Their potencies in inhibiting disease-associated protease-resistant prion protein were compared in several types of TSE-infected cell cultures. In addition, prophylactic antiscrapie activities were determined in scrapie-infected mice. The activity of phthalocyanine was relatively insensitive to the number of peripheral sulfonate groups but varied with the type of metal bound at the center of the molecule. The tendency of the various phthalocyanine sulfonates to oligomerize (i.e., stack) correlated with anti-TSE activity. Notably, aluminum(III) phthalocyanine tetrasulfonate was both the poorest anti-TSE compound and the least prone to oligomerization in aqueous media. Similar comparisons of iron- and manganese-bound porphyrin sulfonates confirmed that stacking ability correlates with anti-TSE activity among cyclic tetrapyrroles.

The abnormal aggregation of protein monomers is commonly associated with the transmissible spongiform encephalopathies (TSEs) and over 20 other diseases, including type II diabetes and Alzheimer's, Parkinson's, and Huntington's diseases. The TSEs or prion diseases are infectious neurodegenerative diseases of mammals that include bovine spongiform encephalopathy, chronic wasting disease of deer and elk, scrapie in sheep, and Creutzfeldt-Jacob disease in humans. The pathogenesis of TSEs involves the conversion of the normally protease-sensitive prion protein (PrP^{sen} or PrP^C) to a protease-resistant amyloidogenic oligomer/multimer, called PrP^{res} or PrP^{Sc} (for a review, see reference 2). Although the full nature of TSE infectivity remains uncertain, PrP^{res} is a key, if not the sole, component.

Anti-TSE compounds are often potent inhibitors of PrP^{res} formation in cell cultures (5) and have strong prophylactic antiscrapie activities in vivo. Among the most potent classes of inhibitors are the cyclic tetrapyrroles, which include porphyrins and phthalocyanines (5, 10, 11, 17, 18). The known phthalocyanine inhibitors contain four sulfonic acid groups at the periphery of their large aromatic ring system and may have metal ions bound to the four central nitrogens (Fig. 1) (5). The type of metal can strongly influence the potency of PrP^{res} inhibition in vitro. The only phthalocyanine tested in animals, the metal-free tetrasulfonate (H₂PcS₄), substantially prolonged the lives of scrapie-infected mice (18). H₂PcS₄ can also block the binding of PrP^{sen} to PrP^{res}, an activity that might account for the anti-TSE mechanism of action of various cyclic tetrapyrroles (15).

In addition to its effects on PrP^{res} formation, cyclic tetrapyrroles can also block other types of disease-associated protein aggregation. For instance, H₂PcS₄ suppresses the formation of α -synuclein amyloid, a pathological factor in Parkinson's disease (13). Tetrasulfonated porphyrins inhibit the aggregation of insulin (16), and hemin analogs delay fibril formation by the A/ β peptide associated with Alzheimer's disease (7). These observations raise the possibility that cyclic tetrapyrroles have similar mechanisms of action in slowing the formation of a variety of pathological protein aggregates.

The structural requirements for efficient anti-TSE cyclic tetrapyrroles and their influence on PrP^{res} formation remain unclear. The phthalocyanine tetrasulfonate (PcS₄) inhibitors carry four negatively charged SO₃³⁻ groups with the potential to bond electrostatically with complementary positive centers on PrP molecules. The presence of negative charges on the periphery is not a critical determinant of the anti-TSE activity of cyclic tetrapyrroles because an iron porphyrin {iron(III) tetra-(4-*N*-methylpyridyl)porphine [Fe(III)TMPyPo]} (Fig. 1) and a tetra-anilinium porphyrin, each with four positively charged peripheral groups, are also active both in vivo and in vitro (5, 11, 18). Moreover, the often marked differences in in vitro anti-PrP^{res} activities among the various metal-PcS₄ complexes do not correlate with several variables in the chemical properties of metals bound to cyclic tetrapyrroles, i.e., residual charge, affinity for axial ligands, and preferred stereochemistry. However, a metal-sensitive property of cyclic tetrapyrroles that may be relevant to inhibition is the ability to oligomerize via various types of π stacking (1, 6, 21).

In the present study, the effects of metal occupancy and the extent of sulfonation on the anti-TSE activities of cyclic tetrapyrroles were investigated in vivo and in vitro. The results correlated with the influence of the structure and the dissolving medium on cyclic tetrapyrrole oligomerization and π stacking.

* Corresponding author. Mailing address: RML, NIAID, 903 S. 4th St., Hamilton, MT 59840. Phone: (406) 363-9264. Fax: (406) 363-9286. E-mail: beaughey@niaid.nih.gov.

[†] Present address: Department of Neurobiology, Biogen Idec, 14 Cambridge Center, Cambridge, MA 02142.

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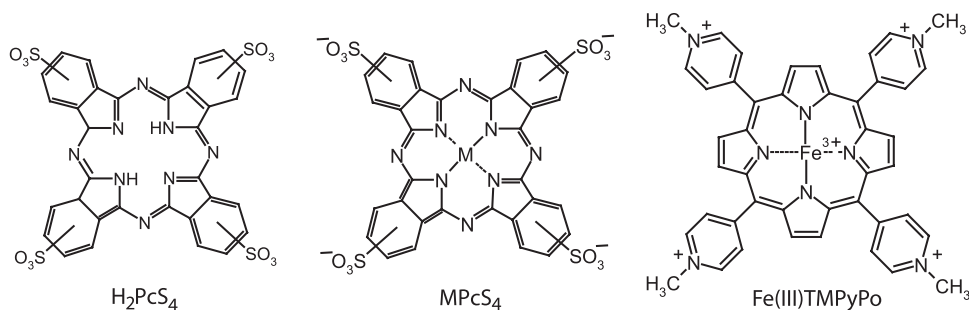


FIG. 1. Structures of H_2PcS_4 ; metal (unspecified) PcS_4 ($MPcS_4$); and $Fe(III)TMPyPo$.

These findings support the idea that oligomerization is important in the anti-TSE mechanism of cyclic tetrapyrroles and related PrP-res inhibitors.

MATERIALS AND METHODS

Phthalocyanine sulfonates. Phthalocyanine sulfonates were obtained from Frontier Science (Logan, UT) or Midcentury Chemicals (Posen, IL) as mixtures of regioisomers in undefined relative amounts. For example, with PcS_1 , which has only one sulfonate, isomers have either a 3-sulfonate or a 4-sulfonate. For PcS_2 , with two sulfonates, sulfonation may also occur on adjacent or opposite rings. The absence of defined locations for the sulfonates of the PcS_4 structures in Fig. 1 reflects the large number of regioisomers possible for a tetrasulfonate. To minimize possible ambiguities by use of different preparations for a given phthalocyanine sulfonate, the same preparation was used for cell culture, mouse, and spectral studies.

Cell culture PrP-res inhibition assays. In vitro assays for inhibition of PrP-res formation and the determination of the effective concentrations giving 50% inhibition (EC_{50} s) of PrP-res formation were performed in N2a murine neuroblastoma cells chronically infected with the RML (20) or 22L (8) scrapie strains or in a rabbit epithelial cell line (Rov9) that expresses ovine PrP-sen and that is infected with sheep scrapie (22) by either Western blotting or dot blotting (9, 12).

Scrapie infection and phthalocyanine treatments. Transgenic mice overexpressing hamster PrP-sen (Tg7 mice) have been described previously (19). Tg7 mice are highly susceptible to infection with hamster scrapie strain 263K and thus represent a relatively rapid assay system for scrapie disease inhibition (18). Brains from hamsters infected with scrapie strain 263K were Dounce homogenized in 0.32 M sucrose, the cellular debris was removed by low-speed centrifugation, and the supernatant was stored as a 10% (wt/vol) brain homogenate at $-80^\circ C$. Prior to infection the homogenate was thawed, briefly sonicated, and diluted 1:10 in phosphate-buffered saline (PBS) containing 2% fetal bovine serum (1% [wt/vol] brain homogenate); and 0.05 ml was used to infect weanling Tg7 mice intraperitoneally (i.p.) (17). The homogenate used for i.p. administration had an i.p. titer of $1 \times 10^{4.6}$ 50% infectious doses per 0.05 ml in Tg7 mice. Immediately following infection, the mice were treated i.p. with 0.05 ml of the individual phthalocyanine compounds. All of the compounds were dissolved in either water or saline, with the exception of H_2PcS_1 , which was sufficiently soluble only in dimethyl sulfoxide (DMSO). DMSO alone does not have significant anti-TSE activity (17). The concentrations of the compounds used were as follows: H_2PcS_4 , H_2PcS_2 and H_2PcS_1 , 5 mg/ml; $Ni(II)PcS_4$, $V(IV)OPcS_4$, and $Al(III)PcS_4$, 10 mg/ml; and $Fe(III)PcS_4$, 40 mg/ml. Treatment was continued three times per week over a 4-week period (12 treatments in total) and then halted. Control mice were not treated with any compounds. In separate experiments, Tg7 mice were treated with $Cu(II)PcS_4$, $Fe(III)TSP$, or $Mn(III)TSP$ dissolved in PBS to evaluate their anticrapie activities (see Table 2). These mice were inoculated with 0.05 ml of 1% scrapie strain 263K-infected brain homogenate either i.p. or intracerebrally (i.c.). All mice were monitored and euthanized when they exhibited clear signs of early scrapie infection, including tremors, ataxia, and somnolence. Where necessary, diagnoses of scrapie were confirmed by the detection of PrP-res in the brains of infected animals by using Western blot analysis.

Spectral methods. UV and visible spectra were collected by using an OLIS (Online Instruments) conversion of a Cary 16 spectrophotometer.

RESULTS

Sulfonation and metal effects on inhibition of PrP-res accumulation in vitro. The relative abilities of differently sulfonated and metal-bound phthalocyanines to inhibit PrP-res accumulation were compared by using a murine neuroblastoma cell line (N2a) chronically infected with either the RML or 22L strain of scrapie (8, 20) and the Rov9 cell line infected with sheep scrapie (12, 22). After the cells were seeded at a low density, a series of concentrations of phthalocyanine was added to the culture medium. The cells were grown to near confluence, harvested, and analyzed for PrP-res by using immunoblotting procedures, as described previously (8). The EC_{50} of the inhibitor (the concentration of inhibitor that gave 50% of the PrP-res found in the controls) was estimated from semi-quantitative analyses of the immunoblot signals (Table 1).

The extent of sulfonation of metal-free phthalocyanine had little effect on the EC_{50} values observed in a given infected cell type. The $Ni(II)$ and $Fe(III)$ complexes exhibited EC_{50} values similar to those for the metal-free compound. Other metal ions increased the EC_{50} values (i.e., reduced the anti-TSE potency of the phthalocyanine). The rank order of EC_{50} s was, in general, H_2PcS_4 , $Ni(II)PcS_4$ < $Cu(II)PcS_4$, $V(IV)OPcS_4$, $Zn(II)PcS_4$, $Mn(III)PcS_4$ \ll $Al(III)PcS_4$. $Fe(III)PcS_4$ was among the most potent in the murine cells, while it was of intermediate potency in the Rov9 cells.

TABLE 1. Phthalocyanine sulfonate inhibition of PrP-res formation in scrapie-infected cell cultures

Compound	Mean $EC_{50} \pm SD$ (μM) ($n = 3-6$)		
	Mouse scrapie strain 22L (N2a cells)	Mouse scrapie strain RML (N2a cells)	Sheep scrapie strain (Rov9 cells)
H_2PcS_1	0.8 ± 0.2	0.7 ± 0.5	NT ^a
H_2PcS_2	0.7 ± 0.1	0.4 ± 0.2	NT
H_2PcS_4	1.1 ± 0.6	1.2 ± 0.6	NT
$Ni(II)PcS_4$	0.7 ± 0.0	0.2 ± 0.0	0.9 ± 1.5
$Fe(III)PcS_4$	0.7 ± 0.1	0.4 ± 0.2	4.5 ± 1.3
$Mn(III)PcS_4$	2.5 ± 1.2	NT	4.5 ± 1.4
$Cu(II)PcS_4$	3.0 ± 0.4	NT	5.0 ± 0.7
$V(IV)OPcS_4$	3.5 ± 1.2	2.3 ± 0.1	2.6 ± 1.5
$Zn(II)PcS_4$	6.1 ± 4.1	NT	3.7 ± 1.0
$Al(III)PcS_4$	10.0 ± 1.2	>10	>20

^a NT, not tested.

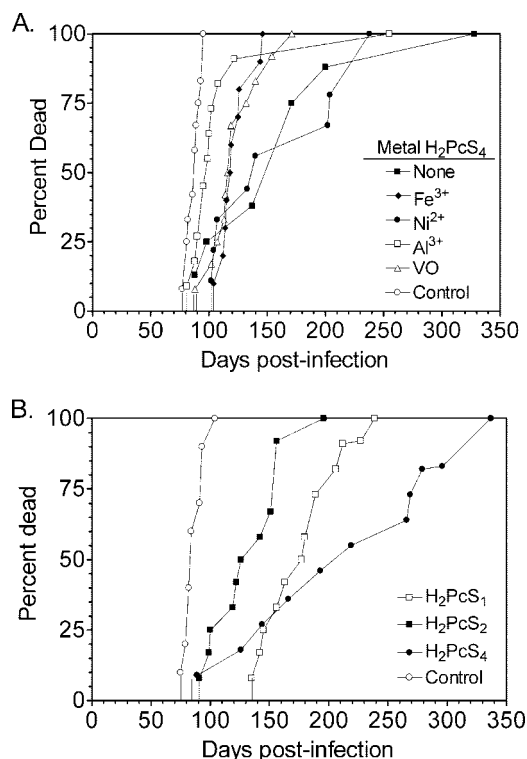


FIG. 2. Effect of prophylactic treatment with differentially metal-bound (A) and sulfonated (B) phthalocyanines on scrapie incubation periods in mice. Tg7 mice were infected i.p. with the 263K scrapie strain. "Control" designates mock-treated animals. Starting at the time of infection, mice were treated i.p. with the different compounds three times a week for 4 weeks. Comparisons of the relative efficacies of H₂PcS₁ and H₂PcS₄ (0.25 mg/dose) by Dunnett's, Tukey's, or Bonferroni's multiple-comparison tests showed no significant difference between the two compounds (*P* > 0.05), while analysis by the Newman-Keuls test showed only a marginal difference (*P* < 0.05). In some cases, the relative efficacies were not directly comparable because the phthalocyanine doses varied as follows: Ni(II)PcS₄, V(IV)OPcS₄ and Al(III)PcS₄, 0.5 mg; Fe(III)PcS₄, 2 mg.

Sulfonation and metal effects on in vivo antiscrapie activities of cyclic tetrapyrroles. To assess the abilities of the various sulfonated and metal-bound phthalocyanines to affect the progression of TSE disease in vivo, the compounds were tested in a rodent model of scrapie. Tg7 mice, which overexpress hamster PrP-sen and which are highly susceptible to hamster scrapie (19), were infected i.p. with hamster scrapie strain 263K. Starting at the time of infection, the mice were treated i.p. with the different phthalocyanines three times a week for 4 weeks, and disease incubation times were monitored. As shown in Fig. 2A, the nickel-bound and metal-free compounds significantly delayed disease incubation times compared to the times for the untreated controls (*P* < 0.01 by one-way analysis of variance [ANOVA] with Dunnett's posttest), with the metal-free phthalocyanine doing so at half or less of the doses of the metal-bound compounds. While none of the other metal-bound phthalocyanines delayed disease significantly (*P* > 0.05), in the majority of animals the V(IV)OPcS₄ and Fe(III)PcS₄ compounds appeared to delay disease at least 2 weeks longer than Al(III)PcS₄. The fact that the metal-free and Ni(II) compounds were most effective in vivo while Al(III)PcS₄ was least effective correlated with their relative abilities to inhibit PrP-res formation in vitro.

As one of the more effective in vitro PrP-res inhibitors, Cu(II)PcS₄ was tested against scrapie strain 263K in Tg7 mice by using several testing regimens (Table 2). As a prophylactic treatment, Cu(II)PcS₄ administered i.p. for 4 weeks following i.p. scrapie challenge delayed the time to disease onset by about fourfold. However, as a postexposure treatment, it was ineffective against an established i.p. infection when it was administered i.p. or an established i.c. infection when it was administered i.c.

The differently sulfonated phthalocyanines were also tested in Tg7 mice (Fig. 2B). In vivo, H₂PcS₁ and H₂PcS₄ significantly delayed the disease incubation times compared with the times for the untreated controls (*P* < 0.01 by one-way ANOVA with Dunnett's posttest), whereas H₂PcS₂ had a marginally significant beneficial effect (*P* < 0.05). It should be noted that due to relative insolubility in aqueous media, H₂PcS₁ was dissolved in DMSO prior to inoculation, possibly affecting its relative ac-

TABLE 2. Effects of different cyclic tetrapyrrole treatments on scrapie strain 263K incubation periods in Tg7 mice

Compound	Dose	Dosing regimen ^a	Scrapie infection route	Incubation periods (days)	Mean ± SD
None	None		i.p.	85, 76, 93, 91, 83, 83, 85, 97	86.6 ± 6.7
Cu(II)PcS ₄	25 mg/kg, i.p.	M, W, F for 6 wk starting 2 wk prior to inoculation	i.p.	335, 354, 353, 365, 396, 294, 378, 332	350.9 ± 31.2
Cu(II)PcS ₄	25 mg/kg, i.p.	M, W, F until death starting 50 days after inoculation	i.p.	98, 97, 83, 91, 87, 83, 87, 82, 83	87.9 ± 6.2
None ^b	50 µl PBS, i.c.	Days 14, 21, 28, 35, 42	i.c.	42, 42, 44, 44, 45, 45, 46, 46, 46, 47, 47, 48, 51, 52, 53	46.5 ± 3.3
Cu(II)PcS ₄	50 µl 0.5 mg/ml, i.c.	M, W, F for 3 wk starting 2 wk after inoculation ^c	i.c.	51, 42, 51, 58, 79, 48, 45, 48	52.8 ± 11.6
Cu(II)PcS ₄	50 µl 0.5 mg/ml, i.c.	Days 14, 21, 28, 35, 42	i.c.	41, 47, 46, 47, 44, 47, 45, 45	45.3 ± 2.1
Fe(III)TSP ^b	50 µl 0.5 mM, i.c.	Days 14, 21, 28, 35, 42	i.c.	53, 54, 58, 59, 59, 61, 62, 63, 63, 64, 65, 65, 71	61.3 ± 4.8
Mn(III)TSP	50 µl 0.5 mM, i.c.	Days 14, 21, 28, 35, 42	i.c.	46, 47, 48, 48, 48, 49, 50, 51	48.4 ± 1.6

^a M, Monday; W, Wednesday; F, Friday.

^b Combined data from two separate but identically conducted experiments.

^c Treatments were stopped at 3 weeks due to observed toxicity.

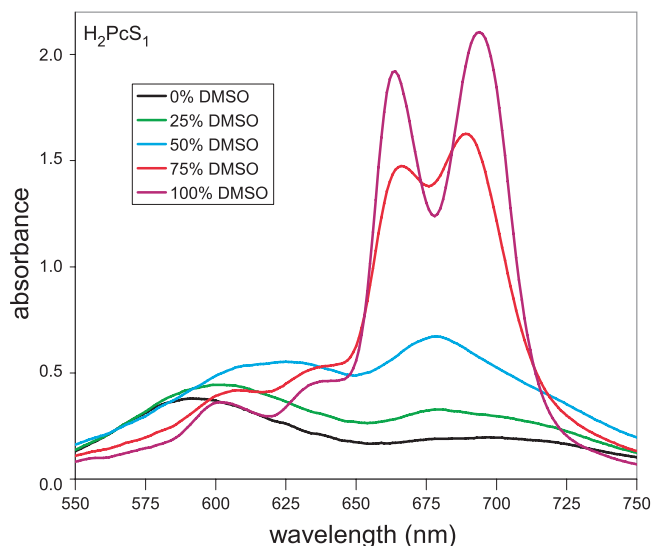


FIG. 3. Effect of solvent polarity on visible absorbance spectra of H_2PcS_1 . H_2PcS_1 solutions ($50 \mu M$) were prepared with the designated percentage of DMSO. The balance of the solvent was PBS (10 mM sodium phosphate, pH 6.9, 130 mM sodium chloride). The spectra of DMSO and PBS alone gave no significant absorbance in this spectral window (data not shown).

tivity. However, given that H_2PcS_1 and H_2PcS_4 exhibited similar effects in vivo ($P > 0.05$ or $P < 0.05$ by multiple one-way ANOVA tests; see the legend to Fig. 2B), these data are consistent with the observations in vitro and provide evidence that the number of sulfonates does not strongly influence anti-TSE activity.

Fe(III) complexed with *meso*-tetra(4-sulfonylphenyl)porphyrin [Fe(III)TSP] can substantially ($P < 0.01$ versus the results for the untreated animals) improve survival times either when it is administered i.p. prior to an i.p. scrapie inoculation or when it is administered i.c. beginning ~2 weeks after an i.c. scrapie inoculation (11) (Table 2). However, Mn(III)TSP proved ineffective in the same test.

Self-association tendencies of phthalocyanine sulfonates.

To study the possibility that the self-association of the phthalocyanines correlates with anti-TSE activity, relative aggregation tendencies were compared by using visible spectroscopy. The spectra of various sulfonates in graded DMSO-PBS mixtures were determined. The ability of DMSO to stabilize these compounds as monomeric species and the ability of aqueous media to promote their aggregation are well established (21, 24). Spectra can vary widely with changes in aggregation state and, hence, with mixtures of PBS and DMSO solvents. Aggregates characteristically exhibit much broader, less intense band maxima than the monomers from which they are formed (21). Depending on the geometric arrangement of the molecules in the aggregate, wavelength shifts toward the blue or red may be seen compared with the absorbance maximum of the monomer. For example, such solvent effects are shown for H_2PcS_1 in PBS, DMSO, and mixtures of these solvents (Fig. 3). The H_2PcS_1 spectrum in PBS exhibited a band at ~590 nm for one type of aggregate that was blue shifted and less intense compared with the spectrum of the monomer in pure DMSO. Another aggregate type yielded spectra with a broad low-in-

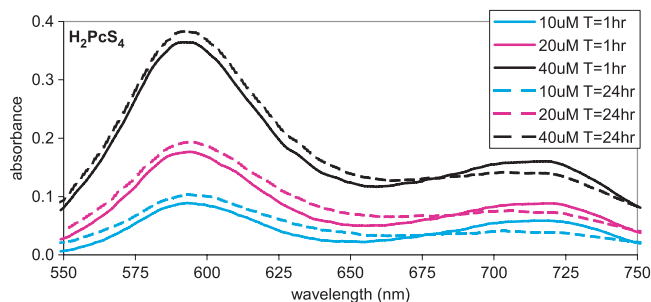


FIG. 4. Absorbance spectra of H_2PcS_4 in PBS at the designated concentrations and times (T) after solution preparation.

tensity band that was red shifted beyond 700 nm compared to the spectrum of the monomer (Fig. 3). Similar bands near 590 nm and 720 nm were observed for H_2PcS_4 in PBS. These bands were affected modestly by both concentration and the time that they were allowed to stand after solution preparation (Fig. 4). Blue-shifted species are described as being of the H type, wherein cyclic tetrapyrroles are oriented face to face, whereas aggregates with red shifts are described as being of the J type due to an edge-to-edge or slipped face-to-face orientation (21). Thus, the spectra in Fig. 4 indicate that H_2PcS_4 formed more H-type aggregate than J-type aggregate at all concentrations, but did so to a greater extent at the higher concentrations. At each concentration, slow conversions from the J type to the H type were evident. However, the spectra of solutions of H_2PcS_4 in DMSO exhibited no significant changes at comparable concentrations or times of standing (data not shown).

Relative to the similarities of the spectra of the differently sulfonated metal-free phthalocyanines, the spectra obtained for PcS_4 molecules with different metals varied widely. Such differences are evident for the Ni(II), Mn(III), V(IV)O, and Al(III) complexes in the spectra in DMSO (Fig. 5). As with H_2PcS_4 , when the solvent was changed from DMSO to PBS, the Ni(II), Mn(III), and V(IV)O complexes gave striking spectral changes that were indicative of self-association in PBS by the criteria described above (Fig. 6). In contrast, the Al(II-I) PcS_4 spectra exhibited a major band near 670 nm and a much less intense band near 600 nm under all solvent conditions (Fig. 6), which are characteristic of the spectra for the soluble monomer. The solvent-induced subtle red shifts found with Al(III) PcS_4 on going from PBS to DMSO may be attributed

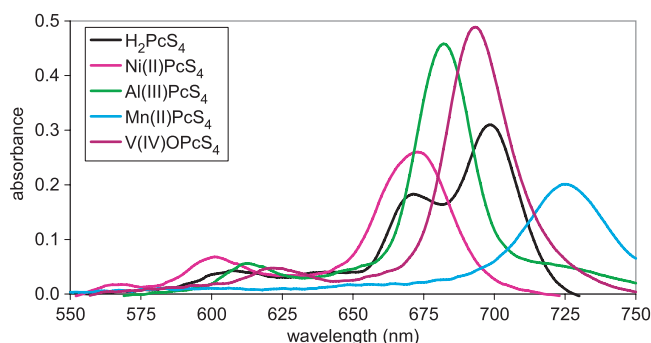


FIG. 5. Absorbance spectra of H_2PcS_4 and its Ni(II), V(IV)O, Mn(III), and Al(III) complexes at $5 \mu g/ml$ in DMSO.

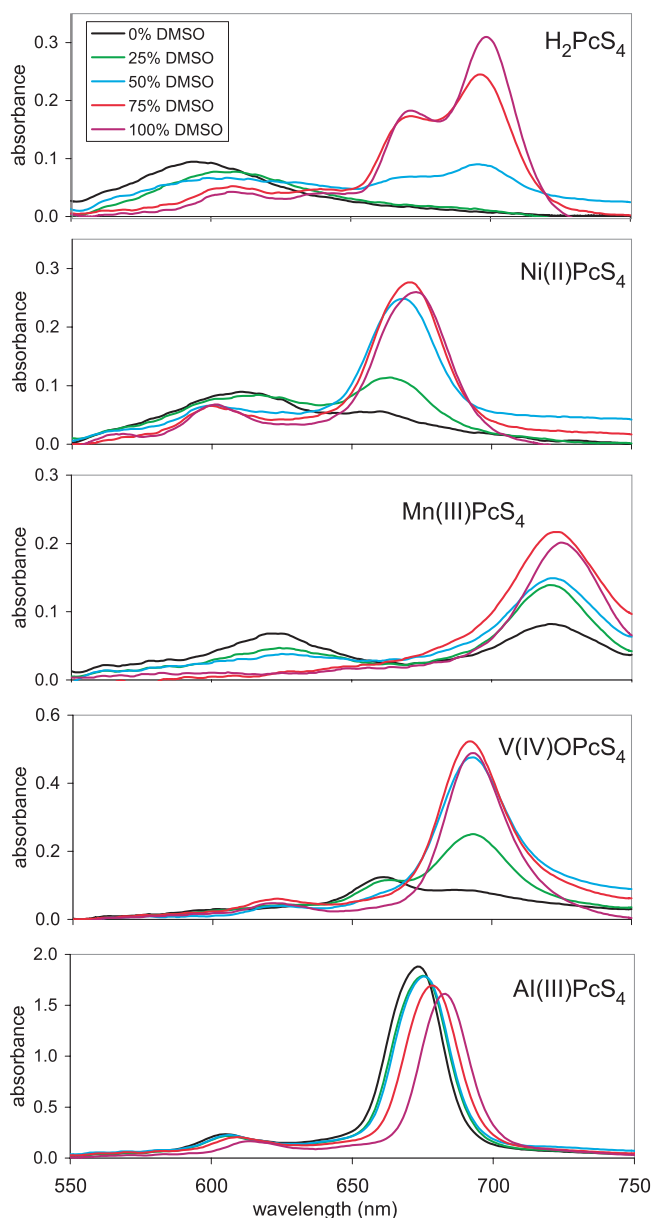


FIG. 6. Absorbance spectra of H_2PcS_4 and its Ni(II), V(IV)O, Mn(III), and Al(III) complexes in PBS, DMSO, and PBS-DMSO mixtures. The Al(III) complex was used at a concentration of $10 \mu M$, and the others were used at concentrations of $5 \mu M$. Data for a higher concentration of Al(III)PcS₄ are shown to provide stronger evidence of its relative lack of a tendency to self-associate, because the oligomerization of molecules is more likely at higher concentrations.

solely to differences in solvation. Because monomeric Al(II)PcS₄ is a much less potent PrP-res inhibitor than the other much more aggregation-prone phthalocyanines, these results suggest that self-association is important in the inhibition of PrP-res formation.

Induction of phthalocyanine sulfonate aggregation by a cationic cyclic tetrapyrrole. To further investigate aggregation behavior, Fe(III)TMPyPo (Fig. 1) was incorporated into phthalocyanine sulfonate solutions as another spectral probe and a potential nidus for the assembly of phthalocyanine oligo-

mers. Fe(III)TMPyPo is a cationic cyclic tetrapyrrole that is capable of forming ion pairs and, possibly, π -stacked oligomers with anionic phthalocyanine sulfonates (14). Spectra for solutions in PBS containing a constant concentration of Fe(III)TMPyPo but different levels of H_2PcS_4 or Al(III)PcS₄ were obtained (Fig. 7). With increasing phthalocyanine concentrations, the Fe(III)TMPyPo Soret band near 425 nm, which is in a region of low absorbance by the phthalocyanines, lost intensity as the absorbance from 575 nm to 800 nm increased. Although a detailed elucidation of the spectrum and composition of each individual heteroaggregate present was not attempted, the changes in isobestic points (points of intersection of spectral curves) observed in the spectra at 1:1 and 2:1 phthalocyanine/porphyrin ratios indicate the formation of heterodimers and heterotrimers with each of the phthalocyanines. More importantly, heteroaggregates with even higher phthalocyanine/porphyrin ratios were indicated by further changes in the isobestic point with increasing concentrations of H_2PcS_4 but not with increasing concentrations of Al(III)PcS₄. Observations similar to those made for H_2PcS_4 were found for Ni(II)PcS₄ (data not shown). In DMSO, which monomerizes phthalocyanine sulfonates, Fe(III)TMPyPo promoted the higher-order aggregation of both Ni(II)PcS₄ and H_2PcS_4 (data not shown). No interaction with Al(III)PcS₄ was evident even at high concentrations of Al(III)PcS₄ relative to those of Fe(III)TMPyPo (data not shown). These results are consistent with Al(III)PcS₄ having substantially less of a propensity to form higher-order aggregates than H_2PcS_4 or Ni(II)PcS₄, even when it is "seeded" by a cationic cyclic tetrapyrrole.

DISCUSSION

Previous studies have shown that H_2PcS_4 inhibits PrP-res formation and has strong prophylactic activities in vivo (5, 17, 18). Furthermore, in vitro studies have shown that differential metal binding by PcS₄ can affect its inhibition of PrP-res formation (5). Here, we provide evidence that the metal-dependent tendency of various cyclic tetrapyrroles to self-associate correlates with these activities in vivo and in vitro but that the degree of sulfonation of PcS₄ is not as critical a determinant of activity.

Our attempts to correlate the in vitro and in vivo effects are complicated by the fact that different strains and host species were used in the different experimental systems. Species and strain effects have been known to be influential in the activities of some types of inhibitors (12) and may partially explain some of the current observations. Accordingly, one should be cautious about drawing mechanistic parallels between the in vitro and the in vivo systems. Nonetheless, a common observation in each of the various experimental systems is that active cyclic tetrapyrroles have a propensity to self-associate or stack, while those that are least active are much less prone to self-association.

A better understanding of the structure-activity relationships of cyclic tetrapyrroles may help to identify more effective anti-TSE compounds. Increasing the number of negatively charged sulfonate groups on phthalocyanines enhances their solubility in aqueous media and influences their distribution in vivo (1, 6, 21). However, the fact that H_2PcS_1 , H_2PcS_2 , and H_2PcS_4 each showed strong anti-TSE activities in vitro and in vivo indicates

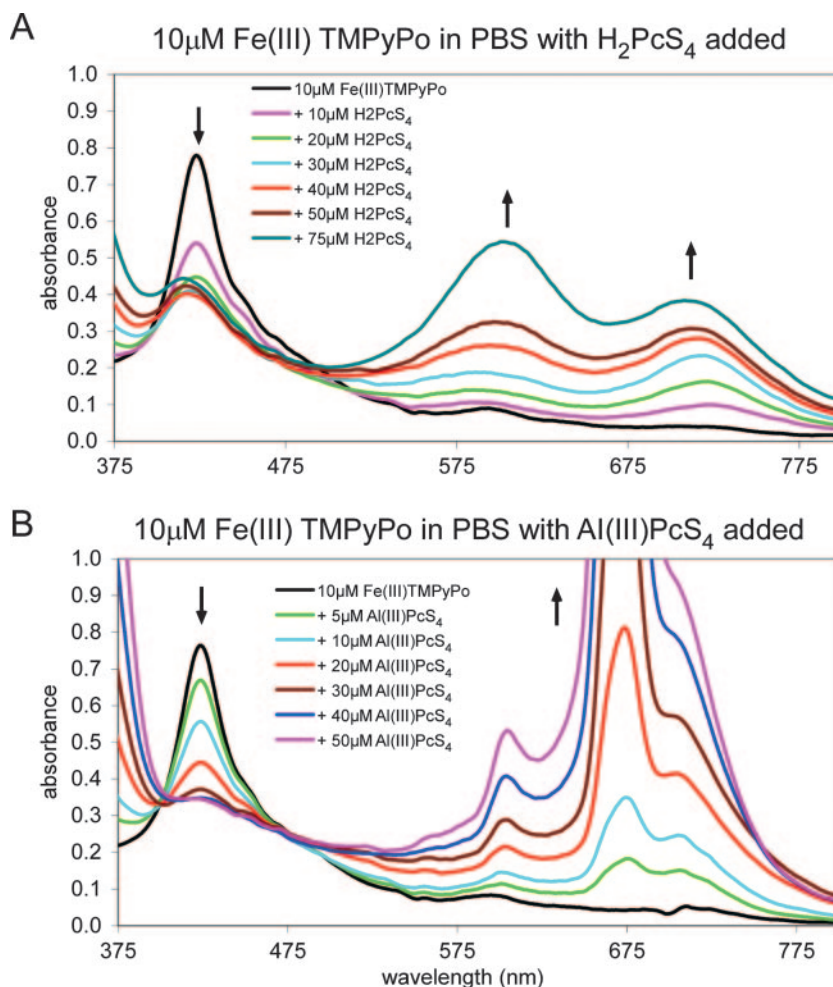


FIG. 7. Absorbance spectra of solutions of Fe(III)TMPyPo with the designated concentrations of H₂PcS₄ (A) and Al(III)PcS₄ (B). The arrows denote directions of absorbance changes as concentrations of added H₂PcS₄ or Al(III)PcS₄ increased.

that the number of sulfonate groups bearing negative charges at the periphery of metal-free phthalocyanine macrocycles is not a critical determinant of anti-TSE activity. This finding suggests that electrostatic bonding interactions between negative sulfonates and positive centers on a target binding site on PrP contribute much less to the strength of the binding than do other types of bonding, such as π - π interactions involving the phthalocyanine aromatic system. The similarity of the UV-visible absorption spectra of the different metal-free phthalocyanine sulfonates is consistent with the fact that the number of sulfonates has little effect on the π -electron systems of these highly aromatic cyclic tetrapyrroles. Furthermore, the spectral data provided evidence that the tendencies to form stacked aggregates in PBS are similar for these metal-free phthalocyanines. Thus, one property that the three differentially sulfonated phthalocyanines have in common is an ability to enter into π bonding of the type required for stacking.

The substantial influence of different metals on the *in vivo* and *in vitro* anti-TSE activities of PcS₄ compounds raises the question of how metal ion occupancy affects the behavior of this molecule. Several well-established differences in coordination chemistry among the metals in the phthalocyanine prep-

arations appeared not to be critical determinants of activity. As noted above, one such variable is the residual positive charge at the metal. The displacement of two protons from the central nitrogens of H₂PcS₄ leaves two negative charges to neutralize two positive charges associated with an incoming metal ion (Fig. 1). As a result, there is no residual charge on the metal in the Ni²⁺, Cu²⁺, and Zn²⁺ complexes of PcS₄ and one positive charge with the Fe³⁺, Mn³⁺, or Al³⁺ complexes. With V(IV)-OPcS₄, wherein V⁴⁺ is bonded to an oxygen atom, there is no charge remaining on the vanadium. The results obtained suggest that the residual charge on the metal (Table 1) is not a critical determinant of inhibitor efficacy. Another variable is the preferred coordination stereochemistry among these metals. Ni(II) and Cu(II) prefer square planar complexes, with the metal atom being coplanar with the four nitrogens to which it is bonded. Fe(III), Al(III), and V(IV) each prefer square pyramidal structures, wherein the metal lies outside the plane defined by the four nitrogens. However, if two axial ligands bind to Fe(III), one on each side of the macrocycle, the iron atom can assume coplanarity with the four nitrogens. A third variable not obviously essential for anti-TSE activity is a marked difference in affinities of the

metal for axial ligands among the metal PcS_4 s; Ni(II) and Cu(II) are expected to bind to ligands much less avidly than the other metals.

One property of cyclic tetrapyrroles that does appear to correlate with anti-PrP-res activity is their tendency to form stacked oligomers. Of the cyclic tetrapyrroles tested, Al(III)- PcS_4 has much less of a tendency to self-associate and is by far the least effective PrP-res inhibitor in vivo and in cell culture. Previous studies have also shown that Al(III) PcS_4 is a much weaker inhibitor of PrP-res formation in cell-free conversion reactions than H_2PcS_4 and Fe(III) PcS_4 (5). Additional support for a relationship between PrP-res inhibition and aggregation tendency is found in studies of Mn(III) and Fe(III) complexes of TSP, another cyclic tetrapyrrole with four sulfonic acid groups on the periphery of the molecule (5). The previous study showed the Mn(III)TSP is a poor inhibitor in cell cultures compared to Fe(III)TSP. Furthermore, as shown in Table 2, Fe(III)TSP exhibits therapeutic activity in mice, whereas under the same conditions Mn(III)TSP does not. Others have shown that Mn(III)TSP is essentially monomeric in aqueous media at physiological pH, whereas Fe(III)TSP exists predominantly as dimers or larger aggregates (23). Furthermore, we have seen less inhibition of PrP-res accumulation in vitro by Mn(III) than Fe(III) complexes of deuteroporphyrinIX-2,4-disulfonate, a sulfonated cyclic tetrapyrrole structure related to hemin (data not shown). Moreover, spectral analyses in graded PBS-DMSO were consistent with the Mn(III) deuteroporphyrinIX-2,4-disulfonate having less of a tendency to oligomerize than the Fe(III) complex. Thus, analyses of several types of cyclic sulfonated tetrapyrroles suggest the importance of oligomerization in anti-TSE activity.

The spectra of mixtures of different PcS_4 s with Fe(III)-TMPyPo indicated the formation of heteroaggregates between a positively charged molecule and one or more negative phthalocyanines. With H_2PcS_4 and Ni(II) PcS_4 , evidence for the stacking of many phthalocyanines per porphyrin was observed. Such stacking occurred to a greater extent in PBS than in DMSO. In contrast, Al(III) PcS_4 showed no tendency to form heteroaggregates in DMSO and in PBS formed heterodimers and heterotrimers [possibly sandwiches with Fe(III)TMPyPo in the middle] but not higher-order aggregates. In the sense that Fe(III)TMPyPo can serve as a nidus for the stacking of the inhibitory PcS_4 molecules, it may be acting in a manner analogous to the binding site on PrP that mediates the anti-TSE activity of cyclic tetrapyrroles. H_2PcS_4 has been shown to inhibit the conversion interaction between PrP-sen and PrP-res directly, apparently by binding to one or both forms of PrP (4, 5). Anti-TSE activity may depend on the ability of multiple cyclic tetrapyrroles to bind to PrP as a preformed aggregate or to stack sequentially onto a bound monomer. In this case, Al(III) PcS_4 molecules may bind individually to PrP but have little tendency to attract additional Al(III) PcS_4 s, accounting for its low anti-TSE activity. Such relationships are consistent with a recently proposed general mechanism of anti-TSE activity (3).

The solvent effects on phthalocyanine associations observed in these studies, as well as other reports (21), provide evidence that differences within tissue microenvironments may modify the equilibrium constants and the kinetics of formation and dissociation of aggregates. A specific geometric arrangement

that would be required for an aggregate to possess activity cannot be reliably proposed based solely on the present evidence. However, the further application of UV-visible and other spectral techniques may allow the clarification of the structure and bonding interactions of inhibitory cyclic tetrapyrrole oligomers when they are bound to PrP. Related future studies in which individual phthalocyanine sulfonate regioisomers are used may help to refine more sharply the structural requirements for maximal anti-TSE activity.

The present data are consistent with those from previous studies (17, 18) in showing that certain phthalocyanine sulfonates and other anti-TSE cyclic tetrapyrroles appear to be well tolerated by rodents receiving long-term dosing regimens. These observations remain consistent with the possibility that the development of effective pre- and postexposure prophylactic treatments against a variety of TSE/prion diseases may be possible with cyclic tetrapyrroles or related compounds.

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Hemin Interactions and Alterations of the Subcellular Localization of Prion Protein^{*S}

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Kil S. Lee, Lynne D. Raymond, Brianna Schoen, Gregory J. Raymond, Lauren Kett, Roger A. Moore, Lisa M. Johnson, Lara Taubner, Jonathan O. Speare, Henry A. Onwubiko, Gerald S. Baron, Winslow S. Caughey, and Byron Caughey¹

From the Laboratory of Persistent Viral Diseases, Rocky Mountain Laboratories, NIAID, National Institutes of Health, Hamilton, Montana 59840

Hemin (iron protoporphyrin IX) is a crucial component of many physiological processes acting either as a prosthetic group or as an intracellular messenger. Some unnatural, synthetic porphyrins have potent anti-scrapie activity and can interact with normal prion protein (PrP^C). These observations raised the possibility that hemin, as a natural porphyrin, is a physiological ligand for PrP^C. Accordingly, we evaluated PrP^C interactions with hemin. When hemin (3–10 μM) was added to the medium of cultured cells, clusters of PrP^C formed on the cell surface, and the detergent solubility of PrP^C decreased. The addition of hemin also induced PrP^C internalization and turnover. The ability of hemin to bind directly to PrP^C was demonstrated by hemin-agarose affinity chromatography and UV-visible spectroscopy. Multiple hemin molecules bound primarily to the N-terminal third of PrP^C, with reduced binding to PrP^C lacking residues 34–94. These hemin-PrP^C interactions suggest that PrP^C may participate in hemin homeostasis, sensing, and/or uptake and that hemin might affect PrP^C functions.

Iron protoporphyrin IX, a natural cyclic tetrapyrrole (cTP),² is vital to cellular homeostasis in either the Fe³⁺ (hemin) or Fe²⁺ (heme) oxidation state (supplemental Fig. 1). In hemoglobin and myoglobin the reversible binding of oxygen to the reduced iron of the heme permits oxygen transport and storage (1). Heme also plays key roles in the electron transport function of various cytochromes and in the catalytic reactions of hydrogen peroxide by catalases and peroxidases (1, 2). Hemin may serve as intracellular messengers that modulate gene expression, the opening of ion channels, micro-RNA processing, and other physiological processes (3–5). Although hemin is essential for cell maintenance, excess free hemin can trigger several toxic effects such as permeabilization of cellular membranes (6) and oxidation of proteins, lipids, and nucleic acids (7).

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^S The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. S1 and S2.

¹ To whom correspondence should be addressed: Rocky Mountain Laboratories, 903 S. 4th St., Hamilton, MT 59840. Tel.: 406-363-9264; Fax: 406-363-9286; E-mail: bcaughey@nih.gov.

² The abbreviations used are: cTP, cyclic tetrapyrrole; PrP^C, cellular prion protein; rPrP^C, recombinant PrP^C; PrP^{Sc}, scrapie PrP; GPI, glycosylphosphatidylinositol; PBS, phosphate-buffered saline; BSA, bovine serum albumin; TMB, 3,3',5,5'-tetramethylbenzidine.

A variety of synthetic cTPs, including both porphyrins and phthalocyanines (supplemental Fig. 1), have potent activity against transmissible spongiform encephalopathies or prion diseases (8–11). The anti-transmissible spongiform encephalopathy mechanism of action of cTPs appears to be inhibition of the formation of abnormal prion protein (PrP^{Sc}) (10), which is the primary component of the infectious agent of these diseases. PrP^{Sc} is generated post-translationally from the normal cellular prion protein (PrP^C) (12, 13). Some inhibitory cTPs have been shown to bind to PrP^C (10, 14, 15) and may thereby inhibit PrP^{Sc} formation. These findings suggest that PrP^C may also bind to physiological cTPs, such as hemin.

PrP^C is a glycoprotein linked to the outer leaflet of the plasma membrane by its C-terminal glycosylphosphatidylinositol (GPI) anchor (16). The unstructured N-terminal half of PrP^C contains a domain consisting of four or more repeats of the octapeptide sequence PHGGGWGQ. These repeats can coordinate copper and other divalent ions (17). High concentrations of copper can induce the endocytosis of PrP^C, suggesting that PrP^C may be involved in copper transport or homeostasis (18, 19). Interestingly, molecules such as glycosaminoglycans and other anti-transmissible spongiform encephalopathy compounds also bind to the N terminus and alter the subcellular trafficking of PrP^C (20–23). This suggests that the modulation of endocytosis of PrP^C through its N-terminal domain is important in a conserved physiological function of PrP^C.

Based on these observations, we evaluated the possibility of hemin being a physiological ligand of PrP^C. Here we show that hemin promotes PrP^C clustering, internalization, and degradation in cultured cells. In cell-free reactions, the binding of PrP^C to hemin alters the aggregation state and inherent peroxidase activity of the latter.

EXPERIMENTAL PROCEDURES

Preparation of Recombinant PrP (rPrP^C)—Cell pellets of *Escherichia coli* expressing hamster PrP corresponding to residues 23–231 or 90–231 in the pET41 vector (EMD Biosciences) were lysed with BugBusterTM and lysonase (EMD Biosciences) in the presence of EDTA-free protease inhibitors (Roche Applied Science). Inclusion bodies were washed twice with 0.1 × BugBusterTM in water and pelleted by centrifugation. The enriched rPrP^C was further purified by minor modifications to the method of Zahn *et al.* (24). The protein was eluted with 10 mM sodium phosphate (pH 5.8), 500 mM imidazole, and 10 mM Tris. Pooled fractions were dialyzed against 10 mM sodium acetate or PBS. The construct containing residues 23–106 was

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purified in the same manner except the protein was eluted from the nickel column using 10 mM sodium acetate at pH 3.5, and fractions that contain the protein were further purified on an SP-Sepharose column using a salt gradient in sodium acetate at pH 5.0. The protein concentration of rPrP^C was determined by absorbance at 280 nm. Purity of the final protein preparations was estimated at ≥99% when analyzed by SDS-PAGE, Western blot, and matrix-assisted laser desorption ionization-mass spectrometry (data not shown).

Preparation of Solutions—Hemin (Mann Research Laboratories Inc.), biliverdin (Frontier Scientific), and bilirubin (Frontier Scientific) were dissolved in Me₂SO at 10 mM. Further dilutions were carried out in PBS or serum-free Opti-MEM (Invitrogen). Hemin stock solutions were also prepared in 0.5 M NaOH at 10 mM to investigate the effect of the μ -oxo-dimer of hemin, which is known to form at basic pH.

UV-visible Absorption Spectroscopy—For spectroscopic analysis, rPrP^C and hemin were mixed in PBS (pH 7.4) containing 1 mM EDTA prior to measurement of absorbance. Measurements were made on a SpectraMAX 190 plate reader (Molecular Devices). The spectra were acquired between 300 and 800 nm.

Cell Culture—N2a5E4E is a mouse neuroblastoma (N2a) cell line that overexpresses mouse PrP^C as described previously (25). N2aGFP-GPI is an N2a cell line that was stably transfected with a GFP-GPI (glycosylphosphatidylinositol-anchored green fluorescent protein) expression vector (26). CF10 cells generated from PrP^C null mice (27) were transduced with a murine retroviral vector (pSFF) encoding full-length hamster PrP^C or hamster PrP^C lacking residues 34–94 (28). Human neuroblastoma cells (NB1) express endogenous levels of PrP^C. All cell lines were maintained at 37 °C in a humidified atmosphere of 5% CO₂ in Opti-MEM supplemented with 10% fetal bovine serum (Invitrogen) and penicillin/streptomycin (100 units/ml, 100 μ g/ml; Invitrogen).

PrP^C Binding to Hemin-Agarose—A confluent tissue culture flask (25 cm²) containing cells described above was rinsed three times with PBS and lysed with 600 μ l of PBS containing 0.5% Triton X-100 and 0.5% sodium deoxycholate. Cell debris and nuclei were removed by centrifugation at 2,700 \times g for 5 min, and 600 μ l of postnuclear supernatant was recovered. To the postnuclear supernatant, a protease inhibitor mixture (Complete, Roche Applied Science) was added according to the manufacturer's instructions. The final concentration of NaCl was adjusted to 0.5 M. Additional Triton X-100 and sodium deoxycholate were added to final concentrations of 1%. Sarkosyl (1%) was also added to improve the dissolution of the cell membranes. The final volume was adjusted to 1200 μ l after the addition of all reagents and then incubated for 10 min at room temperature to allow the dissolution of membranes. Hemin-agarose beads (Sigma) were washed three times with PBS prior to use. Washed beads containing the equivalent of 0.1 μ mol of hemin were added to 200 μ l of cell lysate and incubated for 10 min at room temperature. After incubation, the unbound fraction was collected and precipitated with 800 μ l of methanol. The beads were washed three times with the same buffer used for the binding step. The methanol precipitate and the beads were resuspended in 100 μ l of SDS-PAGE sample buffer and

boiled, and 5 μ l was subjected to SDS-PAGE with staining for proteins with GelCode Blue (Pierce) or to immunoblot analysis for PrP^C using antibody D13 (InPro) for mouse and hamster PrP^C and 3F4 for human PrP^C.

Immunodetection of Cell Surface PrP^C—N2a5E4E cells were plated at low density in a 96-well plate and grown to confluence. At confluence, cells were washed once with serum-free Opti-MEM and treated with hemin, biliverdin, or bilirubin at 0, 1, 3, and 10 μ M for 1 h at 37 °C. After treatment, the cells were fixed with 4% paraformaldehyde in PBS for 10 min followed by two washes with PBS. Then the cells were incubated with antibody D13 (InPro) diluted in PBS at 1:1000 for 1 h. After three washes of 5 min each, cells were incubated with secondary antibody conjugated with alkaline phosphatase diluted in PBS at 1:2000 for 1 h. Cells were washed three times and incubated with Atto-phos substrate (Promega) for 7–15 min until a yellow color was visible. Fluorescence intensity was measured in a SpectaMAX Gemini EM plate reader (Molecular Devices) using 450 nm excitation filter and 520 nm emission filter. The relative fluorescence intensity was calculated based on the signal obtained from untreated cells.

Immunofluorescence—N2a5E4E cells were washed twice with serum-free Opti-MEM and treated with 3 μ M hemin for 1 h. After treatment, cells were fixed with 4% paraformaldehyde in PBS for 10 min followed by two washes with PBS. Cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min. To block nonspecific antibody binding, cells were incubated with 10% normal goat serum and 0.1% Triton X-100 in PBS (blocking solution) for 10 min. An antibody against PrP^C, SAF-32 (Cayman Chemicals), was diluted in blocking solution (1:200) and added to the cells. After 1 h of incubation, the cells were washed three times with PBS and incubated with secondary antibody anti-mouse IgG conjugated with Alexa 488 fluorescent dye (1:1000) for 1 h. Cells were washed three times and observed by confocal microscopy. All images were acquired with the same confocal parameters.

Biotinylation and Isolation of Cell Surface Proteins—N2aGFP-GPI cells were plated in 24-well plates and cultured for 3 days. At confluence, cells were washed three times with serum-free Opti-MEM and treated with hemin at 0, 1, 3, and 10 μ M for 1 h at 37 °C. The cells were washed three times with PBS containing 1 mM CaCl₂ and 1.2 mM MgSO₄ (PBS Ca²⁺/Mg²⁺) on ice. Then 150 μ l of 1 mg/ml NHS sulfo-LC biotin was added per well and incubated for 5 min at room temperature. NHS sulfo-LC biotin reacts predominantly with primary amino groups. After biotinylation, the cells were washed three times with PBS Ca²⁺/Mg²⁺ containing 100 mM glycine. The cells were then lysed with 200 μ l of PBS containing 0.5% Triton X-100, 0.5% sodium deoxycholate, and a protease inhibitor mixture (Complete, Roche Applied Science) (lysis buffer). Cell lysates were incubated with 20 μ l of streptavidin Dynabeads (Invitrogen) for 30 min at room temperature and then washed three times with the lysis buffer. The beads were resuspended in 40 μ l of SDS-PAGE sample buffer. The samples were subjected to SDS-PAGE and Western blot analysis using the designated antibody for PrP^C (D13, InPro), GFP (monoclonal anti-GFP, Roche Applied Science), or NCAM (anti-NCAM, Chemicon). Biotinylated proteins in general were stained with Neutravidin

conjugated with alkaline phosphatase (Pierce). To check the effect of hemin on PrP^C turnover, biotinylation of N2a5E4E cells was performed as described above but prior to the hemin treatment.

Detergent Insolubility Assay—All cells described were cultured and treated with hemin as described in the section above. The cells were then lysed with 200 μ l of lysis buffer. Nuclei and cell debris were removed by centrifugation at $2,700 \times g$ for 5 min at 4 $^{\circ}$ C, and then Sarkosyl was added to a final concentration of 0.5–1% (29). After 10 min of incubation on ice or at 37 $^{\circ}$ C, the detergent-insoluble material was recovered by ultracentrifugation at $360,000 \times g$ for 30 min at 4 $^{\circ}$ C. Supernatant proteins were subjected to methanol precipitation. Pellets that were generated from ultracentrifugation or methanol precipitation were dissolved in SDS-PAGE sample buffer and subjected to immunoblot analyses using antibodies described in the section above.

Metabolic Labeling—Tissue culture flasks (25-cm²) were seeded with equal numbers of human neuroblastoma NB1 cells and grown until they were 80–90% confluent. The cells were preincubated for 1 h with 5 ml of 10 μ M hemin in serum-free Opti-MEM followed by a 30-min incubation in 2 ml of methionine-free MEM containing hemin. Then 500 μ Ci of [³⁵S]methionine was added to each flask and incubated for 30 min. Cells were rinsed twice with PBS and incubated in serum-free Opti-MEM containing hemin for the designated chase time.

Peroxidase Activity—The peroxidase activity was measured by oxidation of 3,3',5,5'-tetramethylbenzidine (TMB) (Pierce) or 2,2'-azino bis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (Pierce) by H₂O₂. Hemin (8 μ M) was mixed with various concentrations of rPrP^C, prior to the addition of substrate. After substrate addition the reaction was monitored for absorbance at 650 nm on a SpectraMAX 190 plate reader (Molecular Devices).

RESULTS

Hemin-induced PrP^C Clustering and Internalization—Several different inhibitors of PrP^{Sc} formation, *e.g.* pentosan polysulfate (21), copper (18), suramin (30), and phosphorothioate oligonucleotides (22), affect the intracellular localization of PrP^C. Because various synthetic cTPs inhibit PrP^{Sc} formation, we wondered if hemin, as a natural cTP and potential physiological ligand for PrP^C, can also affect PrP^C localization. This was tested initially by using an immunofluorescence assay for PrP^C detection in fixed and permeabilized cells. To enhance PrP^C detection, a neuroblastoma cell line that expresses a high level of PrP^C, N2a5E4E, was used. Without hemin treatment, both cell surface and intracellular perinuclear staining was observed. Hemin (3 μ M) treatment of N2a5E4E cells for 1 h decreased the immunofluorescence of PrP^C on the cell surface and caused some residual surface staining to appear more punctate than in the control cells (Fig. 1). No staining was observed when the primary antibody SAF-32 was omitted from the staining protocol. Furthermore, SAF-32 did not stain CF10 cells generated from PrP^C null mice (data not shown) confirming the specificity of the antibody against PrP^C. Finally, similar results were obtained with antibody D13 (data not shown). These hemin effects on PrP^C localization were not likely

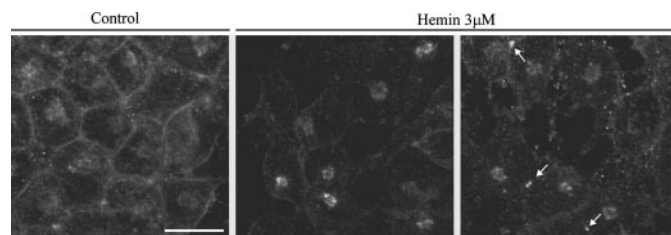


FIGURE 1. Hemin effects on PrP^C subcellular localization. N2a5E4E cells were treated with or without hemin (3 μ M) for 1 h and subjected to immunofluorescence staining of PrP^C using antibody SAF-32. All images were acquired using the same confocal parameters. In control cells, a typical distribution of PrP^C was observed in the plasma membrane and the perinuclear region (*left panel*). Hemin treatment decreased the fluorescence intensity on the cell surface (*middle panel*), leaving residual punctate cell surface staining in some cells (*right panel, arrows*). Bar = 20 μ m.

because of cytotoxicity because the treatment at $\leq 10 \mu$ M for at least 4 h did not induce any signs of toxicity as judged by morphology or a cytotoxicity assay using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (data not shown). Altogether, these results suggested that hemin induced both the aggregation and internalization of PrP^C.

Selective Effects of Hemin on PrP^C Aggregation—The formation of PrP^C aggregates was also evaluated using a detergent insolubility assay (29). Upon hemin treatment, PrP^C solubility decreased in a dose-dependent manner in both mouse and human cell lines (Fig. 2, *a, b, e, and f*). To eliminate the possibility of PrP^C aggregation being due to overexpression, the solubility of endogenous PrP^C expressed in N2aGFP-GPI cells was also analyzed, and similar results were observed (supplemental Fig. 2). Because hemin can interact with many proteins, we investigated the selectivity of the effects of hemin on the aggregation of PrP^C compared with other cell surface proteins such as NCAM (neural cell adhesion molecule), which interacts with PrP^C (31, 32), and GFP-GPI protein, which follows default trafficking pathways of GPI anchored, lipid-raft-associated proteins (26). No hemin-induced alteration of NCAM or GFP-GPI solubility was observed, indicating a degree of specificity for the effects of hemin on PrP^C solubility (Fig. 2*c* and supplemental Fig. 2).

Quantifying Hemin-induced PrP^C Internalization—To estimate the extent of internalization of PrP^C after hemin treatments, relative amounts of cell surface PrP^C were quantified using a fluorogenic immunoassay described under “Experimental Procedures.” To increase the PrP^C detection in this assay, N2a5E4E cells were used. The PrP^C specificity of the assay was indicated by the lack of fluorescence signal when the primary antibody (D13) was omitted and when the assay was applied to primary neuronal cells derived from PrP^C null mice. The treatment of N2a5E4E cells with hemin for 1 h caused a dose-dependent reduction of cell surface PrP^C (Fig. 3) reaching $\sim 10\%$ of control levels at 10 μ M hemin. It is known that basic solutions (*e.g.* NaOH) favor the formation of μ -oxo-dimers of hemin. NaOH-treated hemin was slightly more potent than Me₂SO-solubilized hemin at causing PrP^C internalization at 3 μ M, but both solutions were effective overall. In contrast to hemin, its linear tetrapyrrole metabolites biliverdin and bilirubin did not significantly reduce cell surface PrP^C (Fig. 3).

Hemin Interactions with Prion Protein

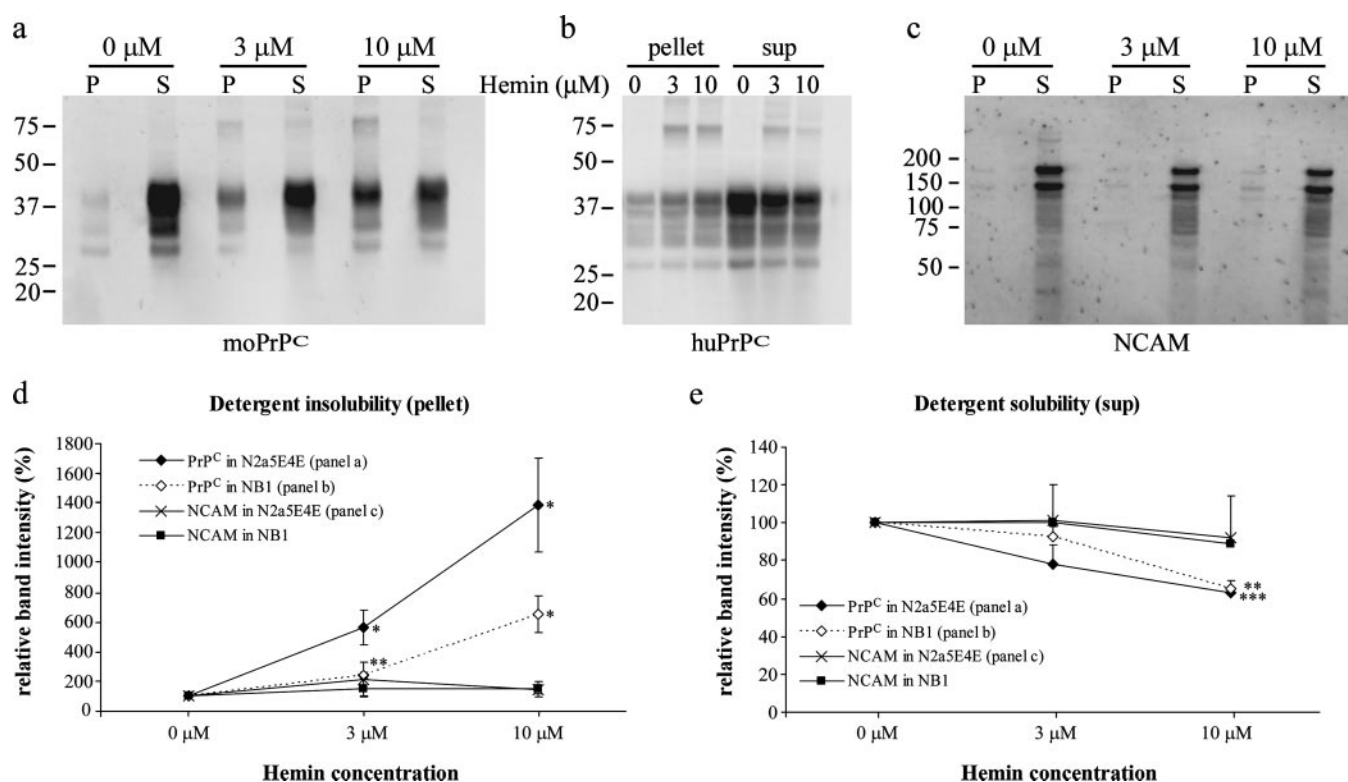


FIGURE 2. Hemin effects on the detergent insolubility of PrP^C. *a*, after a 1-h hemin treatment of N2a5E4E cells, detergent-insoluble material (P) was pelleted by ultracentrifugation of the detergent cell lysate, and the supernatant (S) was precipitated with methanol. The amount of PrP^C in each fraction was analyzed by immunoblot using antibody D13. *b* and *c*, the same procedure was performed to test the solubility of human PrP^C in NB1 cells (using antibody 3F4) (*b*) and NCAM in N2a5E4E cells (*c*). *sup*, supernatant. *d* and *e*, relative mean intensities \pm S.E. ($n = 4$) of the bands in the pellet (*d*) and supernatant (*e*) fractions from samples like those shown in *a*–*c*. Statistical significance of the difference between the mean of hemin-treated *versus* untreated samples by Student's *t* test is indicated by * ($p \leq 0.05$), ** ($p \leq 0.01$), or *** ($p \leq 0.001$).

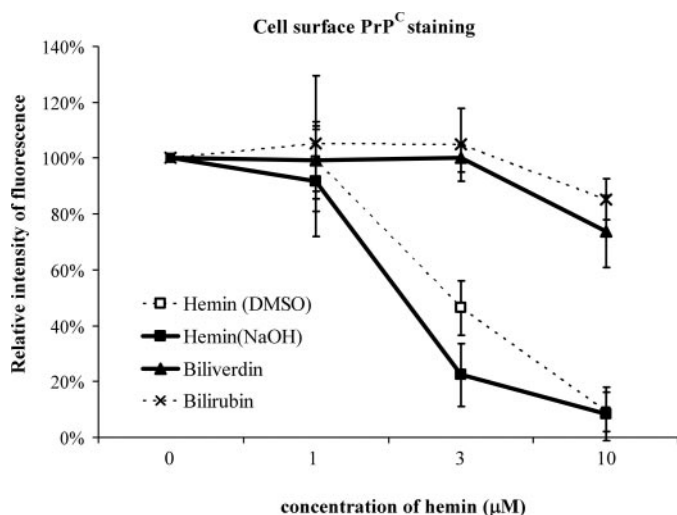


FIGURE 3. Reduction of cell surface PrP^C with hemin treatment. N2a5E4E cells were treated with bilirubin, biliverdin, and hemin at the designated concentrations for 1 h and subjected to the immunostaining of PrP^C (using D13) without permeabilization. The degree of staining was determined by measuring fluorescent substrate cleaved by secondary antibody conjugated to alkaline phosphatase. The percentage of internalization was calculated relative to the fluorescence intensity of untreated samples. Bilirubin (×) and biliverdin (▲) did not have a significant effect, but hemin dissolved in Me₂SO (DMSO; □) or in NaOH (■) caused a dose-dependent reduction of cell surface PrP^C. The error bars represent S.D. calculated from six independent experiments. Two-sample *t* tests with equal variances indicated that the difference between the means hemin (Me₂SO)- and hemin (NaOH)-treated samples was significant ($p = 0.0026$) only at 3 μM.

Selectivity of Hemin Effects on PrP^C Internalization—To further test the selectivity of hemin effects on PrP^C internalization, N2aGFP-GPI cells that express endogenous levels of PrP^C and a recombinant GFP-GPI were treated with hemin, and cell surface proteins were then biotinylated, captured with streptavidin-coated magnetic beads, and subjected to SDS-PAGE. The gels were either immunoblotted for the detection of individual proteins or stained with a Neutravidin-alkaline phosphatase conjugate to reveal the overall profile of biotinylated cell surface proteins. Consistent with previous assays, the cell surface PrP^C signal decreased in a dose-dependent manner (Fig. 4, *a* and *b*), but the extent of internalization was lower than the previous assay using the N2a5E4E cells. This discrepancy could be related to different expression levels of PrP^C in the two cell types because the N2aGFP-GPI cells express a lower, endogenous level of PrP^C, whereas the N2a5E4E cells overexpress PrP^C. In contrast, the banding patterns and intensity of many other cell surface proteins were not visibly altered with hemin treatment (Fig. 4*a*). We also examined hemin effects on NCAM and GFP-GPI. As shown in Fig. 4, *a* and *b*, no alteration of NCAM was observed, whereas GFP bands increased with hemin treatment. GFP-GPI is expressed under a cytomegalovirus promoter whose activity can be enhanced by histone acetylation or demethylation (33). Given that hemin can regulate both histone acetylation and methylation (34), the increase of GFP-GPI on the cell surface could be due to increased expression, which was confirmed by a Western blot assay on total cell

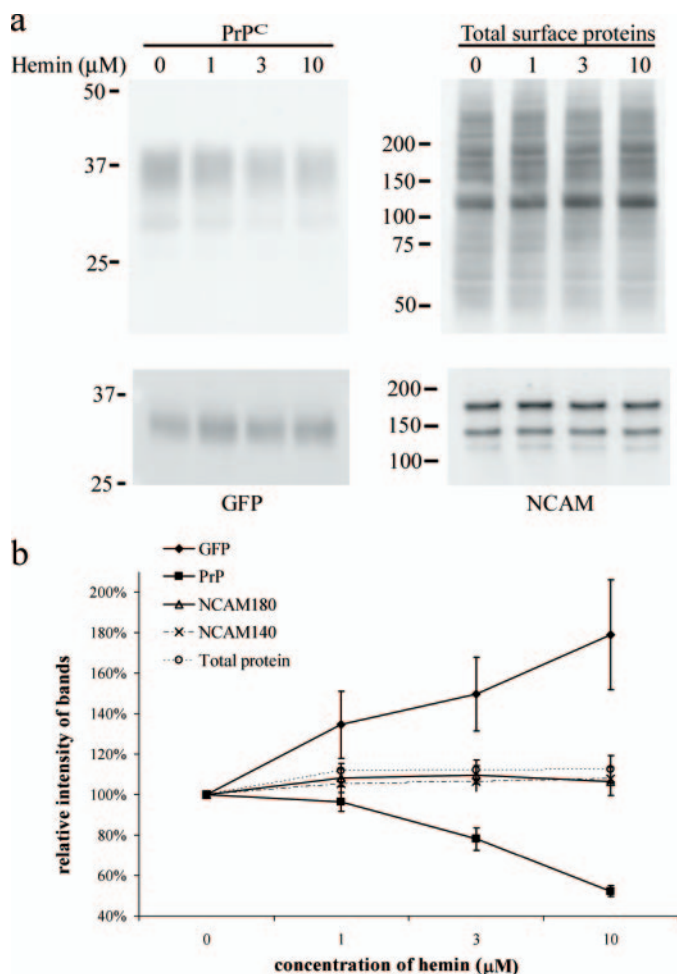


FIGURE 4. Selective effect of hemin on PrP^C internalization. After a 1-h treatment with hemin, cell surface proteins of N2aGFP-GPI cells were biotinylated with a membrane-impermeant reagent and isolated using streptavidin-coated magnetic beads. Biotinylated proteins were subjected to SDS-PAGE and electroblotting. The blots in *a* were stained for PrP^C, GFP, and NCAM using appropriate antibodies (D13 in the case of PrP^C) or for total biotinylated proteins using Neutravidin-alkaline phosphatase. Quantification of the bands from four independent experiments (each experiment had duplicate samples) is shown in *b*. The relative intensity of each band was calculated based on the intensity of untreated control sample (*y* axis). The error bar shows means \pm S.E.

lysates (data not shown). Collectively, these data provide evidence that the effect of hemin on PrP^C internalization was relatively selective.

Hemin-induced Degradation of PrP^C—In the immunofluorescence studies of PrP^C using N2a5E4E cells, we observed that in some cells, intracellular fluorescence increased as the cell surface staining decreased. However, in other cells, the overall fluorescence intensity decreased (Fig. 1) suggesting that the internalized PrP^C might have been degraded. To directly analyze whether degradation of PrP^C is induced by hemin treatment, cell surface proteins of N2a5E4E cells were pulse-labeled with biotin for 5 min, incubated with or without hemin (10 μM) for 1 h to allow for turnover, and isolated on streptavidin beads for immunoblot analysis. With hemin treatment, the biotinylated PrP^C bands decreased compared with those in untreated cells, whereas the overall banding patterns and intensity of other biotinylated proteins was not noticeably affected (Fig. 5, *a*

and *b*). These results clarify that the decrease of PrP^C signal in response to hemin treatment is not because of reduced expression but to enhanced degradation.

Hemin effects on the biosynthesis and turnover of PrP^C were also evaluated by pulse-chase [³⁵S]methionine labeling and radioimmunoprecipitation of PrP^C in human NB1 cells that express endogenous levels of PrP^C. At time 0, immature glycosylated and unglycosylated forms of PrP^C were seen as described previously (Fig. 5*c*, arrows) (35). After a 1-h chase, mature glycosylated forms predominated (Fig. 5*c*, asterisk). In hemin (10 μM)-treated cells, these PrP^C bands disappeared more rapidly with increasing chase periods, showing increased PrP^C turnover relative to that seen in control cells. The quantification of all glycosylated and unglycosylated PrP^C bands from two experiments revealed that the loss of pulse-labeled PrP^C was accelerated in the presence of 10 μM hemin (Fig. 5*d*). Altogether, these data show that hemin selectively alters the subcellular localization and turnover of the PrP^C.

PrP^C Binding to Hemin-Agarose—To evaluate whether hemin can directly interact with PrP^C, hemin-agarose affinity chromatography was performed using N2a5E4E and NB1 cell lysates. Amounts of PrP^C in bound and unbound fractions were analyzed by Western blotting. A single aliquot of hemin-agarose beads was able to fractionate ~50% of PrP^C from the total cell lysate (Fig. 6, *a* and *b*, lane 2). Additional PrP^C (~25%) could be extracted from the lysate with a fresh aliquot of hemin-agarose beads (data not shown). The absence of PrP^C binding to agarose beads without hemin confirmed the specificity of the interaction between hemin and PrP^C (Fig. 6, *a* and *b*, lane 4). To assess the selectivity of PrP^C binding, the other proteins of each fraction were stained nonspecifically with GelCode Blue. A number of other proteins from the cell lysates also bound to hemin-agarose, as expected, but most proteins were much more abundant in the unbound fraction (Fig. 6, *a* and *b*, lanes 5 and 6). Thus, the hemin-agarose showed some selectivity for binding PrP^C. As an additional indication of specificity and to examine whether the octapeptide repeats in PrP^C might be involved in hemin binding, we evaluated the hemin-agarose binding of hamster PrP^C lacking the octapeptide repeats and flanking sequences (HaPrP Δ34–94). Although the binding of wild-type hamster PrP^C was as efficient as the binding of the wild-type mouse and human PrP^C, only ~10% of HaPrP Δ34–94 bound to hemin-agarose (Fig. 6*c*). These results showed that hemin interacts directly or indirectly with PrP^C of multiple species and that PrP^C residues 34–94 strongly influence that interaction.

UV-visible Spectroscopy—To obtain additional evidence of direct interactions between hemin and PrP^C, we used UV-visible spectroscopy. Hemin is sparingly soluble in aqueous media and, when not bound to proteins, tends to form oligomers that absorb strongly at ~390 nm (the Soret band). This absorbance maximum (A_{\max}) can shift to different wavelengths upon interaction with other molecules. Using this spectral property of hemin, we evaluated the binding of hemin to purified rPrP^C. Spectra of hemin alone at various concentrations showed that the A_{\max} was slightly blue-shifted with increasing concentrations (Fig. 7, *a–c*, blue lines). However, when hemin was incubated with rPrP^C at a 1:1 molar ratio, the A_{\max} red-shifted to

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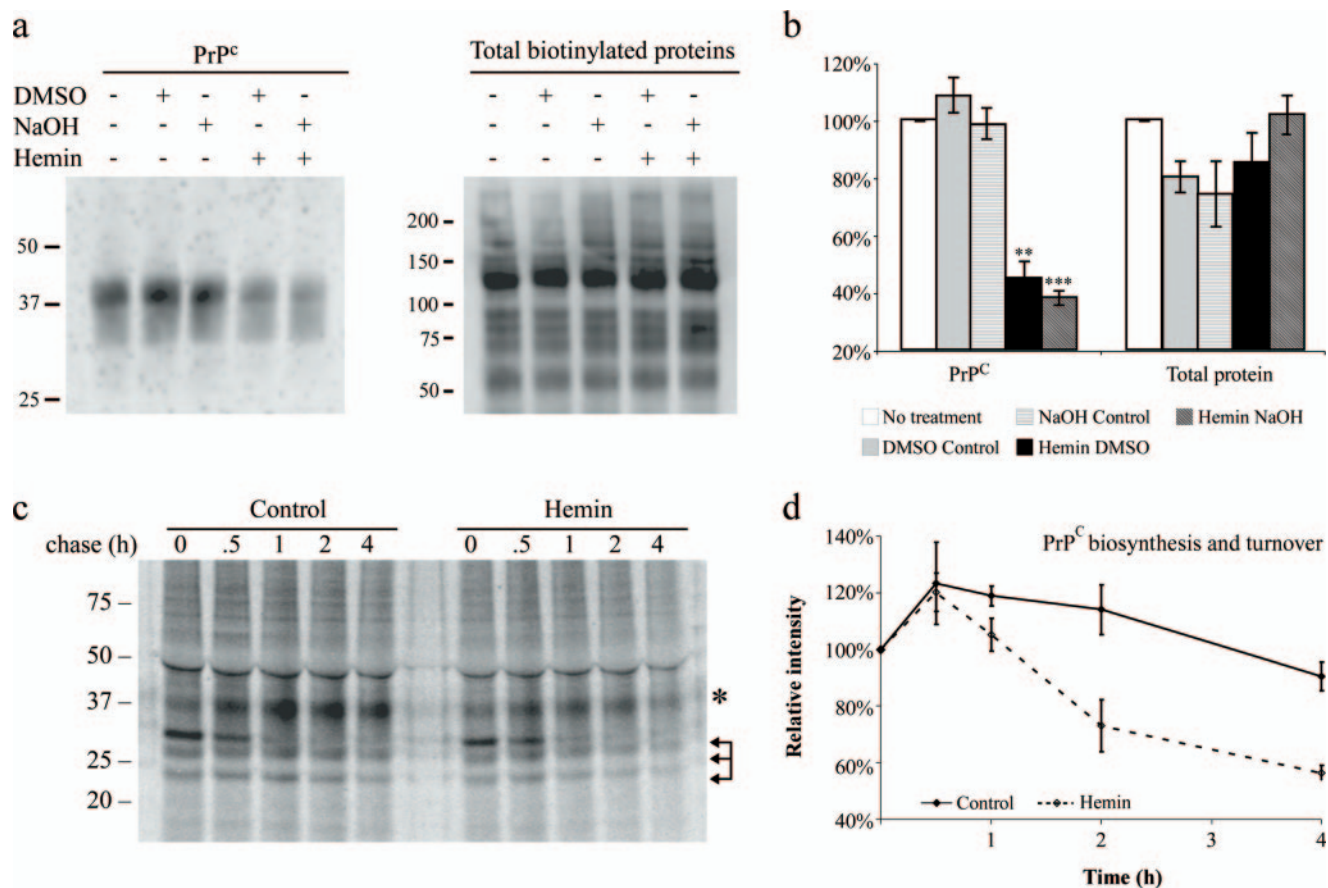


FIGURE 5. Hemin induces turnover of PrP^C. *a*, cell surface proteins were pulse-biotinylated for 5 min using a membrane-impermeant reagent and then the cells were subjected to hemin treatment (10 μ M) for 1 h. Biotinylated proteins were isolated from cell lysates using streptavidin beads and subjected to immunoblotting for PrP^C (using D13) or Neutravidin blotting for total biotinylated proteins. *b*, means \pm S.E. from three independent experiments are like that shown in *a*. Statistical significance of the difference between the mean of hemin-treated versus control samples (*t* test) is indicated by ** ($p \leq 0.004$) or *** ($p \leq 0.0002$). *c*, NB1 cells were pulse-labeled with [³⁵S]Met for 30 min and chased for the designated times in the presence or absence of 10 μ M hemin. The immature glycosylated and unglycosylated forms of PrP^C (arrows) disappear as the fully glycosylated PrP^C band (*) is produced, which decreases more rapidly with hemin treatment. *d*, radiolabeling experiment in *b* was performed in duplicate and mean values of intensities of quantified bands (all PrP^C forms) are presented in the graph with range bars.

411 \pm 3 nm independent of the concentration of the complex (Fig. 7*a*, dotted green line). These results suggested that PrP^C reorganized hemin molecules into distinct oligomeric states. Similar spectral changes were produced with C-terminally truncated rPrP (residues 23–106), which contains the octapeptide repeats (Fig. 7*a*, dotted pink line). However, the N-terminally truncated rPrP (residues 90–231) did not alter the hemin spectrum indicating that the C-terminal residues 90–231 were not required for hemin binding (Fig. 7*a*, dotted orange line).

Bovine serum albumin (BSA) is a well known hemin-binding protein that has nanomolar affinity for hemin (36). When BSA interacted with hemin, only a small red shift of A_{\max} (396 \pm 2 nm) occurred (Fig. 7*b*, dotted pink line). These different effects of rPrP^C and BSA on the hemin spectrum indicated that PrP^C and BSA interact in distinct ways with hemin.

To determine the stoichiometry of the observed hemin-PrP^C interactions, increasing concentrations of hemin were added to a fixed rPrP^C concentration (Fig. 7*c*) and vice versa (not shown). At molar excesses of hemin up to \sim 10:1, the full A_{\max} red shift was maintained, indicating that each PrP molecule could influence the spectrum of multiple hemin molecules. However, with further increases in the hemin:PrP^C ratio, the A_{\max} gradually

shifted back toward the A_{\max} of free hemin, suggesting that saturation of the binding to rPrP^C had occurred. Taken together, these data indicate that multiple hemin molecules can bind directly to PrP^C, primarily via the N-terminal half of the molecule.

Enhancement of the Peroxidase Activity of Hemin by Interactions with rPrP^C—Given the observed interactions between hemin and PrP^C, we sought clues as to whether such interactions might have additional physiological significance. It has been reported that an excess of free hemin can have cytolytic activity because of its inherent peroxidase activity (37). To see if binding to PrP^C might alter such activities of hemin, we compared the peroxidase activity of free hemin and its rPrP^C complex. In an assay using TMB as a substrate, the hemin-rPrP^C complex showed increased peroxidase activity by up to 3-fold compared with hemin alone (Fig. 8). Similar results were also obtained using 2,2'-azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt as a substrate (data not shown). Consistent with previous studies (36), the binding of hemin to BSA also increased its peroxidase activity (Fig. 8). In contrast, no superoxide dismutase or catalase activities of hemin itself or hemin-rPrP^C complexes were observed (data not shown). The

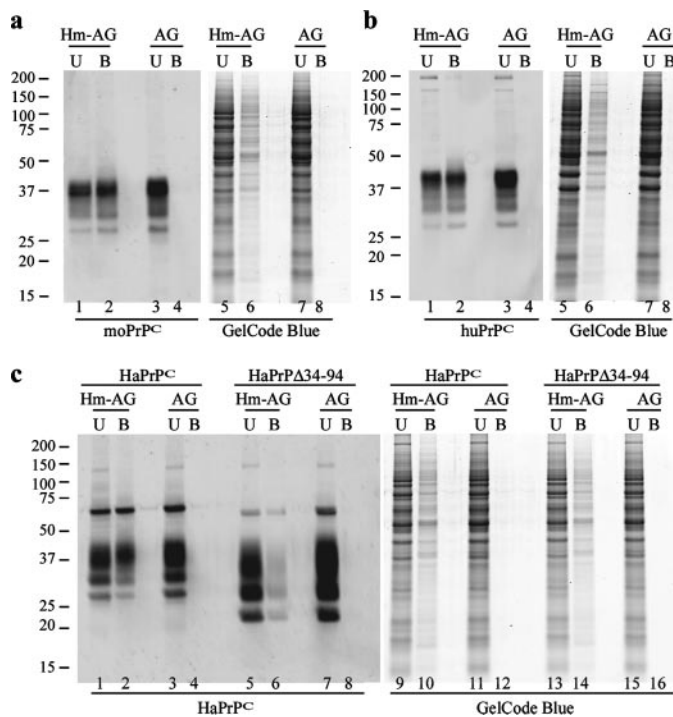


FIGURE 6. PrP^C binding to hemin-agarose. *a*, N2a5E4E cell lysates were incubated with hemin-agarose (Hm-AG) or agarose-only (AG) beads. The amounts of PrP^C or other proteins in bound (B) and unbound (U) fractions were assessed by immunoblotting for PrP^C and GelCode Blue staining of total proteins. *b* and *c*, same procedure was performed using NB1 cells that express human PrP^C (*b*) and CF10 cells that express full-length hamster PrP^C (HaPrP^C) or HaPrP^C lacking residues 34–94 (*c*). The data are representative of the results of two independent binding experiments performed in each cell type.

fact that the peroxidase activity of hemin is altered by binding to PrP^C indicates that the interaction affects the inherent redox properties of this porphyrin.

DISCUSSION

Hemin interacts with a number of proteins stably or reversibly and orchestrates various vital biological activities. Here we have demonstrated that PrP^C is a hemin-binding protein that undergoes aggregation, internalization, and degradation upon exposure to hemin.

Potential Relevance of Hemin Binding and Cellular Trafficking in Biological Activities of PrP^C—PrP^C constitutively cycles between the plasma membrane and endocytic compartments, and its endocytosis can take place via a clathrin-dependent mechanism (38, 39). Copper, at very high concentrations (100 μ M), can enhance the endocytosis of PrP^C, leading to proposals that it functions as a transporter or sensor for metal ions (18, 40). The internalization of PrP^C is also involved in nitric oxide-dependent autoprocessing of glypican-1 (41) and in p53-dependent staurosporine-induced caspase-3 activation (42, 43). These observations suggest that cellular trafficking of PrP^C is closely related to its physiological activities. Thus, the fact that hemin binding alters the PrP^C trafficking suggests that PrP^C may participate in hemin-dependent biological events and/or that hemin binding is relevant in PrP^C functions.

The endocytosis of PrP^C through clathrin-coated pits requires a transmembrane receptor. Recently, the low density lipoprotein receptor-related protein was identified as the transmembrane

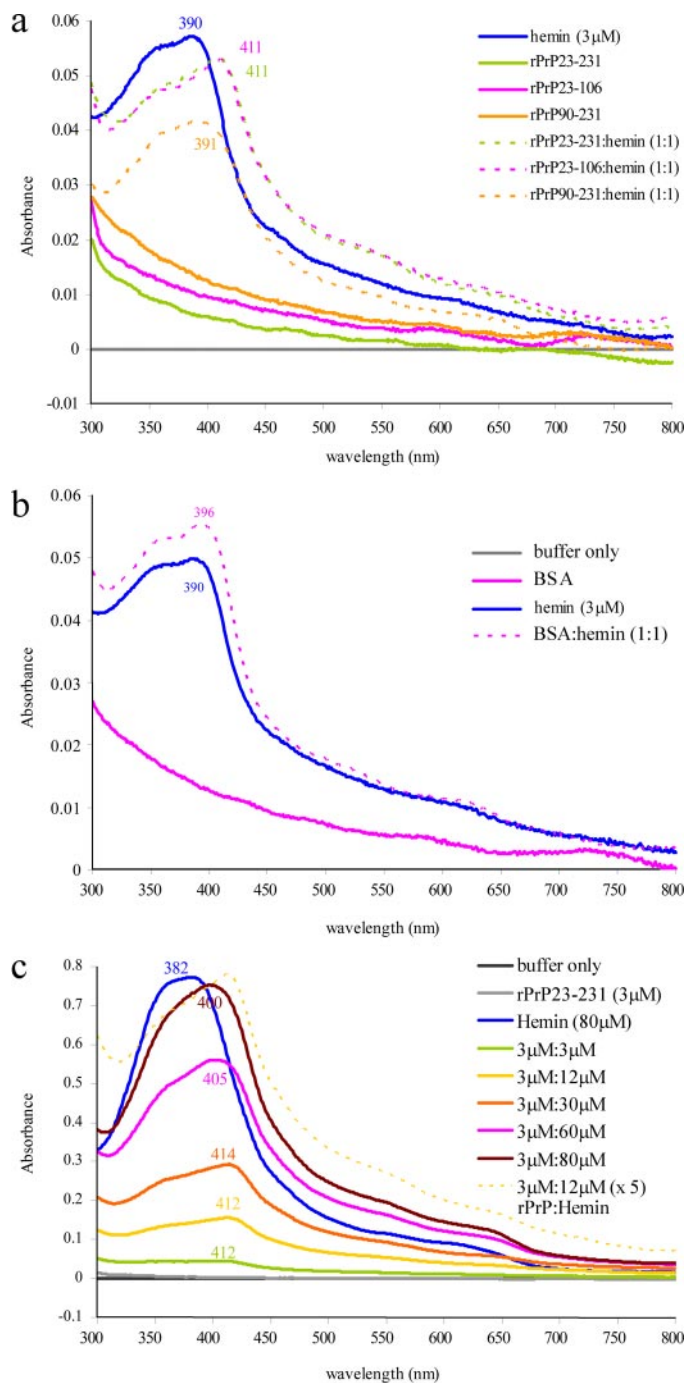


FIGURE 7. Modification of absorption spectra of hemin by recombinant PrP^C. *a*, hemin absorption spectrum is represented by the solid blue line. The addition of equimolar concentrations of either full-length rPrP^C (rPrP residues 23–231; dotted green line) or C-terminally truncated rPrP (rPrP residues 23–106; dotted pink line) red-shifted the A_{\max} of free hemin, whereas N-terminally truncated rPrP (rPrP residues 90–231; dotted orange line) did not alter the hemin spectrum. The numbers indicate the mean of the wavelength in nm at the A_{\max} . *b*, A_{\max} of the hemin spectrum (solid blue line) was slightly red-shifted (dotted pink line) by interactions with BSA. *c*, the red shift of the hemin spectrum was maintained in the presence of rPrP^C at molar excesses of hemin up to 10:1 (compare green lines with the yellow and orange lines). Further molar excesses of hemin gradually reduced the red shift of the hemin A_{\max} (pink and brown lines). For easier comparison to the spectra at highest hemin:PrP ratios, the spectrum of 4:1 hemin:rPrP residues 23–231 (solid yellow line) was multiplied by a factor of 5 (dotted yellow line). The spectra shown are representative of spectra of at least four replicate samples.

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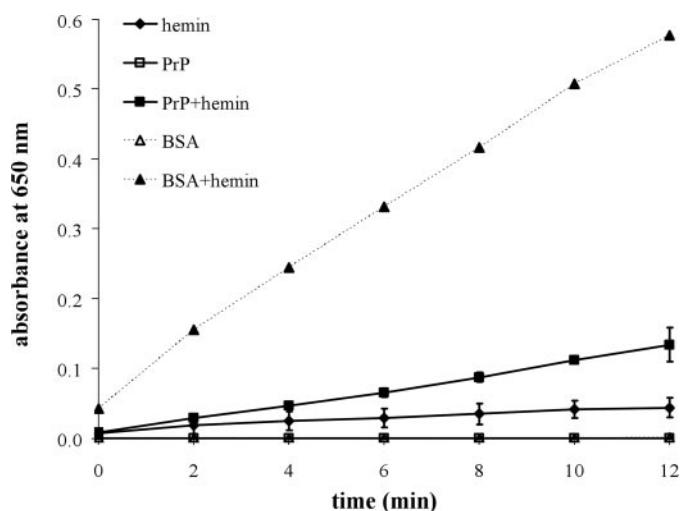


FIGURE 8. **PrP^C binding increases the inherent peroxidase activity of hemin.** Hemin and rPrP^C were mixed prior to reaction with the TMB substrate, and the reaction was monitored by absorbance at 650 nm as a function of time. Hemin (8 μ M) alone had some activity (◆), and this activity was enhanced in the presence of 8 μ M rPrP^C (■). BSA (8 μ M) also increased the peroxidase activity of hemin (▲). BSA (△) and rPrP (□) alone had no activity. The data points show the mean \pm S.D. ($n = 4$).

receptor that mediates copper-induced PrP^C internalization (44). Low density lipoprotein receptor-related protein also participates in the internalization of the hemin-hemopexin complex resulting in cellular hemin uptake (45). At the moment, it is not clear whether PrP^C acts as a receptor for free hemin or interacts with hemin-hemopexin complexes and acts as a co-receptor in compartments such as blood and liver when these complexes are formed. However, given that both copper and hemin are small ligands that have redox properties and appear to bind a similar region of PrP^C, it is possible that both of these ligands induce the internalization of PrP^C by a related mechanism. Further studies will be required to evaluate this possibility.

PrP^C trafficking also influences its conversion into PrP^{Sc} and disruption of the normal trafficking of PrP^C seems to be a common mechanism for several classes of PrP^{Sc} inhibitors (23, 46). Notably, the hemin concentration that effectively altered PrP^C trafficking ($\sim 3 \mu$ M) also reduced the formation of PrP^{Sc} in scrapie-infected cell cultures (data not shown). Therefore, the anti-scrapie activity of hemin in cell culture models might relate to its ability to stimulate the internalization of PrP^C.

Roles of Peroxidase Activity of the Hemin-PrP^C Complex—Hemin-containing peroxidases react with H₂O₂ and promote oxidative modifications of proteins, lipids, and halides (47). It is well known that hemin forms oligomers in aqueous media. The fact that co-incubation of hemin with rPrP^C caused a red shift in the Soret region of the UV-visible spectrum indicates that hemin oligomers reorganize in the presence of PrP^C. As a result, they become more reactive with H₂O₂ as indicated by the PrP^C-induced enhancement of the peroxidase activity of hemin (Fig. 8).

The activity of antioxidant enzymes, such as glutathione peroxidase and superoxide dismutase, is altered in PrP^C null mice and in scrapie-infected brains (48, 49). These findings suggest that PrP^C participates in the modulation of the activity of these enzymes and/or PrP^C itself functions as an antioxidant molecule. Moreover, it has been reported that the flexible N-termi-

nal domain of PrP^C, which we have shown contains hemin-binding sites, is influential in cellular responses to oxidative stress (50, 51). This suggests that the peroxidase activity of hemin-PrP^C complexes may play a protective role against oxidative stress.

However, excessive hemin-containing peroxidases can be damaging (47). Notably, peroxidase activity contributes to the oxidative damage that occurs in neurodegenerative diseases (52, 53). Moreover, lipid peroxidation constitutes an early pathological event of prion disease (54) and redox-active iron can be detected in PrP^{Sc} plaques and surrounding areas (55). Hence, in pathological conditions, peroxidase activity of PrP^C-hemin or PrP^{Sc}-hemin complexes may contribute to the progression of disease.

Induction of PrP^C Aggregation by Redox-active Ligands and Its Implications—Several types of evidence indicate that PrP^C plays a role in neurite outgrowth. Laminin (an extracellular matrix protein) (56), NCAM (a transmembrane adhesion molecule) (31, 32), and STI-1 (a co-chaperone) (57) are important partners that assist in this function of PrP^C. Interestingly, these ligands have distinct binding sites on the PrP^C molecule and interact with PrP^C on the cell surface suggesting that a formation of the macromolecular complex may occur on the plasma membrane in PrP^C-dependent neuritogenesis. On the other hand, these interactions may be disrupted or prevented upon hemin binding to PrP^C because the effect of hemin was selective for PrP^C trafficking, without affecting the aggregation state or internalization of NCAM (Figs. 2 and 4), which is also endocytosed by a clathrin-dependent mechanism in normal circumstances (58). Competition may occur between hemin and NCAM for the same binding site on PrP^C because the N-terminal flexible domain of PrP^C is involved in the binding of both hemin and NCAM (32). Furthermore, the induction of the formation of detergent-insoluble aggregates of PrP^C by hemin (Fig. 2) may limit the binding of large ligands like NCAM or laminin.

The aggregation of PrP^C can also be induced by copper or iron binding with the redox activity of the metal ions in the aggregates preserved (59–62). However, relative to hemin, much higher concentrations of the metals are required to produce effects similar to those of hemin (59, 60, 62). This suggests that hemin binding may be more effective and physiologically relevant than free iron or copper interactions with PrP^C.

PrP^C not only mediates neuronal differentiation but also has protective roles against various oxidative injuries (51, 63). Thus, PrP^C may be a multifunctional protein that requires a mechanism for switching between these functions. Conceivably, the binding of redox-active ligands such as hemin and metal ions can isolate PrP^C from a macromolecular complex involved in neuronal plasticity and help organize PrP^C into supramolecular assemblies that modulate cellular redox activities and/or sense reactive oxygen species. Further studies will be required to fully elucidate the physiological implications of hemin-PrP^C interactions.

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