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14. ABSTRACT No effective treatments have been validated for the transmissible spongiform encephalopathies (TSEs) or prion diseases. To advance the rational basis for the search for anti-TSE therapeutics, we have developed a new unified mechanistic model for the activity of various classes of PrPSc inhibitors which is consistent with a considerable body of evidence from our laboratory and others. Based on this model, we have successfully developed a new 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. Finally, we have discovered that combination drug treatments can substantially improve survival times of animals with established TSE infections of the central nervous system.					
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INTRODUCTION

The main purpose of this work is to identify new prophylaxes and therapeutics for the transmissible spongiform encephalopathies (TSEs) or prion diseases. 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 (1)], 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 pathological prion protein accumulation and to test new ways of administering such compounds to infected animals to improve their survival times.

BODY

Research accomplishments associated with Objective 2: Develop a cell-free conversion system for high throughput use. In our previous work relating to Objectives 1, 2 and 4, we determined that a variety of non-CpG phosphorothioate oligonucleotides (PS-ONs) had potent anti-TSE activity *in vitro* and *in vivo* (4). 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*, **Accounts of Chemical Research** 39:646-653 (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 in Kocisko et al., *Identification of prion inhibitors by a fluorescence-polarization-based competitive binding assay*, **Analytical Biochemistry** 363: 154-156 (2007); see 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 recently 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*, **Methods in Enzymology** 412: 223-234 (2006).

Research accomplishments associated with Objective 4: Test effective anti-PrP^{Sc} compounds from the cell-culture and cell-free models in scrapie-infected animals. Some compounds have delayed scrapie onset in rodents when administered at or near the time of peripheral infection, but few have helped after intracerebral (ic) inoculation. Two compounds effective after ic scrapie inoculation include pentosan polysulfate (PPS) (2) and Fe(III)meso-tetra (4-sulfonatophenyl) porphine (FeTSP) (3), which due to poor blood brain barrier penetration must be administered directly to the brain. PPS, a semi-synthetic carbohydrate polymer approved as an oral therapy for interstitial cystitis (Elmiron®), has been infused into the brains of CJD patients as an experimental therapy (5). FeTSP, a porphyrin, has recently demonstrated anti-scrapie activity when administered via ic injections to mice with established brain infections (3). 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*, **Antimicrobial Agents and Chemotherapy** 50:3447-3449 (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.

KEY RESEARCH ACCOMPLISHMENTS

- Developed a new unified mechanistic model to explain the effects of many of the best classes of anti-TSE compounds on PrP^{Sc} formation.
- Developed a new potentially high-throughput cell-free fluorescence correlation spectroscopy-based screen for compounds with anti-TSE activity.
- Discovered that combination treatments with a sulfated glycan and a porphyrin gave more than additive extensions of survival times in rodents with established infections of the brain.

REPORTABLE OUTCOMES

Caughey B, Caughey WS, Kocisko DA, Lee KS, Silveira JR, Morrey JD, Prions and TSE chemotherapeutics: A common mechanism for anti-TSE compounds, **Accounts of Chemical Research** 39:646-653 (2006)

Kocisko DA, Bertholet N, Moore RA, Caughey B, Vaillant A, Identification of prion inhibitors by a fluorescence-polarization-based competitive binding assay, **Analytical Biochemistry** 363: 154-156 (2007)

Kocisko DA and Caughey B, Searching for anti-prion compounds: cell-based high-throughput in vitro assays and animal testing strategies, **Methods in Enzymology** 412: 223-234 (2006)

Kocisko DA, Caughey B, Morrey JD, Race RE, Enhanced anti-scrapie effect using combination drug treatment, **Antimicrobial Agents and Chemotherapy** 50:3447-3449 (2006)

CONCLUSIONS

We have made significant progress toward the goals of this project on three fronts. To bolster the rational basis for the search for anti-TSE therapeutics, we have 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. Based on this model, we have successfully developed a new 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^{Sc}. Finally, we have discovered that combination drug treatments can substantially improve survival times of animals with established TSE infections of the central nervous system.

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APPENDICES

1. Caughey B, Caughey WS, Kocisko DA, Lee KS, Silveira JR, Morrey JD, Prions and TSE chemotherapeutics: A common mechanism for anti-TSE compounds, **Accounts of Chemical Research** 39:646-653 (2006)
2. Kocisko DA, Bertholet N, Moore RA, Caughey B, Vaillant A, Identification of prion inhibitors by a fluorescence-polarization-based competitive binding assay, **Analytical Biochemistry** 363: 154-156 (2007)
3. Kocisko DA and Caughey B, Searching for anti-prion compounds: cell-based high-throughput in vitro assays and animal testing strategies, **Methods in Enzymology** 412: 223-234 (2006)
4. Kocisko DA, Caughey B, Morrey JD, Race RE, Enhanced anti-scrapie effect using combination drug treatment, **Antimicrobial Agents and Chemotherapy** 50:3447-3449 (2006)

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

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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 Creutzfeld–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

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

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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., 10^3 – 10^4 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 (10^6 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 10^3 – 10^4 .

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

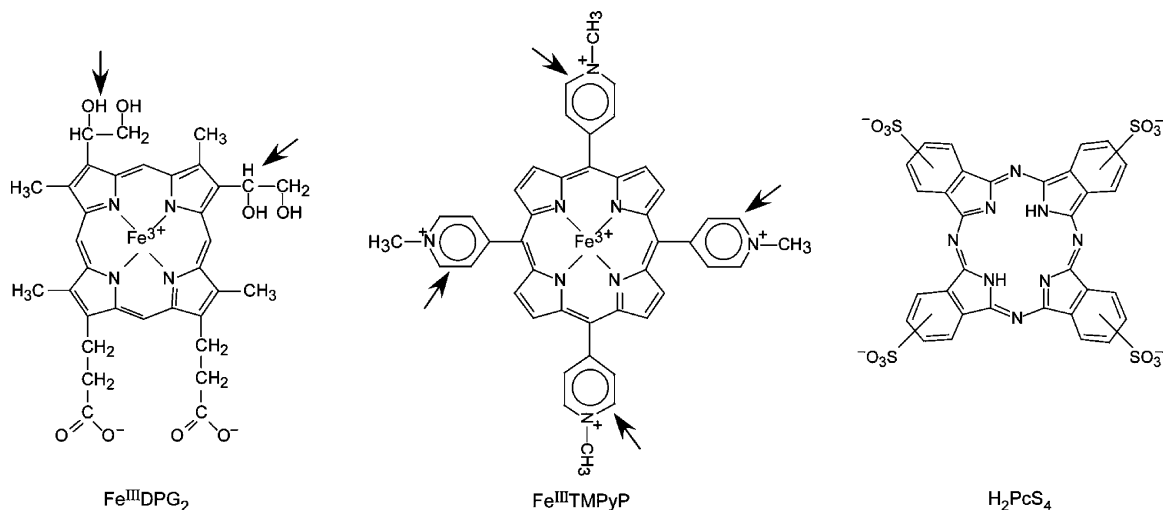


FIGURE 1. Representative cyclic tetrapyrrole (cTP) structures with anti-TSE activity. The cTPs most extensively studied have structures related to these. On the left, iron(III) deuteroporphyrin IX 2,4-bis(ethylene glycol), designated $\text{Fe}^{\text{III}}\text{DPG}_2$, represents one of many deuteroporphyrin 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 PcS_4 '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 PcS_4 or several other metal PcS_4 '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}

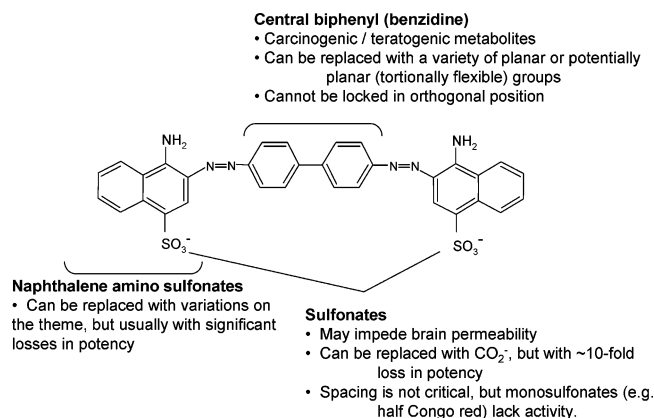


FIGURE 2. Structure–activity relationships of Congo red and analogues.

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-

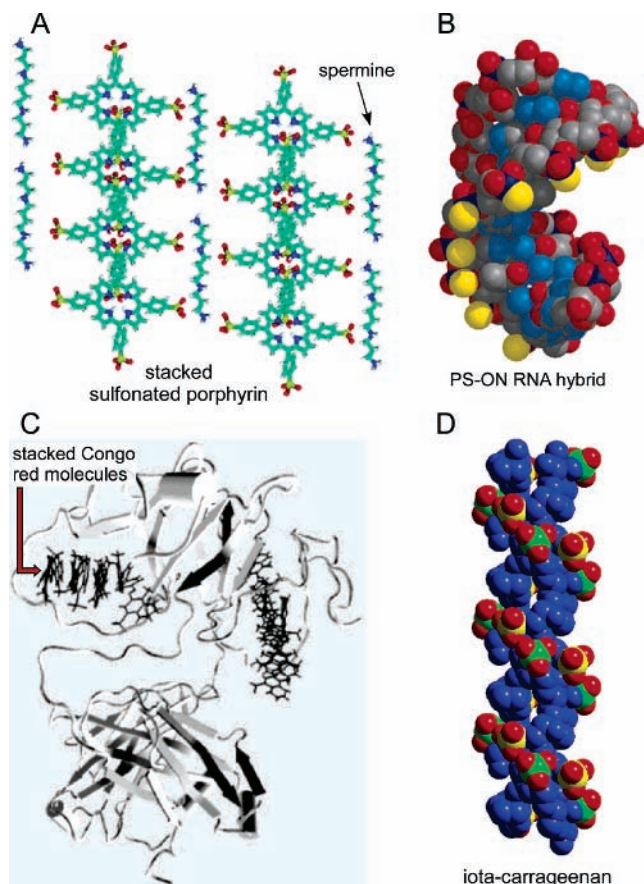


FIGURE 3. Structural similarities among different classes of anti-TSE compounds. Like the phosphorothioated oligonucleotides (PS-ONs) and sulfated glycans, planar π -stacked supramolecular aggregates of sulfonated cTPs and dyes can be extended structures with periodic negative charges and hydrophobic 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

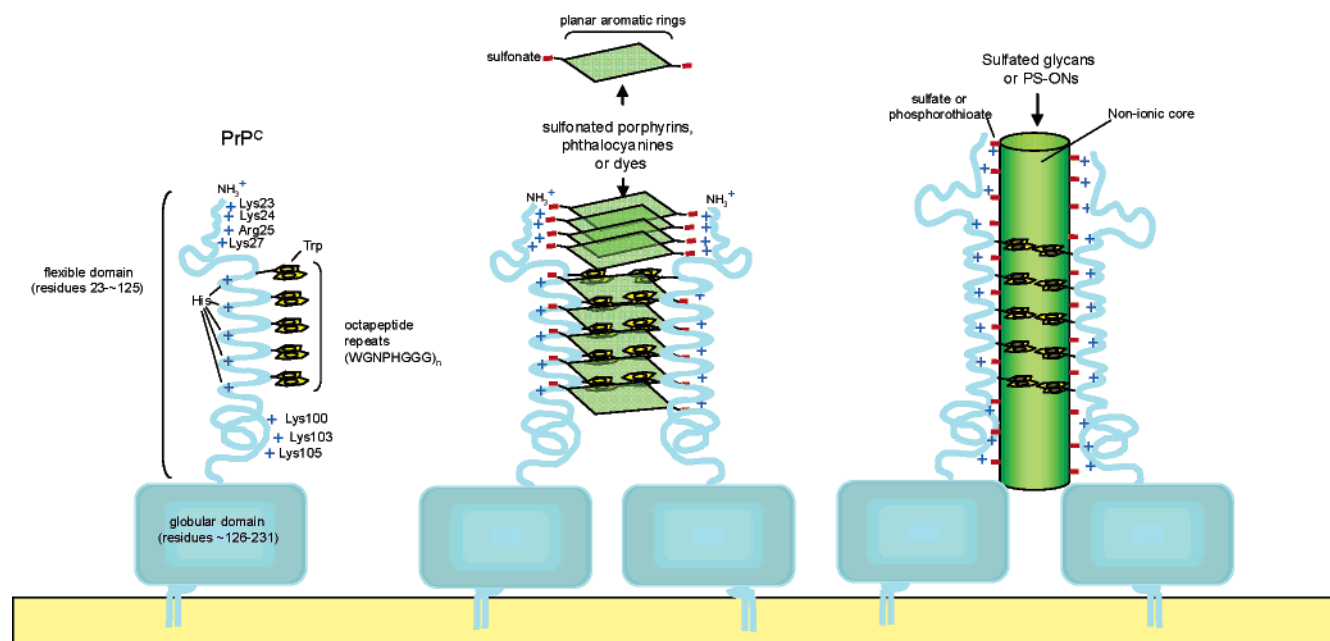


FIGURE 4. Model of possible interactions between PrP^C and various PrP^{Sc} inhibitors that cause PrP^C aggregation. The left panel shows diagrammatic PrP^C structure emphasizing the planar aromatic tryptophan side chains in the octapeptide repeats and cationic residues in regions that have been implicated 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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Notes & Tips

Identification of prion inhibitors by
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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-

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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 IC₅₀ (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 complementary in vitro models.

The relationship between K_i (FP assay), cell culture IC₅₀, 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 A1PCTS	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} IC₅₀ $\leq 10 \mu\text{M}$ 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.

Acknowledgments

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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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[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

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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

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.

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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 μ g/ml ($\sim$ 1 μ 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 μ l 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 μ l of lysing buffer is added; 25 μ l of 0.2 U/ μ l 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 μ l of 100 μ g/ml PK is added after benzonase treatment to give a final concentration of 25 μ g/ml, and the plates are incubated at 37° for 50 min. Immediately after protease treatment, 200 μ l 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 μ l of TBS. The suction is adjusted so that 500 μ l 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- μ m 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).

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The availability of sheep scrapie-infected Rov9 cells allows the comparison of a compounds' 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

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DHIANJALI CHANDRARATNA, 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

Enhanced Antiscrapie Effect Using Combination Drug Treatment

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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.

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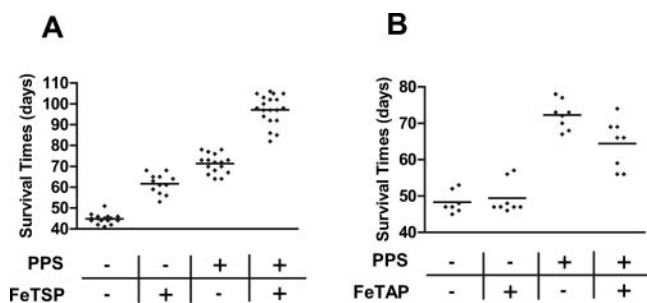


FIG. 1. Combined PPS and porphyrin treatments beginning at 14 days postinfection. Phosphate-buffered saline was the vehicle for all weekly 50- μ l i.c. injections. Injections were phosphate-buffered saline only, 0.5 mM porphyrin only, 0.05 mM PPS only, or 0.5 mM porphyrin and 0.05 mM PPS in the same 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

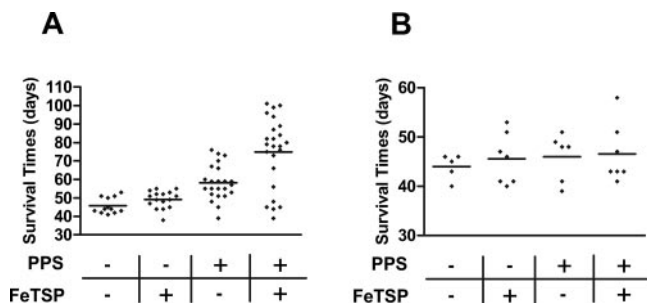


FIG. 2. Combined PPS and FeTSP treatments beginning at 28 or 35 days postinfection. Physiological saline was the vehicle for all weekly 50- μ l i.c. injections. Injections were saline only, 0.5 mM FeTSP only, 0.1 mM PPS only, or 0.5 mM FeTSP and 0.1 mM PPS in the same solution. The line in each scatter 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.

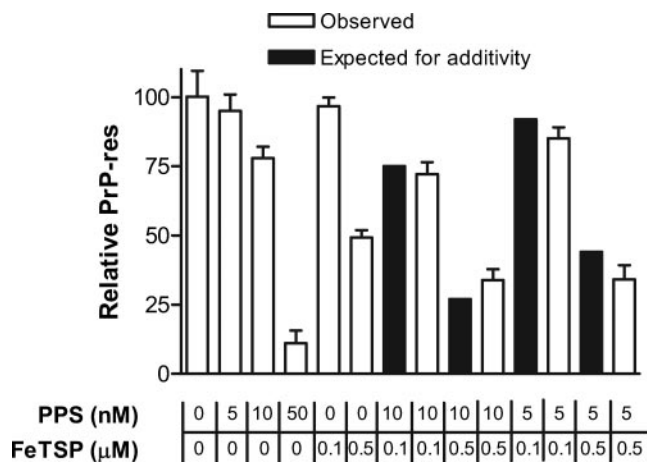


FIG. 3. Additive inhibitory effect with combinations of FeTSP and PPS in scrapie-infected mouse neuroblastoma cells. Cells chronically infected with the 22L scrapie strain were seeded at 5% confluent density and grown to confluence in the presence of the designated inhibitor concentrations. The cells 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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