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14. ABSTRACT: Transmissible spongiform encephalopathies are fatal untreatable neurodegenerative diseases associated with the accumulation of a disease-specific form of prion protein (PrPSc) in the brain. One therapeutic approach is the inhibition of PrPSc accumulation. Indeed, many inhibitors of PrPSc accumulation in scrapie-infected cells also have anti-scrapie activity in rodents. During this year, a cell line derived from deer has been chronically infected with CWD to more effectively search for agents to combat that disease. Additionally, two compounds that inhibit PrPSc accumulation in scrapie-infected mouse cells and CWD-infected cells, iron tetrasulfonatophenyl porphyrin (FeTSP) and a degenerate 40 base phosphorothioate oligonucleotide (Randomer 1), were found to also have anti-scrapie activity in mice. Randomer 1 mixed with infectious brain homogenate delays the onset of disease and also works prophylactically against an ip inoculated scrapie. FeTSP has prophylactic activity as well as therapeutic activity against an existing scrapie brain infection, which places it among a very small group of molecules with such activity. Finally, an antimalarial compound, mefloquine, which is an effective inhibitor of PrPSc in cell culture, did not demonstrate prophylactic anti-scrapie activity in mice. This underscores the need to test promising anti-prion agents in animals prior to human trials.

15. SUBJECT TERMS
Transmissible Spongiform Encephalopathy (TSE), Prion Disease

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INTRODUCTION

This research is aimed at finding therapeutics against the Transmissible Spongiform Encephalopathies (TSEs) or prion diseases, which are currently untreatable. TSEs are neurodegenerative diseases associated with the deposition of a disease specific form of prion protein called PrP^{Sc}. This form of prion protein is a logical target for intervention as the basis for therapy. Currently, only mouse cells lines are available that are chronically-infected with a TSE. In this year of work, a deer cell line has been developed that is chronically infected with CWD. This will allow chemicals to be tested for the ability to inhibit CWD-associated PrP (PrP^{CWD}) which may give insight into their potential use in infected animals. Two different kinds of compounds that inhibit PrP^{Sc} in infected cells were tested for anti-scrapie activity in infected mice. A 40-base degenerate phosphorothioate oligonucleotide developed by REPLICor Inc., was found to have strong anti-scrapie prophylactic activity in mice. This molecule also delayed the onset of scrapie in mice when mixed into infectious brain homogenate prior to inoculation into the brains of mice. A porphyrin, iron tetrasulfonatophenyl porphine, was found to have prophylactic activity as well as therapeutic activity. When treatment of this porphyrin was started after inoculation of scrapie into the brain of mice, there was a significant delay in the onset of clinical symptoms. Finally, a promising anti-prion candidate, mefloquine, which has strong anti-PrP^{Sc} activity in infected cells, did not demonstrate prophylactic activity in an infected mouse. This testing underscores the need to assess in vivo anti-prion activity prior to moving a molecule to clinical use.

BODY

Research accomplishments associated with Task 1: To increase the throughput of a screen for anti-PrP^{Sc} activity in the scrapie-infected neuroblastoma cell model.

In prior years, this task has been completed for two strains of mouse scrapie with the development of a high-throughput cell-culture assay to measure the inhibition of the formation of PrP^{Sc}. Additionally, using sheep scrapie infected cells developed by Didier Vilette, a high-throughput dot blot screening assay has been developed. The best cell culture screen for a given type of TSE would use cells infected with that specific TSE. We have addressed this need by producing a line of deer cells that is chronically infected with PrP^{CWD} (see first manuscript in appendex). Compounds that inhibit PrP^{CWD} in this cell line are more suited for in vivo testing than compounds that don't inhibit PrP^{CWD} or that can only inhibit PrP^{Sc} in mouse cells. Further work is needed to adapt these cells to a 96 well plate assay and higher-throughput use.

Research accomplishments associated with Task 2: To develop a high-throughput cell-free system to measure the ability of compounds to interfere with PrP^{C} to protease-resistant PrP conversion.

Work in this area centers around the interaction between a degenerate 40-base phosphorothioate oligonucleotide (Randomer 1) and PrP (see second manuscript in appendex). These molecules bind very tightly to one another based on fluorescence polarization measurements and the basis of an effective cell-free assay may be to look for compounds that interfere with this interaction. In the initial work, sulfated glycans with in vivo activity tended to compete with this interaction of Randomer 1 bound to PrP. More compounds need to be tested to evaluate the predictive power of such a competitive binding assay.

Research accomplishments associated with Task 3: To screen libraries of compounds for anti-PrP^{Sc} activity.

Many different porphyrins, phosphorothioate oligonucleotides, and anti-malarial compounds were tested for the ability to inhibit PrP^{Sc} in cells. The most effective inhibitors found were tested in animal models (see below).

Research accomplishments associated with Task 4: To test the compounds with the best activity in the anti-PrP^{Sc} screens in TSE-infected animals.

Phosphorothioate oligonucleotides are nucleic acids with a phosphorous atom in place of one of the oxygen atoms in the diester in the backbone of the molecule. The activity of these compounds seemed to increase with the number of bases and peaked at about 40 bases. The 40-base degenerate phosphorothioated oligonucleotide (Randomer 1) was tested in scrapie-infected mice (see second manuscript in appendex). Using these compounds prophylactically, the onset of scrapie was greatly delayed even against a high

dose of scrapie dosed intraperitoneally. Also, there was a substantial delay in disease onset when the compounds were mixed into infectious brain homogenate. Because of the low toxicity seen when this class of compound is dosed to humans and the in vivo anti-prion activity noted, further study of Randomer 1 is warranted.

One porphyrin, iron tetrasulfonatophenyl porhphine, was the only porphyrin of a group tested in vivo that had therapeutic anti-scrapie activity in mice (see third manuscript in appendex). This compound significantly delayed the onset of scrapie in mice that had been inoculated with scrapie into the brain. Because this compound does not cross the blood brain barrier, it was injected directly into the brain. Very few compounds are known with therapeutic anti-scrapie activity so this makes it interesting for further study.

Finally, a promising anti-malarial compound, mefloquine, which is a strong inhibitor of PrP^{Sc} in infected cells, was tested for prophylactic activity in mice. This compound is approved as an antimalarial compound and also crosses the blood brain barrier. This compound failed to demonstrate any significant increase in lifespan of the mice (see fourth manuscript in appendex). This work illustrates the importance of using animal models prior to pursuing clinical trials.

KEY RESEARCH ACCOMPLISHMENTS:

- Developed a deer cell line infected with PrP^{CWD} to test compounds for the potential to fight CWD in vivo
- Found that a 40-base degenerate phosphorothioate oligonucleotide has strong prophylactic anti-scrapie activity in vivo. This compounds interaction with PrP may be the basis for a cell-free assay for in vivo anti-prion activity.
- Found that iron tetrasulfonatophenyl porphine has therapeutic anti-scrapie activity in mice. Treatment started two weeks after inoculation of scrapie into the brains of mice was able to delay the onset of disease.
- Mefloquine, a promising anti-prion compound, did not demonstrate any in vivo activity in infected mice.

REPORTABLE OUTCOMES

Manuscipts:

Raymond GJ, Olsen EA, Raymond LD, Bryant PK, Lee KS, Baron GS, Caughey WS, Kocisko DA, McHolland LE, Favara C, Langeveld JPM, vanZiderveld FG, 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, 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 WS, Race RE, Roper G, Caughey B, Morrey JD. A porphyrin increases survival time of mice after intracerebral prion infection. Antimicrob Agents Chemother 2006;50:759-761.

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.

Invited Presentations:

"New compounds for the treatment of prion diseases." CJD Foundation Family Conference, July 2005. Washington, DC

"Phosphorothioate oligonucleotides as anti-scrapie compounds." US Department of Defense National Prion Research Program Investigators Meeting, December 2005. Chantilly, VA

"Searching for Anti-TSE Compounds using Animal Models." Cambridge Healthtech 10th Annual TSE Conference, March 2006. Baltimore, MD

CONCLUSIONS

We are happy with the progress we are making on this project. During the year, the first line of deer cells infected with PrP^{CWD} was developed which will aid in finding compounds to fight CWD. Compounds that inhibited PrP^{Sc} in mouse and deer cells, Randomer 1 and FeTSP, were found to have in vivo anti-prion activity. Both compounds have promise and warrant further testing. Based on our result from scrapie prophylaxis testing of mefloquine and what has been published on quinacrine, we feel that animal testing continues to be an important step in the process of searching for meaningful TSE therapies.

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Raymond GJ, Olsen EA, Raymond LD, Bryant PK, Lee KS, Baron GS, Caughey WS, Kocisko DA, McHolland LE, Favara C, Langeveld JPM, vanZiderveld FG, 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.

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

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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

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† 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 ($250 \times g$). 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-cm^2 Primaria flasks (BD Biosciences) at approximately 2×10^7 cells per flask and incubated at 37°C in a humidified 5% CO_2 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 $\sim 2 \times 10^7$ cells per flask; $\sim 5 \times 10^6$ 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 $500 \times g$, resuspended in the DOF growth medium, and plated into six-well plates at $\sim 7.5 \times 10^5$ 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 frozen 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 subsequently 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 $250 \times g$, resuspended in OF growth medium and seeded into a 25-cm^2 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-cm^2 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, Pepscan 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 ^{93}WGQGG^{97}, 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-cm^2 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 μg/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 × *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 μg/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-cm^2 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 G^{96}M^{132}S^{138}S^{225}Q^{226}. 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 *m*ule *d*eer *b*rain). 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 homozygous for the PrP genotype encoding amino acid residues G^{96}M^{132}S^{138}S^{225}Q^{226}. 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

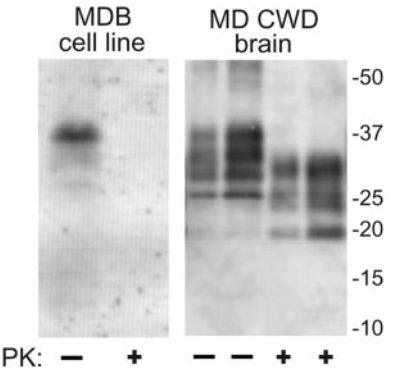

FIG. 1. Expression of PrP-sen by the transformed cell line derived from mule deer brain (MDB). The image is an immunoblot of PK-treated (+) and untreated (−) cell extracts using antibody 12B2 to detect PrP. PK-treated and untreated PrP^{CWD} purified (34) from CWD-affected MD brain (20- and 60-ng samples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

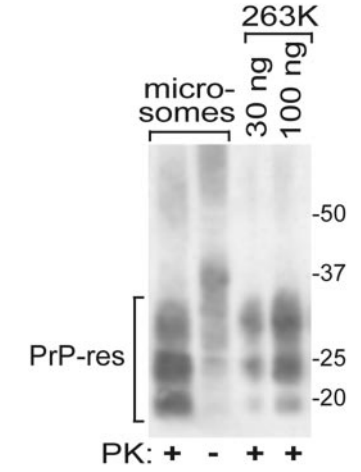

FIG. 2. PrP^{CWD} in microsomes isolated from the CWD-affected MD brain used to infect the MDB cell line. Aliquots of the microsome preparations were incubated with PK (+) or without PK (−) and analyzed on an immunoblot using antibody 12B2 to detect PrP molecules. In this blot, the upper glycoform bands 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 $G^{96}M^{132}S^{138}S^{225}Q^{226}$ 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 consistent PrP^{CWD} production overall through many in vitro passes.

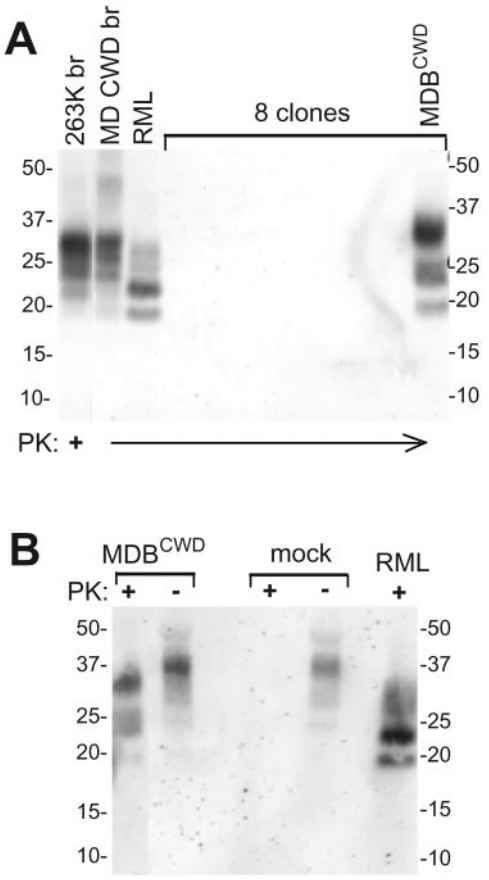

FIG. 3. Immunoblot analysis of mule deer brain cell clones after exposure to CWD microsomes. The MDB-transformed cell line was incubated with microsomes prepared from a CWD-positive mule deer brain. At the third pass, selected lines (see Materials and Methods) were dilution cloned and expanded. At the 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.

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

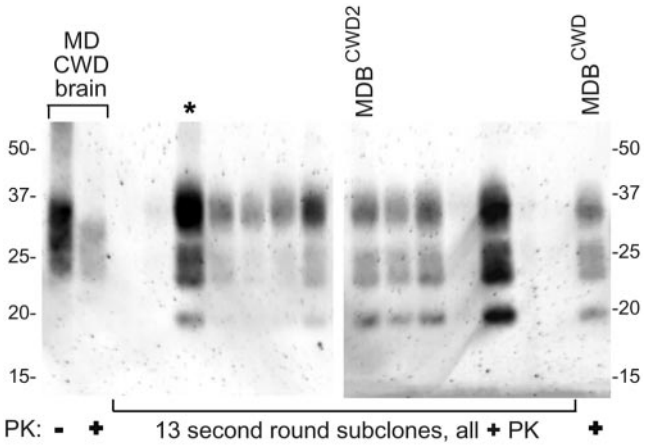

FIG. 4. Immunoblot analysis of MDB^{CWD} subclones from the second-round dilution subcloning. Subclone lysates were PK treated for detection of PrP^{CWD}. The original MDB^{CWD} clone is shown in the last lane on the right. This blot shows 13 representatives of the 30 total second-round subclones that 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 "MDB^{CWD2}" 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.

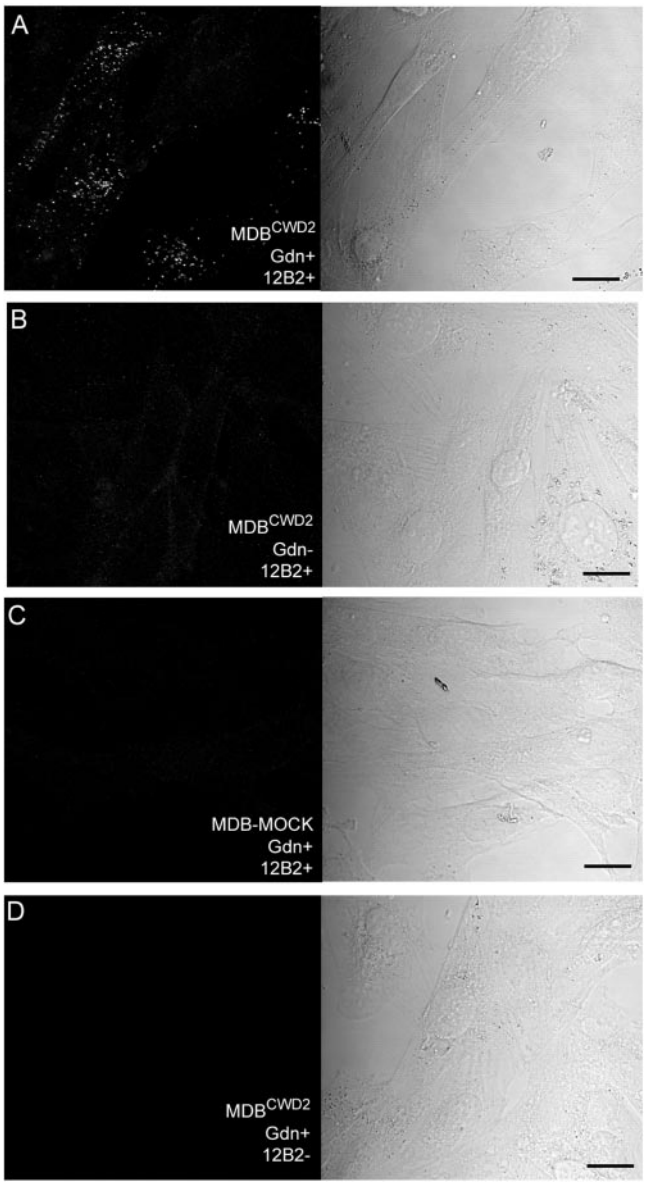

FIG. 5. Immunofluorescence staining of PrP^{CWD} in MDB^{CWD2} cells. MDB^{CWD2} cells were stained with anti-PrP antibody 12B2 after treatment with GdnSCN (Gdn+, panel A, left). The extensive punctate structures are typical of PrP-res staining. When GdnSCN treatment was omitted (Gdn−), only a few faint punctate structures were observed (panel B, left). Only very faint fluorescence was apparent in mock-infected cells (MDB-MOCK, panel C, left). The MDB^{CWD2} 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.

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 MDB^{CWD2} cell line (Fig. 5D). The PrP^{CWD} accumulation pattern observed in MDB^{CWD2} 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 MDB^{CWD2} cell line. To assess the lineage of the MDB^{CWD2} 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 MDB^{CWD2} cell pellets (data not shown). Therefore, these data support the conclusion that the MDB^{CWD2} is a fibroblast-like cell line.

Inhibition of PrP^{CWD} accumulation in MDB^{CWD2} cells. To investigate the utility of MDB^{CWD2} 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-

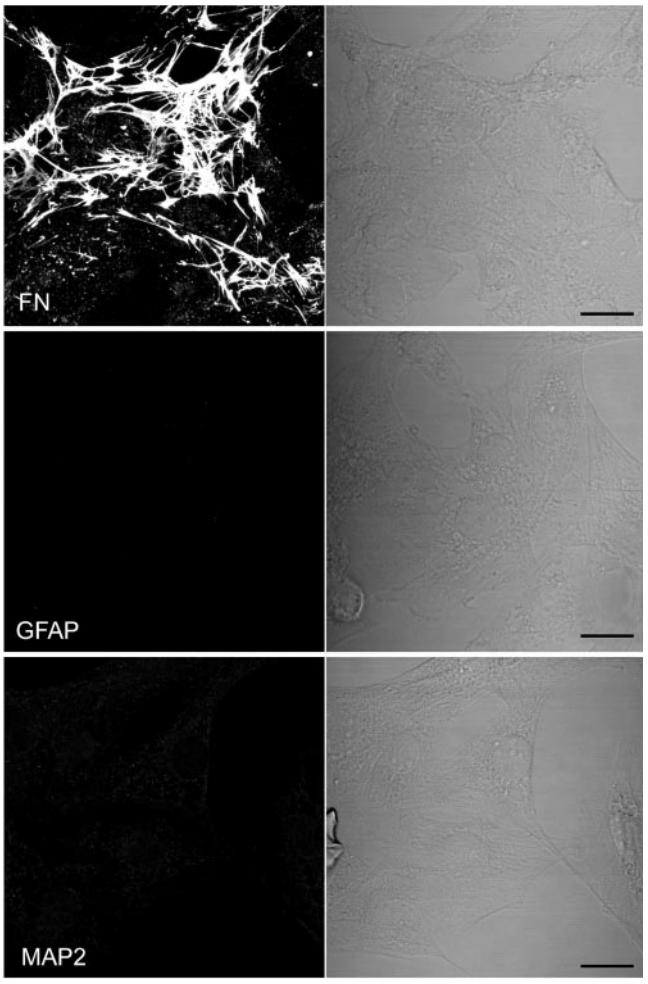

FIG. 6. Immunostaining of MDB^{CWD2} cells. MDB^{CWD2} cells were stained with antibodies against human fibronectin (FN), GFAP, and MAP2, which are markers for fibroblast cells, astrocytes, and neuronal cells, respectively. Reconstructed confocal microscopic images of Z-series acquired with an interval of 0.54 μm are shown in the left panel, and differential interference contrast images are shown in the right panel. Bar, 20 μm.

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 ~10 ng/ml (~3 nM, based on an average, but heterogeneous, molecular mass of ~3,800 Da) and <0.3 μM, 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 μg/ml PPS or 1 μM In-TSP was added directly to untreated MDB^{CWD2} 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 ≤3 μM for In-TSP. PPS started to show minor cytotoxicity at 1 μg/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 MDB^{CWD2} cells were serially passed 1:10 five times in the presence of 0.3 μg/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 MDB^{CWD2} 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

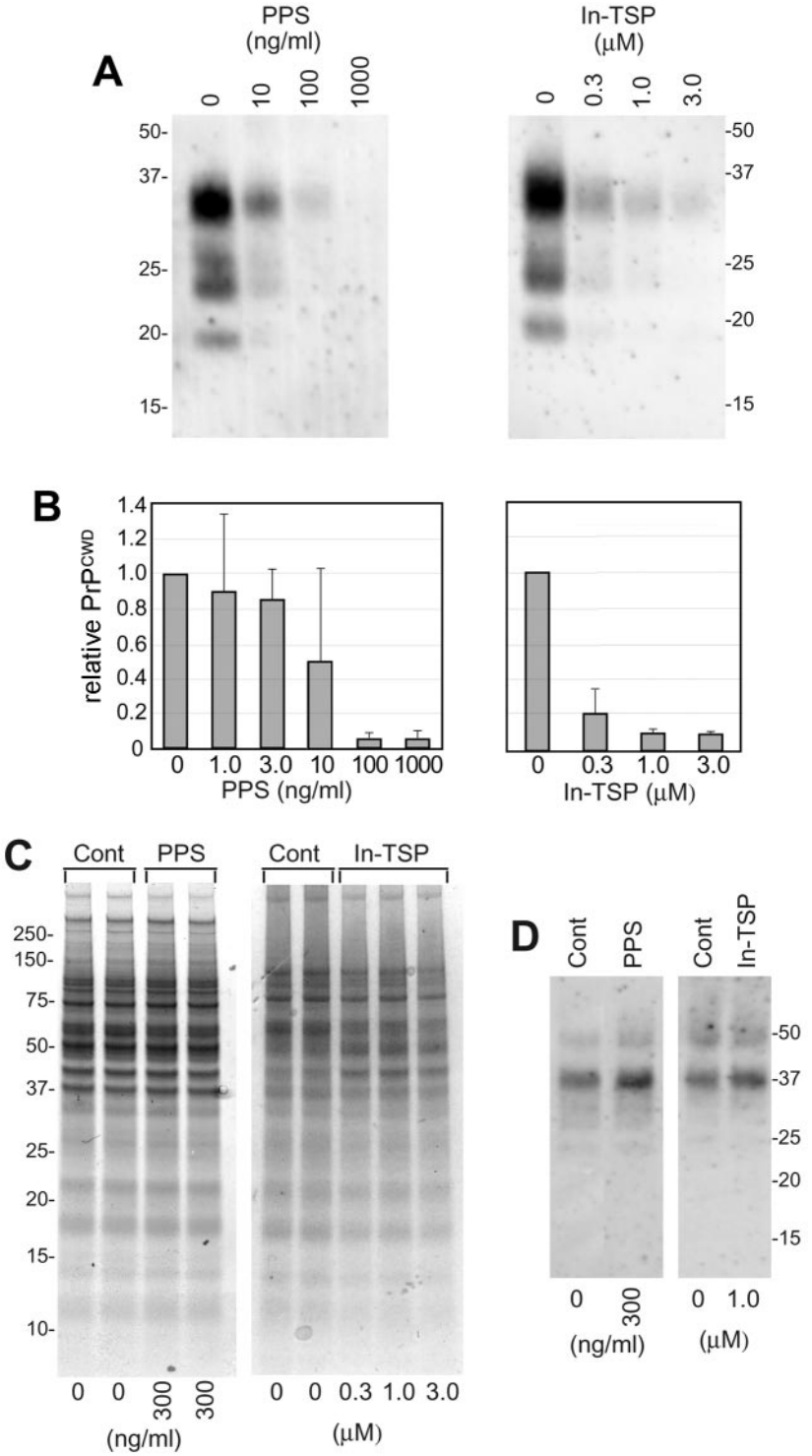

FIG. 7. Effects of PPS and In-TSP on PrP^{CWD} biosynthesis in MDB^{CWD2} cells. Upon plating at 1:10 dilution, cells were treated with the designated amounts of the compounds and grown until near confluence (~4 days). (A) Cell lysates were analyzed for PrP^{CWD} by immunoblotting using antibody 12B2. 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.

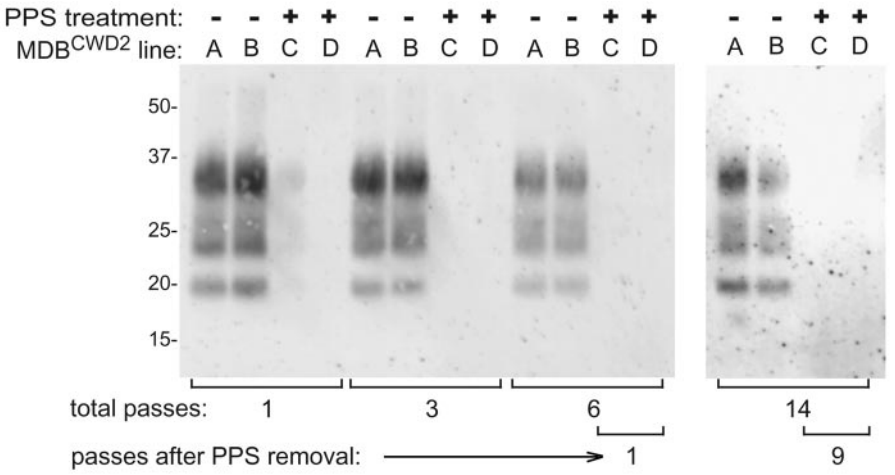

FIG. 8. Lack of recovery of PrP^{CWD} after PPS treatment of MDB^{CWD2} cells. Duplicate lines of MDB^{CWD2} cells were passed at ~1:10 dilution in medium for the designated number of passes (A, B) or in medium containing 0.3 μg/ml PPS for 5 passes and unsupplemented medium thereafter (C, D). One-half 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 $G^{96}M^{132}S^{138}S^{225}Q^{226}$ 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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Potent Antiscrapie Activities of Degenerate Phosphorothioate Oligonucleotides

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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 Creutzfeld-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 μg/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

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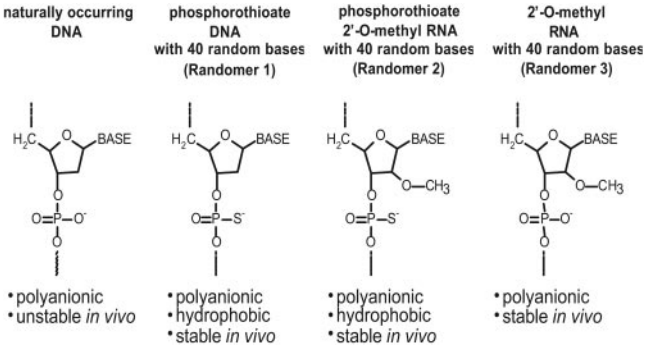

FIG. 1. Structures of nucleic acids tested. The phosphorothioate modification, which reduces enzymatic degradation and increases the hydrophobicities of ONs, and 2′-O-methylation, which only stabilizes ONs, were used to alter the chemical properties of ONs in this study.

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 μg protein for Randomer 1 and 2 and 2 μg 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 glycophosphatidylinositol (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 μg of DNA and 6 μl 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 (×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 μl 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 μl 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 μl 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 μl 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 ± SD IC_{50} for 22L scrapie		Avg ± SD IC_{50} (nM) for sheep scrapie
	nM	ng/ml	
6-base Randomer 1	55,000 ± 11,000	110,000 ± 22,000	>25,000[b]
10-base Randomer 1	3,100 ± 400	10,000 ± 1,000	17,000 ± 4,000
11-base Randomer 1	3,800 ± 700	14,000 ± 3,000	8,400 ± 4,800
12-base Randomer 1	920 ± 80	3,600 ± 300	4,300 ± 400
13-base Randomer 1	1,200 ± 500	5,100 ± 2,100	2,200 ± 1,000
14-base Randomer 1	630 ± 180	2,900 ± 800	3,600 ± 700
15-base Randomer 1	280 ± 90	1,400 ± 400	3,300 ± 200
16-base Randomer 1	290 ± 50	1,500 ± 300	2,900 ± 600
17-base Randomer 1	78 ± 3	440 ± 20	730 ± 80
18-base Randomer 1	70 ± 30	420 ± 180	780 ± 30
19-base Randomer 1	65 ± 5	410 ± 30	620 ± 150
20-base Randomer 1	58 ± 11	380 ± 70	410 ± 80
25-base Randomer 1	24 ± 3	200 ± 30	150 ± 100
28-base Randomer 1	25 ± 3	230 ± 30	48 ± 8
30-base Randomer 1	27 ± 5	270 ± 50	58 ± 18
Randomer 1	21 ± 10	270 ± 130	51 ± 14
50-base Randomer 1	11 ± 5	180 ± 80	28 ± 2
80-base Randomer 1	18 ± 7	480 ± 190	31 ± 2
120-base Randomer 1	9 ± 1	360 ± 40	33 ± 2
40-base DNA	15,000 ± 3,000		61,000 ± 22,000
Randomer 2	20 ± 8		35 ± 6
Randomer 3	90,000 ± 11,000		~100,000[c]
Poly(A) Randomer 1	31 ± 13		28 ± 2
Poly(T) Randomer 1	23 ± 2		21 ± 5
Poly(C) Randomer 1	20 ± 4		31 ± 9
Poly(G) Randomer 1	67 ± 9		NT[d]
Poly(AC) Randomer 1	23 ± 5		47 ± 2
Poly(TC) Randomer 1	9 ± 1		8 ± 1
Poly(AG) Randomer 1	12 ± 5		59 ± 22
Poly(TG) Randomer 1	25 ± 4		12 ± 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 μM.
[c] Inhibition of ~45% at 100 μM.
[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 IC_{50}s 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 ≤100 μM 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

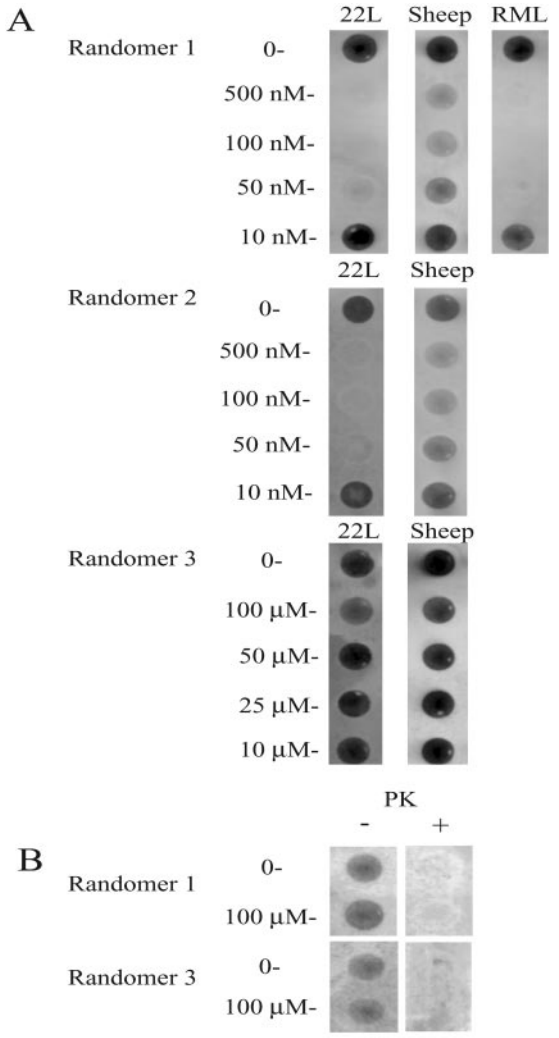

FIG. 2. Randomers 1 and 2 but not Randomer 3 were potent inhibitors of 22L PrP-res and sheep scrapie PrP-res in cells. (A) The three sets of panels are dot blots of the indicated type of PrP-res from cells grown from low density to confluence in the presence of the indicated amounts of Randomer in cell 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 ± SD K_D for mouse rPrP-sen		Avg ± SD K_D (nM) for hamster rPrP-sen
	nM	ng/ml	
6-base Randomer 1	2,400 ± 300	4,800 ± 600	2,800 ± 100
10-base Randomer 1	420 ± 30	1,400 ± 100	430 ± 30
20-base Randomer 1	70 ± 16	460 ± 110	87 ± 31
Randomer 1	16 ± 10	210 ± 130	12 ± 6
80-base Randomer 1	63 ± 8	1,700 ± 200	56 ± 30
Randomer 2	13 ± 4	170 ± 50	25 ± 1
Randomer 3	250 ± 60	3,300 ± 800	210 ± 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 μM 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 ~25 to 28 bases. This was especially apparent when the IC_{50}s 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 eightfold 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 ± SD K_i (nM)					
	Randomer 1-FL		Randomer 2-FL		Randomer 3-FL	
	Mouse	Hamster	Mouse	Hamster	Mouse	Hamster
Randomer 1	16 ± 4	31 ± 10	29 ± 3	48 ± 16	16 ± 5	58 ± 20
Randomer 2	20 ± 1	37 ± 18	29 ± 2	53 ± 18	26 ± 12	52 ± 14
Randomer 3	130 ± 30	220 ± 70	170 ± 50	200 ± 60	62 ± 4	140 ± 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 ± 3	150 ± 70
Dextran sulfate, 8,000 MW	MC	MC	MC	MC	31 ± 3	100 ± 40
Dextran sulfate, 10,000 MW	MC	120 ± 70	MC	83 ± 15	14 ± 3	48 ± 16
Dextran sulfate, 500,000 MW	7.7 ± 4.4	3.5 ± 2.3	0.7 ± 0.1	2.5 ± 0.8	1.1 ± 0.8	1.7 ± 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), ~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), ~17,000 MW	3,300 ± 700	2,600 ± 400	1,900 ± 500	MC	42 ± 18	120 ± 70
Pentosan polysulfate, ~5,000 MW	1,600 ± 300	2,100 ± 600	1,700 ± 400	2,400 ± 700	100 ± 20	360 ± 20
Chondroitin sulfate A (Sigma C9819)	NC	NC	NC	NC	NC	NC
Chondroitin 6-sulfate (Sigma C4384), ~60,000 MW	NC	NC	NC	NC	NC	NC

[a] Abbreviations: FL, fluorescence label; MW, molecular weight; NC, no competition at up to 100 μg/ml competitor; MC, minimal competition (some competition was observed, but >100 μg/ml competitor was required to achieve the IC_{50}).

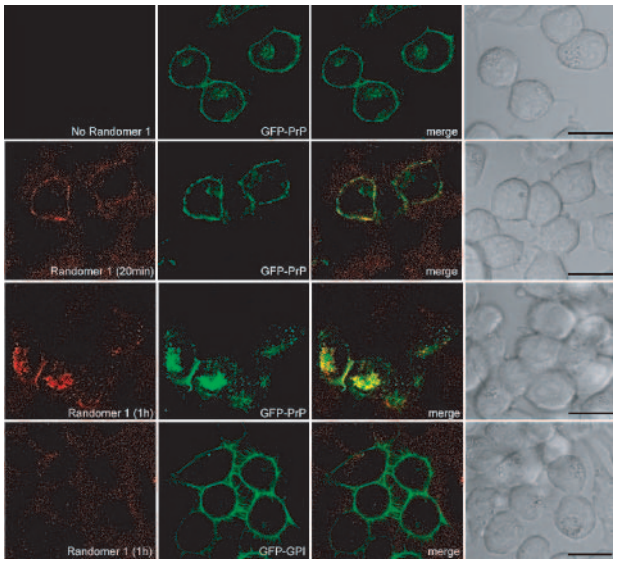

FIG. 3. Enhanced binding and uptake of rh-Randomer 1 in N2a cells expressing GFP-PrP. N2a cells transiently expressing GFP-PrP (green) or GFP-GPI, a green GPI-linked GFP control, were incubated for the designated times with 100 nM rh-Randomer 1 (red). The yellow fluorescence in the merged images indicates 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 μm.

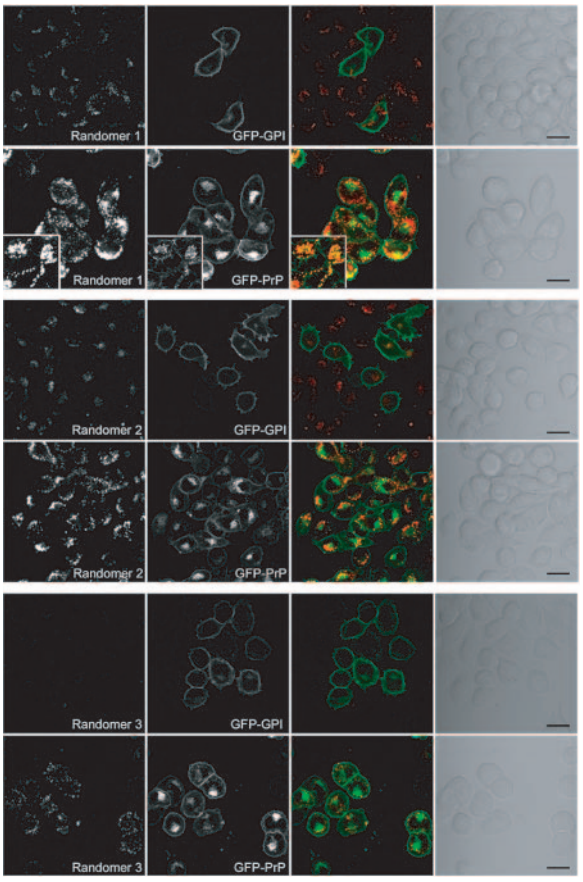

FIG. 4. Comparison of GFP-PrP-dependent uptake of rh-Randomers 1, 2, and 3 in N2a cells. N2a cells transiently expressing GFP-GPI (rows 1, 3, and 5) or GFP-PrP (rows 2, 4, and 6) were incubated for 1 day with 100 nM rh-Randomer 1 (rows 1 and 2), 100 nM rh-Randomer 2 (rows 3 and 4), or 400 nM rh-Randomer 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 μm.

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 μg/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 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

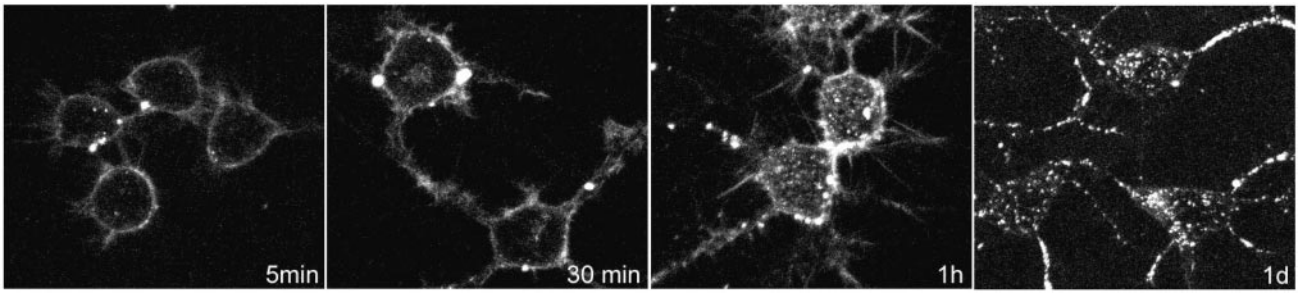

FIG. 5. Uptake of rh-Randomer 1 in SN56 cells. SN56 cells were incubated with 100 nM of rh-Randomer 1, and images were acquired by confocal microscopy after the designated times. Binding of rh-Randomer 1 on the cell surface was observed prior to the internalization. d, day.

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 μM Randomer 1 was mixed with 1% scrapie brain homogenate, a significant 9-day increase in the survival time was observed

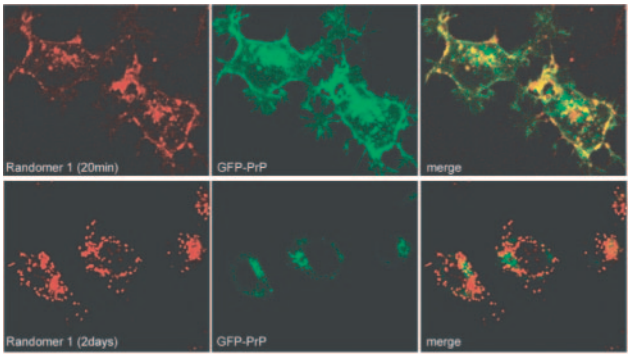

FIG. 6. Colocalization of rh-Randomer 1 with GFP-PrP at the cell surface in SN56 cells. SN56 cells expressing GFP-PrP (green) were incubated with rh-Randomer 1 (red) for 20 min or 2 days. After 20 min of incubation, an extensive colocalization between rh-Randomer 1 and GFP-PrP was observed. After 2 days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

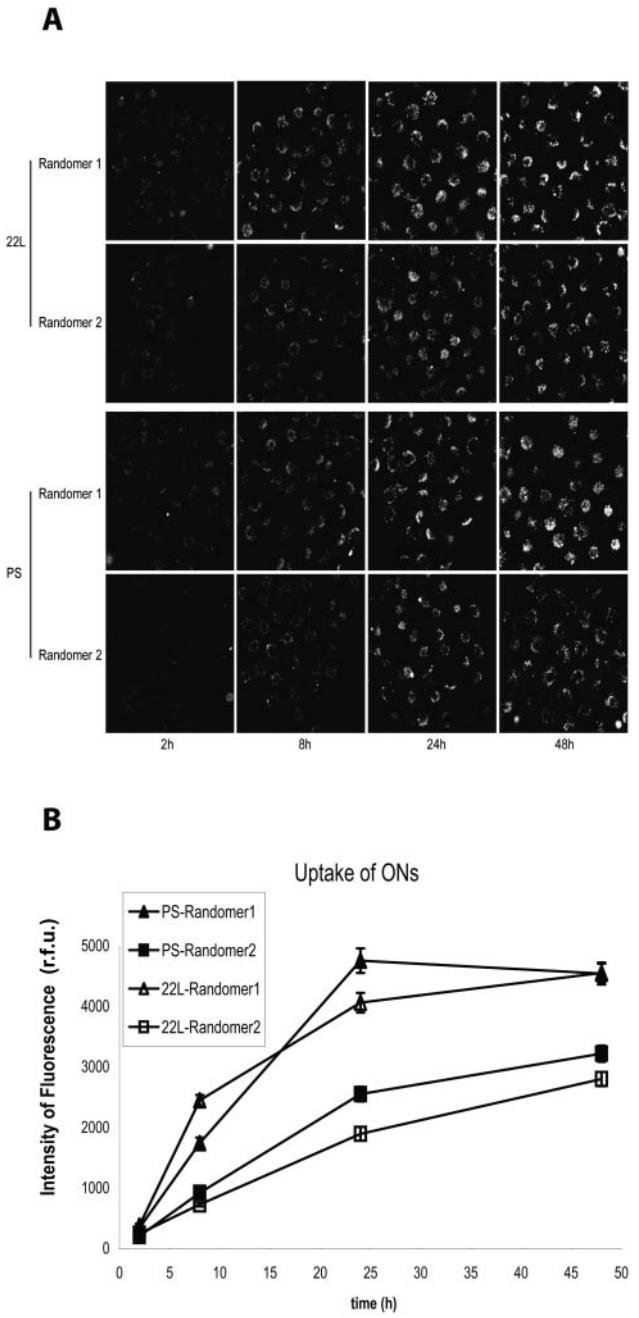

FIG. 7. Uptake of Randomers 1 and 2 in scrapie-infected and cured N2a cells. (A) Images of 22L-infected (22L) or pentosan polysulfate-cured (PS) N2a cells were acquired after 2, 8, 24, or 48 h of incubation with 100 nM rh-Randomer 1 or 2 by confocal microscopy. (B) To quantitate the uptake of Randomers, 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 μM Randomer 1 (Table 5). A second experiment added a 100 μM 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 μM and 100 μM 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 aPPTs 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 ~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 μl of 1% 263K brain homogenate

Treatment after inoculation	Survival times (days)	Avg ± SD survival time (days)	Significance between groups (unpaired *t* test)
4 wk s.c. 5% dextrose	77, 83, 83, 85, 87, 90, 91, 94, 98	87.6 ± 6.4	
4 wk s.c. Randomer 1	181, 201, 238, 254	218.5 ± 33.4	$P < 0.0001$ vs s.c. control
10 wk s.c. Randomer 1	175, 186, 226[a]	195.7 ± 26.8	$P < 0.0001$ vs s.c. control
4 wk i.p. 5% dextrose	75, 76, 76, 76, 77, 77, 79, 82, 83, 86, 103	80.9 ± 8.1	NS,[c] $P > 0.05$ vs s.c. control
4 wk i.p. Randomer 1	276, 281, 300, 462	329.8 ± 88.8	$P < 0.0001$ vs i.p. control
10 wk i.p. Randomer 1	260, 326[b]	293	$P < 0.0001$ 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 μM (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 phosphorothiate 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 μl)	Survival times (days)	Avg ± SD survival time (days)	Significance between groups (Tukey test)
Expt. 1			
1% BH[a]	48, 50, 50, 52, 48, 44, 47, 48	48.4 ± 2.4	
1% BH + 100 nM Randomer 1	49, 50, 52, 51	50.5 ± 1.3	NS[b]; $P > 0.05$ vs 1% BH
1% BH + 10 μM Randomer 1	58, 58, 57, 58, 57	57.6 ± 0.5	$P < 0.001$ vs 1% BH
Expt. 2			
1% BH	47, 46, 48, 45, 45, 45	46.0 ± 1.3	
0.1% BH	51, 51, 53, 54, 52, 55	52.7 ± 1.6	$P < 0.001$ vs 1% BH
0.01% BH	54, 59, 60, 59, 57	57.8 ± 2.4	$P < 0.01$ vs 0.1% BH
0.001% BH	66, 65, 58, 62, 62, 61	62.3 ± 2.9	$P < 0.01$ vs 0.01% BH
1% BH + 10 μM Randomer 1	58, 57, 58, 57, 58, 58, 57	57.6 ± 0.5	$P < 0.001$ vs 1% BH; NS, $P > 0.05$ vs 0.01% BH
1% BH + 100 μM Randomer 1	62, 62, 61, 62, 64, 61, 67	62.7 ± 2.1	$P < 0.001$ vs 1% BH; NS, $P > 0.05$ vs 0.001% BH
1% BH + 100 μM Randomer 3	48, 48, 45, 46, 45	46.4 ± 1.5	NS, $P > 0.05$ vs 1% BH
Expt. 3			
0.01% BH	53, 54, 55, 57, 58, 58	55.8 ± 2.1	
0.001% BH	61, 63, 65, 69, 71, 75	67.3 ± 5.3	$P < 0.001$ vs 0.01% BH
1% BH + 10 μM Randomer 2	56, 56, 59, 62, 62, 56, 56, 54	57.6 ± 3.0	NS, $P > 0.05$ vs 0.01% BH
1% BH + 50 μM Randomer 2	70, 60, 67, 67, 71, 70, 77, 76	69.8 ± 5.4	$P < 0.001$ vs 0.01% BH; NS, $P > 0.05$ vs 0.001% BH
1% BH + 100 μM Randomer 2	72, 70, 70, 70, 67, 70, 72	70.1 ± 1.7	$P < 0.001$ vs 0.01% BH; NS, $P > 0.05$ vs 0.001% BH
1% BH + 10 μM 40-base Randomer 1 analog, poly(C)	52, 52, 52, 53, 54, 55, 55	53.3 ± 1.4	NS, $P > 0.05$ vs 0.01% BH
1% BH + 50 μM 40-base Randomer 1 analog, poly(C)	54, 55, 55, 56, 56, 56, 56, 56, 57	55.7 ± 0.9	NS, $P > 0.05$ vs 0.01% BH
1% BH + 100 μM 40-base Randomer 1 analog, poly(C)	55, 56, 57, 58, 58, 59, 60	57.8 ± 1.7	NS, $P > 0.05$ vs 0.01% BH

[a] BH, brain homogenate.
[b] NS, not significant.

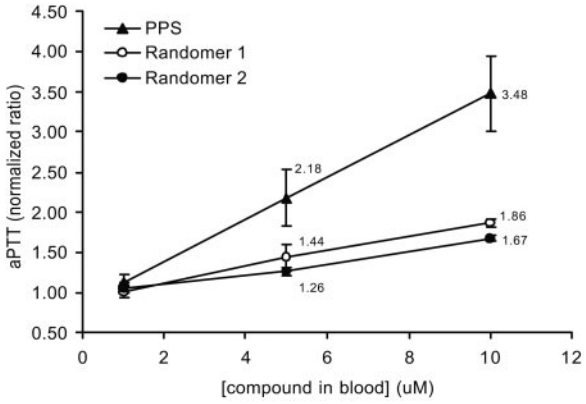

FIG. 8. aPPTs of Randomers 1 and 2 and PPS. Normalized aPTT ratio represents the fold increase in aPPT compared to that obtained with normal saline.

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 antiscrapie 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 antiscrapie 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 antiscrapie 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 antiscrapie 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 antiscrapie activities of PS-ONs. PS-ONs may block or compete with interactions between PrP molecules and endogenous cellular 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 antiscrapie 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 LD_{50}s) 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 antiscrape 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 μg (~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 ≥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 encephalopathy, 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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A Porphyrin Increases Survival Time of Mice after Intracerebral Prion Infection

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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 tetrapyrroles, 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, 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 nonscrapie 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.

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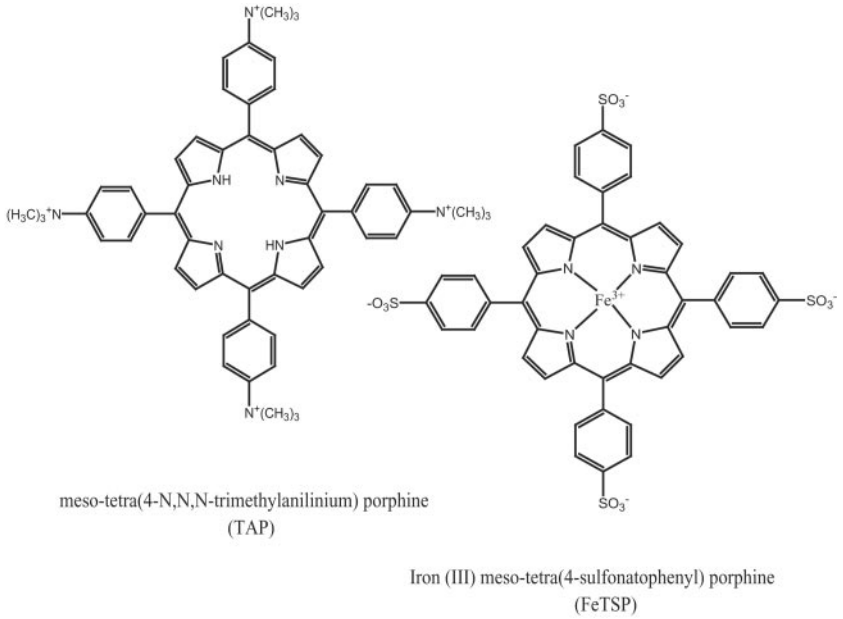

FIG. 1. Structures of the two types of porphyrins tested. Metal-free TAP is shown on the left. The central metal ion of these porphyrins is coordinated with the nitrogen atoms as is shown for FeTSP on the right.

i.p. scrapie challenge and continuing three times per week until near death was ineffective (average survival time ± 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 antiscrapie 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 μl 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.

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

Inoculum (50 μl)[a]	Survival times[b] (days)	Mean survival time ± 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.

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 antiscrapie 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 μl 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 3. Effect of compounds injected into the brain of Tg7 mice[a]

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

[a] Compounds were dosed after i.c. inoculation with 50 μl of 1% 263K-infected brain homogenate. A control group of eight Tg7 mice dosed i.c. with 50 μl 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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Mefloquine, an Antimalaria Drug with Antiprion Activity In Vitro, Lacks Activity In Vivo

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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 μM, 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.) scapie infection in mice as a further evaluation of its potential as an anti-TSE drug.

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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 (IC_{50}) of 1.2 μM. 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 (μM) (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

[a] NR, IC_{50} 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 μl of 1% 263K brain homogenate[a]

Treatment	Dosing regimen	Survival times (days)	Mean survival time ± SD
None		73, 76, 77, 79, 79, 88, 88, 89	81.1 ± 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 ± 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 μl 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 IC_{50} 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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