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

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

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

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

Nucleic acids are a distinct class of polyanions that interact with PrP molecules. DNA binds to recombinant PrP molecules and, depending on the relative concentrations of peptide and nucleic acid, can promote or inhibit PrP-sen aggregation in cell-free reactions (9, 11, 18, 32, 33). Interestingly, the addition of vertebrate RNA but not DNA to cell-free conversion reactions of PrP-sen to PrP-res enhances PrP-res formation, but the mechanism of this effect is not known (12). Also, prophylactic treatments of mice with a specific immunomodulatory CpG deoxynucleotide (cpg1826) can prolong scrapie survival times by a mechanism that was hypothesized to involve stimulation of innate immunity (38). While natural nucleic acids ($\leq 10 \mu\text{g/ml}$) have not been found to affect PrP-res formation in scrapie-infected neuroblastoma cells (8), we show here that degenerate single-stranded phosphorothioated analogs of natural nucleic acids (the structures are provided in Fig. 1) bind to PrP-sen and potentially 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 μ g protein for Randomer 1 and 2 and 2 μ g protein for Randomer 3). Serial dilutions of unlabeled Randomers or other polyanions were then used to challenge the Randomer-PrP-sen interaction. Competition was monitored by determination of the reduction in fluorescence polarization. The reported averages and standard deviations of the K_D values and K_i values (the concentration achieving 50% competition of bound, labeled Randomer) were from at least three independent measurements.

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

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

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

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

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

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

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

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

^b Inhibition of ~30% at 25 μ M.

^c Inhibition of ~45% at 100 μ M.

^d NT, not tested.

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

RESULTS

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

DISCUSSION

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

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

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

Treatment after inoculation	Survival times (days)	Avg $\pm$ SD survival time (days)	Significance between groups (unpaired <i>t</i> test)
4 wk s.c. 5% dextrose	77, 83, 83, 85, 87, 90, 91, 94, 98	87.6 $\pm$ 6.4	
4 wk s.c. Randomer 1	181, 201, 238, 254	218.5 $\pm$ 33.4	$P < 0.0001$ vs s.c. control
10 wk s.c. Randomer 1	175, 186, 226 ^a	195.7 $\pm$ 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 $\pm$ 8.1	NS; ^c $P > 0.05$ vs s.c. control
4 wk i.p. Randomer 1	276, 281, 300, 462	329.8 $\pm$ 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 μ M (data not shown), i.e., >400-fold higher than the corresponding value for Randomer 1 binding to recombinant hamster PrP-sen shown in Table 2. Moreover, the similar internalization of rh-Randomers in scrapie-infected and PPS-cured N2a cells (Fig. 7) also suggests that PS-ON interactions with PrP-res are minimal and that the antiscrapie activities of PS-ONS are mediated primarily by binding to PrP-sen. By binding selectively to PrP-sen, PS-ONS might prevent interactions between PrP-sen and PrP-res that are critical in the conversion of PrP-sen to PrP-res. Nucleic acids are known

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

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

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

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

^a BH, brain homogenate.^b NS, not significant.

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

only by the fully degenerate nature of Randomers but also by the comparable PrP-res inhibitory activities of equivalently sized homo- and heteropolymers in vitro. This argues that the antiscurapic 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 antiscurapic 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 antiscurapic 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 antiscurapic 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 antiscurapic activities of PS-ONs. PS-ONs may block or compete with interactions between PrP molecules and endogenous cel-

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

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

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

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

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

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

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

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

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REFERENCES

1. Agrawal, S., J. Y. Tang, and D. M. Brown. 1990. Analytical study of phosphorothioate analogues of oligodeoxynucleotides using high-performance liquid chromatography. *J. Chromatogr.* **509**:396–399.
2. Ben Zaken, O., S. Tzaban, Y. Tal, L. Horonchik, J. D. Esko, I. Vlodavsky, and A. Taraboulos. 2003. Cellular heparan sulfate participates in the metabolism of prions. *J. Biol. Chem.* **278**:40041–40049.
3. Beringue, V., K. T. Adjou, F. Lamoury, T. Maignien, J. P. Deslys, R. Race, and D. Dormont. 2000. Opposite effects of dextran sulfate 500, the polyene antibiotic MS-8209, and Congo red on accumulation of the protease-resistant isoform of PrP in the spleens of mice inoculated intraperitoneally with the scrapie agent. *J. Virol.* **74**:5432–5440.
4. Birkett, C. R., R. M. Hennion, D. A. Bembridge, M. C. Clarke, A. Chree, M. E. Bruce, and C. J. Bostock. 2001. Scrapie strains maintain biological phenotypes on propagation in a cell line in culture. *EMBO J.* **20**:3351–3358.
5. Cashman, N. R., and B. Caughey. 2004. Prion diseases—close to effective therapy? *Nat. Rev. Drug Discov.* **3**:874–884.
6. Caughey, B., K. Brown, G. J. Raymond, G. E. Katzeinstein, and W. Thresher. 1994. Binding of the protease-sensitive form of PrP (prion protein) to sulfated glycosaminoglycan and Congo red. *J. Virol.* **68**:2135–2141.
7. Caughey, B., and P. T. Lansbury. 2003. Protofibrils, pores, fibrils, and neurodegeneration: separating the responsible protein aggregates from the innocent bystanders. *Annu. Rev. Neurosci.* **26**:267–298.
8. Caughey, B., and G. J. Raymond. 1993. Sulfated polyanion inhibition of scrapie-associated PrP accumulation in cultured cells. *J. Virol.* **67**:643–650.
9. Cordeiro, Y., F. Machado, L. Juliano, M. A. Juliano, R. R. Brentani, D. Foguel, and J. L. Silva. 2001. DNA converts cellular prion protein into the beta-sheet conformation and inhibits prion peptide aggregation. *J. Biol. Chem.* **276**:49400–49409.
10. Cunningham, C. C., J. T. Holmlund, J. H. Schiller, R. S. Geary, T. J. Kwok, A. Dorr, and J. Nemunaitis. 2000. A phase I trial of c-Raf kinase antisense oligonucleotide ISIS 5132 administered as a continuous intravenous infusion in patients with advanced cancer. *Clin. Cancer Res.* **6**:1626–1631.
11. Deleault, N. R., J. C. Geoghegan, K. Nishina, R. Kascasak, R. A. Williamson, and S. Supattapone. 2005. PrPres amplification reconstituted with purified prion proteins and synthetic polyanions. *J. Biol. Chem.* **280**:26873–26879.
12. Deleault, N. R., R. W. Lucassen, and S. Supattapone. 2003. RNA molecules stimulate prion protein conversion. *Nature* **425**:717–720.
13. Diring, H., and B. Ehlers. 1991. Chemoprophylaxis of scrapie in mice. *J. Gen. Virol.* **72**(Pt 2):457–460.
14. Doh-Ura, K., K. Ishikawa, I. Murakami-Kubo, K. Sasaki, S. Mohri, R. Race, and T. Iwaki. 2004. Treatment of transmissible spongiform encephalopathy by intraventricular drug infusion in animal models. *J. Virol.* **78**:4999–5006.
15. Dormont, D. 2003. Approaches to prophylaxis and therapy. *Br. Med. Bull.* **66**:281–292.
16. Farquhar, C. F., and A. G. Dickinson. 1986. Prolongation of scrapie incubation period by an injection of dextran sulphate 500 within the month before or after infection. *J. Gen. Virol.* **67**(Pt 3):463–473.
17. Gabizon, R., Z. Meiner, M. Halimi, and S. A. Ben Sasson. 1993. Heparin-like molecules bind differentially to prion-proteins and change their intracellular metabolic fate. *J. Cell Physiol.* **157**:319–325.
18. Gabus, C., E. Derrington, P. Leblanc, J. Chnaiderman, D. Dormont, W. Swietnicki, M. Morillas, W. K. Surewicz, D. Marc, P. Nandi, and J. L. Darlix. 2001. The prion protein has RNA binding and chaperoning properties characteristic of nucleocapsid protein NCP7 of HIV-1. *J. Biol. Chem.* **276**:19301–19309.
19. Heikenwalder, M., M. Polymenidou, T. Junt, C. Sigurdson, H. Wagner, S.

- Akira, R. Zinkernagel, and A. Aguzzi. 2004. Lymphoid follicle destruction and immunosuppression after repeated CpG oligodeoxynucleotide administration. *Nat. Med.* **10**:187–192.
20. Hornemann, S., C. Korth, B. Oesch, R. Riek, G. Wider, K. Wuthrich, and R. Glockshuber. 1997. Recombinant full-length murine prion protein, mPrP(23-231): purification and spectroscopic characterization. *FEBS Lett.* **413**:277–281.
21. Iversen, P. L., B. L. Copple, and H. K. Tewary. 1995. Pharmacology and toxicology of phosphorothioate oligonucleotides in the mouse, rat, monkey and man. *Toxicol. Lett.* **82-83**:425–430.
22. Kocisko, D. A., G. S. Baron, R. Rubenstein, J. Chen, S. Kuizon, and B. Caughey. 2003. New inhibitors of scrapie-associated prion protein formation in a library of 2000 drugs and natural products. *J. Virol.* **77**:10288–10294.
23. Kocisko, D. A., A. L. Engel, K. Harbuck, K. M. Arnold, E. A. Olsen, L. D. Raymond, D. Vilette, and B. Caughey. 2005. Comparison of protease-resistant prion protein inhibitors in cell cultures infected with two strains of mouse and sheep scrapie. *Neurosci. Lett.* **388**:106–111.
24. Ladogana, A., P. Casaccia, L. Ingrosso, M. Cibati, M. Salvatore, Y. G. Xi, C. Masullo, and M. Pocchiari. 1992. Sulphate polyanions prolong the incubation period of scrapie-infected hamsters. *J. Gen. Virol.* **73**(Pt 3):661–665.
25. Lamond, A. I., and B. S. Sproat. 1993. Antisense oligonucleotides made of 2'-O-alkyl RNA: their properties and applications in RNA biochemistry. *FEBS Lett.* **325**:123–127.
26. Lang, R., L. Hultner, G. B. Lipford, H. Wagner, and K. Heeg. 1999. Guanosine-rich oligodeoxynucleotides induce proliferation of macrophage progenitors in cultures of murine bone marrow cells. *Eur. J. Immunol.* **29**:3496–3506.
27. Lee, K. S., A. C. Magalhaes, S. M. Zanata, R. R. Brentani, V. R. Martins, and M. A. Prado. 2001. Internalization of mammalian fluorescent cellular prion protein and N-terminal deletion mutants in living cells. *J. Neurochem.* **79**:79–87.
28. Liang, H., Y. Nishioka, C. F. Reich, D. S. Pisetsky, and P. E. Lipsky. 1996. Activation of human B cells by phosphorothioate oligodeoxynucleotides. *J. Clin. Investig.* **98**:1119–1129.
29. Lipford, G. B., S. Bendigs, K. Heeg, and H. Wagner. 2000. Poly-guanosine motifs costimulate antigen-reactive CD8 T cells while bacterial CpG-DNA affect T-cell activation via antigen-presenting cell-derived cytokines. *Immunology* **101**:46–52.
30. Magalhaes, A. C., G. S. Baron, K. S. Lee, O. Steele-Mortimer, D. Dorward, M. A. Prado, and B. Caughey. 2005. Uptake and neuritic transport of scrapie prion protein coincident with infection of neuronal cells. *J. Neurosci.* **25**:5207–5216.
31. Morris, M. J., W. P. Tong, C. Cordon-Cardo, M. Drobnjak, W. K. Kelly, S. F. Slovin, K. L. Terry, K. Siedlecki, P. Swanson, M. Rafi, R. S. DiPaola, N. Rosen, and H. I. Scher. 2002. Phase I trial of BCL-2 antisense oligonucleotide (G3139) administered by continuous intravenous infusion in patients with advanced cancer. *Clin. Cancer Res.* **8**:679–683.
32. Moscardini, M., M. Pistello, M. Bendinelli, D. Ficheux, J. T. Miller, C. Gabus, S. F. Le Grice, W. K. Surewicz, and J. L. Darlix. 2002. Functional interactions of nucleocapsid protein of feline immunodeficiency virus and cellular prion protein with the viral RNA. *J. Mol. Biol.* **318**:149–159.
33. Nandi, P. K., and E. Leclerc. 1999. Polymerization of murine recombinant prion protein in nucleic acid solution. *Arch. Virol.* **144**:1751–1763.
34. Priola, S. A., A. Raines, and W. S. Caughey. 2000. Porphyrin and phthalocyanine antiscrapie compounds. *Science* **287**:1503–1506.
35. Prusiner, S. B. 1998. Prions. *Proc. Natl. Acad. Sci. USA* **95**:13363–13383.
36. Race, R., M. Oldstone, and B. Chesebro. 2000. Entry versus blockade of brain infection following oral or intraperitoneal scrapie administration: role of prion protein expression in peripheral nerves and spleen. *J. Virol.* **74**:828–833.
37. Schuette, J. M., D. L. Cole, and G. S. Srivatsa. 1994. Development and validation of a method for routine base composition analysis of phosphorothioate oligonucleotides. *J. Pharm. Biomed. Anal.* **12**:1345–1353.
38. Sethi, S., G. Lipford, H. Wagner, and H. Kretzschmar. 2002. Postexposure prophylaxis against prion disease with a stimulator of innate immunity. *Lancet* **360**:229–230.
39. Shinozuka, K., T. Morita, and H. Sawai. 1991. Synthesis and nuclease susceptibility of alpha-oligodeoxyribonucleotide phosphorothioate. *Nucleic Acids Symp. Ser.* **25**:101–102.
40. Shyng, S. L., S. Lehmann, K. L. Moulder, and D. A. Harris. 1995. Sulfated glycans stimulate endocytosis of the cellular isoform of the prion protein, PrP^C, in cultured cells. *J. Biol. Chem.* **270**:30221–30229.
41. Speare, J. O., T. S. Rush III, M. E. Bloom, and B. Caughey. 2003. The role of helix 1 aspartates and salt bridges in the stability and conversion of prion protein. *J. Biol. Chem.* **278**:12522–12529.
42. Todd, N. V., J. Morrow, K. Doh-Ura, S. O'Hare, P. Farling, M. Duddy, and N. G. Rainov. 2005. Cerebroventricular infusion of pentosan polysulphate in human variant Creutzfeldt-Jakob disease. *J. Infect.* **50**:394–396.
43. Wong, C., L. W. Xiong, M. Horiuchi, L. Raymond, K. Wehrly, B. Chesebro, and B. Caughey. 2001. Sulfated glycans and elevated temperature stimulate PrP(Sc)-dependent cell-free formation of protease-resistant prion protein. *EMBO J.* **20**:377–386.