Cationic Polysaccharides as Antiprion Agents

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Cationic polysaccharides were synthesized by conjugation of various oligoamines to oxidized polysaccharides by reductive amination and tested for antiprion activity. Polycations of dextran, pullulan and arabinogalactan grafted with oligoamines of 2 to 4 amino groups were investigated for their ability to eliminate PrP^{Sc}, the protease-resistant isoform of the prion protein, from chronically infected neuroblastoma cells, ScN2a-M. The proteinase K (PK)-resistant PrP elimination depends on both the concentration of the reagent and the duration of exposure. The most potent compound was found to be dextran−spermine that caused depletion of PrP^{Sc} to undetectable levels at concentration of 31 ng/mL after 4 days of exposure. Activity analysis revealed that grafted oligoamine indentity of the polycation plays a significant role in elimination of PK-resistant PrP from chronically infected N2a-M cells, regardless of the polysaccharide used. Dextran−spermine conjugates were modified with oleic acid and with methoxypoly(ethylene glycol) (MPEG) at various degrees of substitution for further studies and their antiprion activity was examined. Substitution of dextran−spermine with MPEG or oleic acid slightly decreases its activity as a function of MPEG/oleic acid content. These findings confirm previous reports that polycations are effective in eliminating PrP^{Sc} in vitro.

Introduction

The transmissible spongiform encephalopathies (TSE), which comprise infectious, familial, and sporadic neurodegenerations such as Creutzfeldt−Jakob disease (CJD) and Gerstmann−Sträussler−Scheinker disease (GSS) of humans, scrapie of sheep, and bovine spongiform encephalopathy, are caused by prions.[1] Prions are thought to propagate in the host cell by the self-perpetuating refolding a normal cell surface glycoprotein, the cellular prion protein PrP^{C}, into an abnormal β-sheet rich conformation. The resulting pathological conformer, PrP^{Sc}, is in turn the only known component of the infectious prion. Prions thus appear to function without the involvement of coding nucleic acids. Patients with prion disease develop progressive neurological dysfunction that results in death, usually within a year of the first clinical symptoms. Prion diseases share certain mechanistic and pathological features with Alzheimer's disease, a much more common cerebral amyloidosis.

PrP^{C} is a copper-binding glycoprotein that is expressed in neurons and glial cells in the CNS, as well as in several peripheral tissues including leukocytes.[2] Its normal function remains uncertain, but its location on the cell surface would be consistent with roles in cell adhesion and recognition, ligand uptake or transmembrane signaling.[2]

Although there is still no therapy for prion diseases, many compounds with disparate chemical structures have been identified that stop the formation of PrP^{Sc} in chronically infected N2a-M cells. In some cases, these chemicals also increased the incubation time of experimental scrapie in rodents. Antiprion compounds include the amyloid-binding dye Congo red,[3] polyene antibiotics,[4] anthracycline,[5] dextran sulfate, pentosan polysulfate and other polyanions, tricyclic derivatives, tetrapyrroles, cysteine proteases inhibitors and certain PrP antibodies. Most of these antiprion compounds appear to act primarily by decreasing the formation of PrP^{Sc} through a variety of mechanisms. For example, blocking antibodies such as the Fabs D18 and R72 and the mAb 6H4 recognize a specific region within PrP and may prevent the productive interaction between the two PrP isoforms.[6] Substituted tricyclic derivatives such as the antimalarial quinacrine seem to block PrP^{Sc} formation by binding to specific cellular targets that play an essential role in prion replication.[6] Likewise, the potent antiprion tetrapyrrole, *N*-methyl pyridine (with an IC_{50} of 0.5 μM [6] in ScN2a cells), may block PrP^{Sc} formation by binding to a cellular accessory factor. Finally, several polyanions may act by preventing the interaction of PrP with cellular heparan sulfates.[7]

An additional mode of action was recently determined by Suppattapone et al. for a group of potent antiprion polycations, the branched polyamines such as polyethyleneimine (PEI), polypropyleneimine (PPI) and polyamine dendrimers. Rather than just inhibiting the de novo formation of PrP^{Sc}, these compounds appear to clear preexisting PrP^{Sc} from prion-infected cells, perhaps by destabilizating PrP^{Sc} in the acidic environment of lysosomes. In ScN2a, the most potent dendrimers had an IC_{50} of 80 ng/mL.[8,9]

Prompted by these results, we examined the antiprion activity of another class of polycations in which oligoamines are conjugates onto natural polysaccharide chains (dextran, pullulan, or arabinogalactan) via reductive amination. This type of polyamine conjugates has

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Scheme 1. Grafting of Spermine Moieties on Dextran

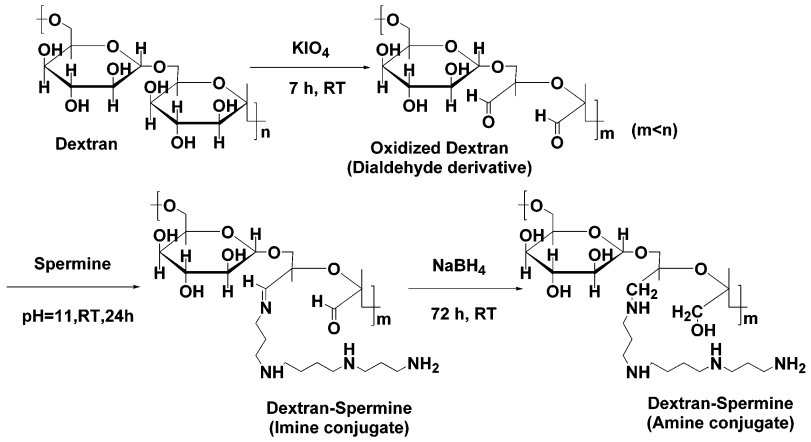
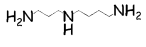
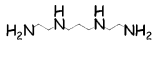
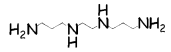
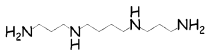


Table 1. Chemical Characterization of Dextran−Oligoamine Conjugates[f]

Code	Oligoamine used for conjugation	Oligoamine structure	Activity[a]	% N[b]	% binding[c]	Oligoamine content[d]	Primary amine[e]
A	Propane-1,3-diamine		>500	10.18	75	26	2.09
B	Spermidine		125	10.05	59	35	1.48
C	N,N-bis(2-aminoethyl)-1,3-propanediamine		125	11.7	51	34	1.06
D	N,N-bis(2-aminopropyl)-1,2-ethylenediamine		500	9.67	40	30	1.28
E	Spermine		31	10.49	48	38	1.23

[a] Approximate concentration of polymer (ng/mL) required for complete eradication of PrP^{Sc} from ScN2a-M after exposure for 4 days. [b] Found nitrogen content (elemental analysis). [c] Percent of substituted saccharide units of a particular conjugate. [d] Oligoamine content (g) in 100 g of corresponding conjugate. [e] Amount of primary amine (mmol/g) in conjugates determined by the TNBS method. M_w of the conjugates was determined by GPC and was found to be 6000 Da, except for spermine conjugate that was 11 000 Da. [f] Appropriate oligoamine was reacted with oxidized dextran (~50% dialdehyde) at 1:1.5 aldehyde/oligoamine mole ratio under the same conditions as described in the Experimental Section.

already been studied extensively. They are water-soluble, relatively nontoxic, biodegradable and biocompatible.[10,11] Of note, dextran−oligoamine derivatives have been tested as gene transfection agents.[12,13] From over 300 polysaccharide-oligoamine derivatives tested, only dextran−spermine was found highly effective in gene transfection, both in vitro and in vivo. Also, spermine derivatives onto pullulan and arabinogalactan were much less effective in gene transfection while PEG and oleate derivatives were active both in cultured cells and following in vivo administration to mice. We have recently studied the toxicity of dextran−spermine in mice after intramuscular and subcutaneous administration and found little if any toxicity.[14]

Here we report that a series of polysaccharide-oligoamine conjugates completely eliminate PrP^{Sc} from ScN2a-M cells at concentration as small as 31 ng/mL. Their antiprion potency depended in part on their amine content; the most potent of these polycations was dextran−spermine.

Results

Activity of Various Dextran−Oligoamine Conjugates against PrP^{Sc} ex Vivo. Cationic polysaccharides were synthesized by conjugation of oligoamine to oxidized polysaccharides by reductive amination as shown in Scheme 1.[12]

The polycations were characterized for their structure (^{1}H NMR), nitrogen content (%N) and primary amine content (TNBS) as shown in Table 1. The polycations had similar % amine content ranging from 9.67 and 11.7. Scrapie-infected mouse neuroblastoma ScN2a-M cells were exposed to various concentrations (0−2000ng/mL) of the polycations for 4 days (because PrP^{Sc} is very stable in cells, several days of treatment are required to eliminate preexisting PrP^{Sc}). Elimination of scrapie was demonstrated by the disappearance of the proteinase K (PK)-resistant core of PrP^{Sc}, as monitored in Western blots (WB) developed with the PrP Ab 3F4. Most effective was dextran−spermine (Figure 1a), which eliminated PrP^{Sc} at 31 ng/mL, whereas dextran−propane-1,3-diamine reduced PrP^{Sc} only slightly even at 500 ng/mL (Figure 1b). The triamines and tetramines: dextran−spermidine, dextran−*N*,*N*-bis(2-aminoethyl)-1,3-propanediamine and dextran−*N*,*N*-bis(2-aminopropyl)-1,2-ethylenediamine were less effective (125, 125 and 500 ng/mL, respectively) than dextran−spermine, but better than the propane-1,3-diamine derivative (not shown).

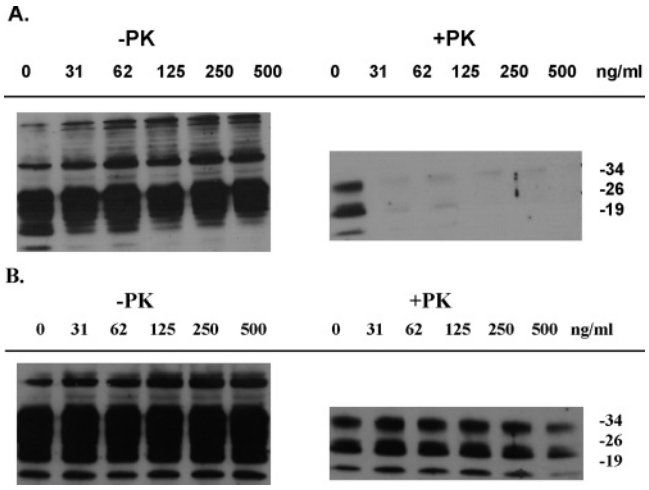


Figure 1. Treatment of scrapie-infected neuroblastoma cells, ScN2a-M, with dextran−oligoamine conjugates. ScN2a-M cells were exposed to various concentrations of tested compound (ng/mL) and harvested after 4 days for analysis. (A) Effect of dextran−spermine on PK-resistant PrP. Apparent molecular masses based on migration of protein standards are 19, 26 and 34 kDa. (B) Effect of dextran−propane-1,3-diamine on PK-resistant PrP. Apparent molecular masses based on migration of protein standards are 19, 26 and 34 kDa.

On the basis of these results, dextran−spermine was selected for further studies.

Activity of Dextran−Spermine with Increasing Spermine Content. To determine whether the nitrogen content directly contributes to the potency of the compounds, we tested dextran−spermine conjugates with various degrees of spermine substitution (prepared by conjugation of increasing amounts of spermine onto oxidized dextran). The compounds also differed in their M_w (see legend to Table 2). The polycations were characterized for their average molecular weight (GPC), structure (^{1}H NMR), nitrogen content (%N) and primary amine content (TNBS) as shown in Table 2. The antiprion activity was determined as described in Figure 1. Interestingly, most efficient was the moderately substituted conjugate, E, whereas both smaller and higher degrees of substitution reduced the antiprion efficiency of the conjugates. The reasons for the reduced potency of G remain to be determined.

Effect of the Polysaccharide Backbone. To examine the effect of the polysaccharide structure on the activity of the agents, spermine grafted on pullulan, arabinogalactan and dextran were examined. Dextran and pullulan are linear chains with glucose units connected by 1,4- and 1,6-glycoside bonds,[15] respectively. Arabinogalactan is a branched polymer, and its units are connected by 1,3-glycoside bonds.[16] The polycations were characterized for their average molecular weight (GPC), structure (^{1}H NMR), nitrogen content (%N) and primary amine content (TNBS) as shown in Table 3. According to WB analysis (data not shown), all three cationic polysaccharides inhibited PK-resistant PrP from ScN2a-M cells at a very low concentration ranging from 31 to125 ng/mL (Table 3).

Activity of Methoxypoly(ethylene glycol) [MPEG]-Conjugated Dextran−Spermine. The purpose of this part of the study was to evaluate the shielding effect of MPEG and its influence on PrP^{Sc} by applying pegylated dextran−spermine conjugates, with the aim of finding effective compounds that can be delivered to the brain. Highly charged polycations can form aggregates with serum components and thus might not cross the blood−brain barrier. Polycations that are shielded with an inert and biocompatible polymer such as PEG are defined as "sterically stabilized" compounds.[17] For this reason dextran−spermine was modified with increasing amounts of MPEG to obtain partially shielded molecules. MPEG (M_w = 2 kDa) was activated with *p*-nitrophenyl chloroformate to obtain *p*-nitrophenyl carbonate derivative. Substitution of dextran−spermine was carried out at room temperature in aqueous solution.[23] The amount of MPEG was fixed at 1%, 5% and 10% (mol/mol) to the primary amines of spermine. The pegylated derivatives were characterized for their average molecular weight (GPC) and structure (^{1}H NMR) as shown in Table 4.

The WB analysis indicated that the MPEG derivatives were active in the elimination of PrP^{Sc} at concentrations ranging from 80 and 500 ng/mL (Figure 2). Increase in MPEG content in the conjugate decreased the antiprion effectiveness of the compound.

Activity of Oleate Dextran−Spermine Derivative as PrP^{Sc} Elimination Agent. The activity of oleate conjugated dextran−spermine was determined. Oleic acid is an unsaturated fatty acid that imparts hydrophobic nature to the conjugate.[18] Addition of oleic acid to the polycation provides a hydrophobic shield to the polymer and condenses into a colloidal dispersion if enough oleate groups are introduced. Dextran−spermine was hydrophobized with increasing amounts of oleic acid by adding oleic acid *N*-hydroxysuccinimide (NHS) ester to concentrated solution of dextran−spermine in a mixture of DDW/THF.[18] The hydrophobized conjugates were characterized for their average molecular weights (GPC) and structure (^{1}H NMR) as shown in Table 4.

Oleate derivatives showed moderate potency in scrapie inhibition (250 ng/mL) relative to unmodified dextran−spermine. No distinct difference in antiprion activity between the hydrophobized conjugates of increasing oleate content was observed (data not shown).

Kinetics of PrP^{Sc} Elimination by Dextran−Spermine as Compared to Pentosan Polysulfate. The following results demonstrate the potency of dextran−spermine to eliminate PK-resistant PrP from ScN2a-M cells after short treatments. Figure 3a summarizes the effect of dextran−spermine on PK-resistant PrP as a function of the exposure time. ScN2a-M cells were treated with either dextran−spermine (2 μg/mL) (Figure 3a) or pentosan polysulfate (PPS) (10 μg/mL) (Figure 3b) for the indicated times, and the elimination of PrP^{Sc} was assessed by WB developed with mAb 3F4. Although the two compounds caused a substantial reduction in PrP^{Sc} levels after 100 h, 2 μg/mL of dextran−spermine eliminated PrP^{Sc} much more quickly (24 h, as compared to 100 h for PPS).

Discussion

Biodegradable polycations prepared by grafting linear (nonbranched) oligoamine residues on natural polysaccharides eliminated PrP^{Sc} from chronically infected ScN2a-M neuroblastoma cells in a dose- and time-dependent manner. The most effective dextran deriva-

Table 2. Chemical Characterization of Dextran−Spermine Conjugates at Different Degrees of Substitution[h]

polymer no.	activity[a]	%N[b]	% binding[c]	oligoamine content[d]	primary amine[e]	M_w[f]	P	% dialdehyde[g]
F	>2000	2.65	8	9.6	0.45	11.600	1.4	12
E	31	10.49	48	38	1.23	11.000	1.2	48
G	500	13.25	72	47.8	1.23	91.500	2.5	105

[a−e] See notes in Table 1. [f] Average molecular weight (M_w) and polydispersity ($P = M_w/M_n$) were determined by GPC. Molecular weight of the FI-70 differs from other conjugates due to slight cross-linking between the polyaldehyde and spermine during conjugation. [g] % Dialdehyde content of the oxidized dextran prior to spermine conjugation as determined by hydroxylamine hydrochloride titration method. [h] Oxidized dextran was reacted with spermine at 1:1.5 aldehyde/oligoamine mole ratio under the same conditions as described in the Experimental Section.

Table 3. Chemical Characterization of Polysaccharide−Spermine Conjugates[g]

polymer no.	polysaccharide used for conjugation	activity[a]	%N[b]	% binding[c]	oligoamine content[d]	primary amine[e]	M_w[f]	P
H	dextran	31	10.49	48	38	1.23	11.000	1.2
I	pullulan	125	8.86	37	31	1.35	17.000	1.4
J	arabinogalactan	125	8.86	37	31	1	17.000	1.5

[a−e] See notes in Table 1. [f] Average molecular weight (M_w) and polydispersity ($P = M_w/M_n$) were determined by GPC. [g] Appropriate oxidized polysaccharide (~50% dialdehyde) was reacted with spermine at 1:1.5 aldehyde/oligoamine mole ratio under the same conditions as described in the Experimental Section.

Table 4. Chemical Characterization of Pegylated and Oleate Dextran−Spermine Derivatives[c]

polymer no.	% substitution to ϵ-NH_2	M_w[a]	P	activity[b]
E	0%	11.000	1.2	31
K	1% $MPEG_{2000}$	10300	3	250
L	5% $MPEG_{2000}$	11000	3.1	500
M	10% $MPEG_{2000}$	7400	2.9	>500
N	5% oleate	22.000	1.6	125−250
O	20% oleate	6.500	2.3	250

[a] Average molecular weight (Mw) and polydispersity ($P = M_w/M_n$) were determined by GPC. [b] See notes in Table 1. [c] %N of dextran−spermine conjugate (no. E) was 10.49% and the amount of primary amines was 1.23 mmol/g as determined by TNBS method.

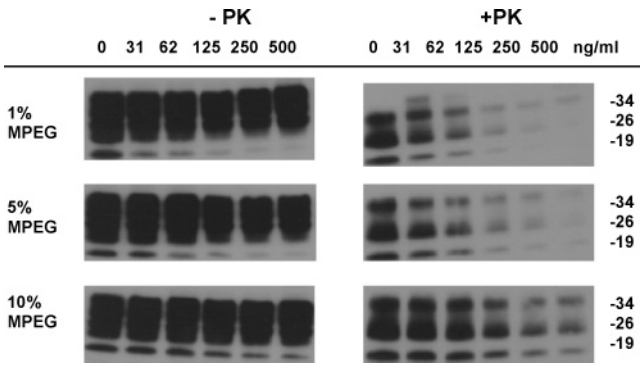


Figure 2. Effect of pegylation on PK-resistant PrP. ScN2a-M cells were incubated with pegylated dextran−spermine (1%, 5% and 10% MPEG) for 4 days. Then cells were harvested for analysis. Apparent molecular masses based on migration of protein standarts are 19, 26 and 34 kDa.

tive was dextran−spermine with one spermine unit per 2.03 saccharide units, which completely eliminated PrP^{Sc} at a concentration of 31 ng/mL. The mechanism through which these molecules influence PrP^{Sc} is still unknown. In particular, whether they interact directly with PrP or if their more significant interaction is with other molecules remains to be determined.

There was a strict correlation between the activity of the compounds and between their overall oligoamine content (Table 1). The oligoamine content integrates several quantitative features of the conjugate, including (i) the M_w of the oligoamine molecule serving as a graft and (ii) the actual degree of conjugation in each particular conjugate. However, we can also discern the

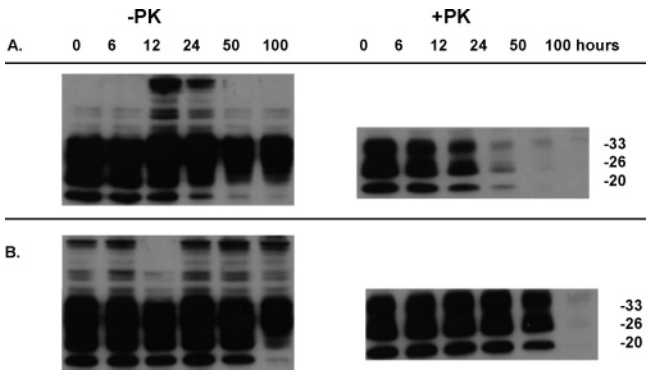


Figure 3. Treatment of scrapie-infected neuroblastoma cells with dextran−spermine and pentosan polysulfate. ScN2a-M cells growing in 12-well plates were incubated with dextran−spermine (2 μg/mL) or PPS (10 μg/mL) for the indicated time periods in DMEM/Opti-MEM supplemented with 5% fetal calf serum (FCS). Cells were harvested after 100 h and analyzed by Western blot using the mAb 3F4. 50% of the sample was digested with proteinase K (+PK) 20 μg/mL at 37 °C for 30 min prior to electrophoresis to eliminate PrP^C. Apparent molecular masses based on migration of protein standarts are 20, 26 and 33 kDa. (A) Effect of dextran−spermine on PK-resistant PrP. (B) Effect of PPS on PK-resistant PrP.

influence of individual parameters on the activity of the compounds. Although several structural parameters influence the antiprion potency of the various conjugates, the most important feature appears to be the identity of the oligoamine grafted onto the sugar backbone, while other parameters played a less significant role.

Influence of the Oligoamine Graft. The identity of the oligoamine used for conjugation had a decisive influence on the antiprion properties of the conjugate. The tetramine spermine yielded by far the most active compounds (e.g. E), whereas other tetramine showed lower antiprion activity in our assay (Table 1).

Effect of the Degree of Spermine Conjugation. Since spermine is clearly the active moiety in antiprion dextran−spermine, it was plausible that increasing the level of spermine conjugation onto dextran would result in increased antiprion potency. Paradoxically, however, increasing the spermine content by just 10% almost completely abolished the activity of the dextran−spermine conjugate (compound G in Table 2). This contra-

dicts the results obtained with dendrimers.[9] We surmise that the surprising inactivity of the more substituted compound is caused by spurious spermine-mediated intermolecular cross-linking caused by the nature of the conjugation process. That the resulting product was cross-linked is shown by its much higher M_w (91 500, as compared to 11 000 for E, Table 2). We hypothesize that in a cross-linked aggregate, many of the active amines would be sequestered in the interior of the aggregate and would thus be prevented to take part in the antiprion activity. More studies will be needed to confirm this hypothesis.

The Importance of the Saccharide Backbone. The nature of the polysaccharide backbone had a relatively minor effect on the efficiency of the conjugates. Dextran−spermine was the most efficient (active dose 31 ng/mL), while spermine grafted in the same manner on pullulan and arabinogalactan (this work) and chitosan (not shown) resulted in slightly less active conjugates (active dose = 125 ng/mL). Branching of the polysaccharide backbone (in arabinogalactan) had no significant influence on the activity of the conjugate. The reason for the superior activity of dextran remains to be determined.

Pegylation and Oleate Substitution Reduce the Antiprion Potency in Cultured ScN2a-M Cells. One important requirement for antiprion drugs is that they cross the blood−brain barrier. With this goal in mind, we coated dextran−spermine with either $MPEG_{2000}$, a hydrophilic, inert and biocompatible polymer at 1%, 5% and 10% mol/mol degrees of substitution relative to the primary amine of the spermine, or with the hydrophobic chain oleic acid at 5% and 20% mol/mol to the primary amine. Previous studies have indeed shown that such coating may help to shield the polycation and thus allow its crossing the blood−brain barrier in vivo. For instance, J. Huwyler has reported brain uptake of PEG−immunoliposomes (antibody-directed liposomes) that were used for delivery of the antineoplastic agent daunomycin to rat brain.[17] PEG-coated liposomes are widely used and are referred to as "sterically stabilized" or "stealth liposomes".[17]

Whether these coated compounds will cross the blood−brain barrier remains to be seen in pharmacological studies. However, the antiprion activity was reduced by both types of coating. For the MPEG conjugates, the activity decreased linearly as the MPEG content increased. A 1% degree of substitution with MPEG already reduced the activity of dextran−spermine by a factor of 8. This steep decrease suggests that it is not the loss of primary amines that determines the reduced activity of these compounds. Rather, our hypothesis for the decreased antiprion activity of the pegylated conjugates is that the long $MPEG_{2000}$ molecules sterically reduce the access of the primary amine to the sites that might be critical for the effectiveness of the compound. Steric hindrance would be expected to be smaller for oleic acid, which is a much smaller M_w of 282. This may be the reason for the more moderate influence of oleic acid substitutions (Table 4).

The reduced activity of the pegylated and oleic acid-substituted dextran−spermine in cells may not predict the activity of these compounds in vivo, as their bioavailability may turn out to be superior to that of their nonshielded analogues.

Possible Mechanisms of Action. The mode and the subcellular sites of action of dextran−spermine remain uncertain. It has been suggested that dendrimers hasten the degradation of prions by destabilizing PrP^{Sc} within strongly acidic organelles such as lysosomes. The possible explanation suggested by these authors is that amino groups on the surface of polyamine might bind to PrP^{Sc} monomers or oligomers that exist in equilibrium with a large aggregate under acidic conditions. Whether this is true for dextran−spermine as well in uncertain. Supattapone et al. have previously shown that branched polyamines destabilize PrP^{Sc} at acidic pH, as evidenced by the reduced protease-resistance of PrP^{Sc} following incubation in vitro with these polycations. In preliminary experiments (not shown), we used their assay to evaluate if dextran−spermine operates through similar mechanisms. Lysates of ScN2a-M cells were exposed to pH 7.3 or 3.5 for 2 h in the presence or absence of the dextran−spermine, E (0−6 μg/mL), and protease-resistance was evaluated after neutralizing the pH. In contrast to the results of Supattapone, there was no detectable influence of dextran−spermine on the stability of PrP^{Sc}. Additional studies will be needed to clarify this issue.

Comparison of Dextran−Spermine Antiprion Activity with Pentosan Polysulfate. To determine the effectiveness of dextran−spermine conjugates, relative to other reagents known to inhibit prions, inhibition of PK-resistant PrP was examined in the presence of a noncytotoxic concentration of dextran−spermine (2 μg/mL) and of the sulfated glycan pentosan polysulfate (PPS)[19] (10 μg/mL) that reduce PrP^{Sc} in prion-infected cultured cells.[20] Sulfated glycans may act by competing with and disrupting a putative interaction of the PrP isoforms with cellular heparan sulfate (HS) which role in the biogenesis of prions is uncharacterized yet. Whether PrP^{C} interacts with cellular HS directly, via its heparin-binding domains, or indirectly through a third factor such as the 37 kDa/67 kDa laminin receptor (LRP/LR) is uknown.[21] Sulfated glycans could compete with HS over prion interactions by mimicking HS specific domains. Kinetic results demonstrated the potency of the cationic polysaccharide conjugate to inhibit and eliminate scrapie from neuroblastoma N2a-M cells within 24 h of treatment, whereas PPS decreased PrP^{Sc} levels within 100 h (Figure 1). The two compounds had no effect on cell viability as indicated by cell growth and change in protein concentration. In comparison to dextran−spermine and PPS, PEI and PAMAM were reported as effective antiprion agents and found potent in clearing PK-resistant PrP from ScN2a-M cells within 8 to 16 h of exposure to 7.5 μg/mL of appropriate compound.[8]

Conclusion

In conclusion, various polycations were prepared by reductive amination between primary amine of certain oligoamines and oxidized polysaccharides. Although the majority of the tested compounds were found to be effective in scrapie elimination, dextran−spermine showed the highest potency in elimination of PrP^{Sc} from the chronically infected neuroblastoma cells, ScN2a-M,

under noncytotoxic concentrations. These results raised the possibility that some of these compounds might be used as therapeutic reagents for prion disease. Further studies focus on modification and formulation of the most effective antiprion agent, dextran−spermine, so it can be active in mice and eradicate PrP^{Sc} in vivo.

Experimental Section

Materials. Dextran of an average molecular weight of 40 kDa was obtained from Sigma Chemical Co. (St. Louis, MO). Arabinogalactan with an average molecular weight of 19 kDa was a gift from Larex International (St. Louis, MO). Pullulan of 40 kDa was received from Sigma Chemical Co. (St. Louis, MO). Potassium periodate (KIO_4), sodium borohydride ($NaBH_4$), poly(ethylene glycol) monomethyl ether ($MPEG_{2000}$), *p*-nitrophenyl chloroformate, oleate-NHS, spermine, spermidine, propane-1,3-diamine, *N*,*N*-bis(2-aminopropyl)-1,2-ethylenediamine and *N*,*N*-bis(2-aminoethyl)-1,3-propanediamine were all purchased from Aldrich (Milwaukee, WI). All solvents and reagents were of analytical grade and were used as indicated.

Water-free methoxy-PEG_{2000} was obtained by azeotropic distillation from toluene and vacuum-dried over P_2O_5. A sage-metering pump model-365 (Orion, NJ) was used for slow and reproducible addition of reactants. Average molecular weights of polycations were estimated by GPC-Spectra Physics instrument (Darmstadt, Germany) containing a pump, column (Shodex KB-803) and refractive index (RI) detector. Average molecular weights were estimated according to pullulan standards (PSS, Mainz, Germany) with molecular weights between 5800 and 212 000. Eluents used were 0.05 M $NaNO_3$ for the uncharged polymers and 5% (w/v) Na_2HPO_4 in 3% (v/v) acetonirile (pH 4) for the cationic conjugates.[22] The degree of conjugation was estimated by elemental microanalysis of nitrogen (%N) using a Perkin-Elmer 2400/II CHN analyzer. ^1H NMR spectra (D_2O) were obtained on a Varian 300-MHz spectrometer in 5 mm o.d. tubes. D_2O-containing tetramethylsilane served as solvent and shift reference. FT-IR spectra were recorded on a Perkin-Elmer 2000 FTIR. Cell culture reagents were from Biological Industries (Beit Haemek, Israel). Opti-MEM was from Gibco. Tissue culture dishes were from Miniplast (Ein Shemer, Israel). Multiwell plates were from Nunc (Roskilde, Denmark). Secondary antibodies were from Jackson Immunoresearch (West Grove, PA).

Methods. Synthesis of Cationic Polysaccharides. Oxidized polysaccharides were prepared and characterized as previously described.[12] In brief, the desired polysaccharide (10 g, 62.5 mmol of glucose units) was dissolved in 200 mL of double deionized water (DDW). Potassium periodate was added at either 1:1, 1:2, or 5:1 mole ratio (glucose/IO_4^-) (eventually leading to the three compounds E, F, and G), and the mixture was stirred in the dark at room temperature for 6−8 h. The resulting polyaldehyde derivatives were purified from iodate (IO_3^-) and unreacted periodate (IO_4^-) by Dowex-1 (acetate form) anion exchange chromatography, followed by extensive dialysis against DDW (3500 cutoff cellulose tubing) at 4 °C for 3 days. Purified polyaldehyde derivatives were freeze-dried to obtain white powder in 70% average yield. FT-IR (KBr) = 1724 cm^{-1} (C=O). The aldehyde content was determined by the hydroxylamine hydrochloride method.[15]

The oligoamine conjugation was conducted as follows: oxidized polysaccharide (1 g, 0.75−6.56 mmol of aldehyde groups) was dissolved in 100 mL of DDW. The dialdehyde solution was slowly added over several hours to a basic solution containing 1.5 equimolar amount of oligoamine dissolved in 50 mL of borate buffer (0.1 M, pH 11). The mixture was stirred at room temperature for 24 h. $NaBH_4$ (1 g, 4 equimolar) was added to reduce the imine bonds to amines, and stirring was continued for 48 h under the same conditions. The reduction was repeated with additional portion of $NaBH_4$ (1 g, 4 equimolar) at the same conditions for 24 h. The resulting light-yellow solution was poured into a dialysis membrane (3500 cutoff cellulose tubing) and dialyzed against DDW at 4 °C for 3 days. The dialysate was lyophilized to dryness and stored under nitrogen atmosphere. Average yield: 50% (w/w). ^1H NMR (D_2O): 1.645 ppm (m, 4H, dextran−$NH(CH_2)_3$-$NHCH_2CH_2CH_2CH_2NH(CH_2)_3NH_2$), 1.804 ppm (m, 4H, dextran−$NHCH_2CH_2CH_2NH(CH_2)_4NHCH_2CH_2CH_2NH_2$), 2.815 ppm (m, 12H, dextran−$NHCH_2CH_2CH_2NHCH_2CH_2CH_2CH_2$-$NHCH_2CH_2CH_2NH_2$), 3.30−4.45 ppm (m, glucose hydrogens), 5.01 ppm (m, 1H, anomeric hydrogen). FT-IR (KBr): 1468 cm^{-1} (CH_2 aliphatic), 1653 cm^{-1} (NH_2, primary amine), 2935 cm^{-1} (C−C, aliphatic) and 3297 cm^{-1} (NH, OH groups). The primary amine content was determined by the TNBS method.[12]

Dextran−Spermine−Methoxypoly(ethylene glycol) (MPEG) Conjugation. Pegylation of dextran−spermine derivatives was obtained as described elsewhere.[23] In brief, dextran−spermine conjugate (100 mg, 123 μmol of ϵ-NH_2), was dissolved in 2.5 mL of DDW. Aqueous solution of $MPEG_{2000}$−*p*-nitrophenyl carbonate (1%, 5% and 10% mol/mol to ϵ-NH_2) was added to the dextran−spermine solution. The mixture was stirred at room temperature for 16 h. The modified derivative was purified from *p*-nitrophenol and unbound MPEG by Sephadex G-25 column chromatography using DDW as eluent. Fractions containing the pegylated derivative were defined by ninhydrin test, collected and freeze-dried to obtain a white powder. Average yield: 80% (w/w). The degree of modification was calculated by spectrophotometric measurement of the released *p*-nitrophenol (UV, λ = 410 nm) after conjugation which was found to be about 95%.

Dextran−Spermine Oleate Conjugation. Oleate conjugates were prepared as described elsewhere.[18] In brief, dextran−spermine conjugate (40 mg, 49.2 μmol of ϵ-NH_2) was dissolved in 1 mL of DDW and in 2 mL of THF. To this solution 5% or 20% mol/mol to ϵ-NH_2 of oleate-NHS dissolved in anhydrous THF (21 mg in 5 mL THF) was added. The mixture was stirred at room temperature overnight and evaporated to dryness. The yellow powder was washed with diethyl ether and vacuum-dried over NaOH. Average yield: 85% (w/w). The degree of substitution with oleate-NHS was determined by ^1H NMR and found to be 95%. ^1H NMR (D_2O): 0.696 ppm (t,3H, terminal methyl group of oleate), 1.095 ppm (m,24H, oleate aliphatic hydrogens), 1.437 ppm (m, 4H, dextran−$NH(CH_2)_3$-$NHCH_2CH_2CH_2CH_2NH(CH_2)_3NH_2$), 1.617 ppm (m, 4H, dextran−$NHCH_2CH_2CH_2NH(CH_2)_4NHCH_2CH_2CH_2NH_2$), 2.15−3.26 ppm (m, 12H, dextran−$NHCH_2CH_2CH_2NHCH_2CH_2CH_2$-$CH_2NHCH_2CH_2CH_2NH_2$), 3.30−4.45 ppm (m, glucose hydrogens), 5.01 ppm (m, 1H, anomeric hydrogen) and 5.14 ppm (m, 2H olefin hydrogens of oleate).

Ex Vivo Biological Activity. Cells. Chronically PrP^{Sc} infected mouse neuroblastoma cells ScN2a-M were stably transfected with a vector that expresses the MHM2-PrP chimeric gene that reacts with mAb 3F4.[7] Cells were grown at 37 °C in DMEM-16 (1 g glucose/liter) supplemented with 10% fetal calf serum (FCS). Treatments with polycations were performed on cells grown in 12-well dishes in DMEM-16/Opti-MEM (1:1) supplemented with 5% FCS.[7]

Antibodies. MAb 3F4 binds to residues Met^{108} and Met^{11124} in chimeric MHM2-PrP but does not recognize the wild-type mouse PrP endogenous to N2a cells.[25] This antibody was used at a dilution of 1:5000 for Western blot.

PrP Analysis. PrP^{Sc} was defined as the PrP fraction resistant to proteolysis catalyzed by proteinase K (20 μg/mL, 37 °C, 30 min). The protease activity was stopped by adding phenylmethylsulfonyl fluoride at 2 mM. SDS−PAGE. Sample preparation and western immunoblotting of the PrP isoforms were carried out as described.[26] In brief, cells were lysed in ice-cold "standard" lysis buffer (0.5% Triton X-100, 0.23% sodium deoxycholate, 150 mM NaCl, 10 mM Tris-Cl, pH 7.5, 10 mM EDTA), and the lysates were immediately centrifuged for 40 s at 14 000 rpm in microcentrifuge. All of the biochemical analyses were performed on this postnuclear fraction. Protein concentration in cell lysates were measured using the Bradford assay (Bio-Rad) where needed.

Lysates were resolved in 12% polyacrylamide gels and electrotransferred to PolyScreen poly(vinylidene difluoride) membranes (PVDF) in a Tris/glycine buffer containing Sarkosyl (48 mM Tris base, 39 mM glycine, 20% methanol, 0.001%

Sarkosyl). The membranes were blocked for 30 min with low fat milk prior to incubation with mAb 3F4. HRP-conjugated secondary antibodies were used at 1:10 000 dilution, and the blots were developed by chemoluminescence.

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References

(1) Collinge, J. Prion diseases of humans and animals: Their causes and molecular basis. *Annu. Rev. Neurosci.* **2001**, *24*, 519−550.
(2) Harris, D. A. Cellular biology of prion diseases. *Clin. Microbiol. Rev.* **1999**, *12*, 429.
(3) Ingrosso, L.; Ladogana, A.; Pocchiari, M. Congo Red Prolongs the Incubation Period in Scrapie-Infected Hamsters. *J. Virol.* **1995**, *69*, 506−508.
(4) Pocchiari, M.; Schmittinger, S.; Masullo, C. Amphotericin-B Delays the Incubation Period of Scrapie in Intracerebrally Inoculated Hamsters. *J. Gen. Virol.* **1987**, *68*, 219−223.
(5) Tagliavini, F.; McArthur, R. A.; Canciani, B.; Giaccone, G.; Porro, M.; Bugiani, M.; Lievens, P. M. J.; Bugiani, O.; Peri, E.; DallAra, P.; Rocchi, M.; Poli, G.; Forloni, G.; Bandiera, T.; Varasi, M.; Suarato, A.; Cassutti, P.; Cervini, M. A.; Lansen, J.; Salmona, M.; Post, C. Effectiveness of anthracycline against experimental prion disease in Syrian hamsters. *Science* **1997**, *276*, 1119−1122.
(6) Supattapone, S.; Nishina, K.; Rees, J. R. Pharmacological approaches to prion research. *Biochem. Pharmacol.* **2002**, *63*, 1383−1388.
(7) Ben-Zaken, O.; Tzaban, S.; Tal, Y.; Horonchik, L.; Esko, J. D.; Vlodavsky, I.; Taraboulos, A. Cellular heparan sulfate participates in the metabolism of prions. *J. Biol. Chem.* **2003**, *278*, 40041−40049.
(8) Supattapone, S.; Nguyen, H. O. B.; Cohen, F. E.; Prusiner, S. B.; Scott, M. R. Elimination of prions by branched polyamines and implications for therapeutics. *Proc. Natl. Acad. Sci. U. S. A.* **1999**, *96*, 14529−14534.
(9) Supattapone, S.; Wille, H.; Uyechi, L.; Safar, J.; Tremblay, P.; Szoka, F. C.; Cohen, F. E.; Prusiner, S. B.; Scott, M. R. Branched polyamines cure prion-infected neuroblastoma cells. *J. Virol.* **2001**, *75*, 3453−3461.
(10) CarrenoGomez, B.; Duncan, R. Evaluation of the biological properties of soluble chitosan and chitosan microspheres. *Int. J. Pharm.* **1997**, *148*, 231−240.
(11) Berscht, P. C.; Nies, B.; Liebendorfer, A.; Kreuter, J. In−Vitro Evaluation of Biocompatibility of Different Wound Dressing Materials. *J. Mater. Sci.-Mater. Med.* **1995**, *6*, 201−205.
(12) Azzam, T.; Eliyahu, H.; Shapira, L.; Linial, M.; Barenholz, Y.; Domb, A. J. Polysaccharide-oligoamine based conjugates for gene delivery. *J. Med. Chem.* **2002**, *45*, 1817−1824.
(13) Azzam, T.; Eliyahu, H.; Makovitzki, A.; Domb, A. J. Dextran-spermine conjugate: An efficient vector for gene delivery. *Macromol. Symp.* **2003**, *195*, 247−261.
(14) Eliyahu, H., Joseph, A., Azzam, T and Domb, A. J. Unpublished results.
(15) Zhao, H.; Heindel, N. D. Determination of Degree of Substitution of Formyl Groups in Polyaldehyde Dextran by the Hydroxylamine Hydrochloride Method. *Pharm. Res.* **1991**, *8*, 400−402.
(16) Groman, E. V.; Enriquez, P. M.; Jung, C.; Josephson, L. Arabinogalactan for Hepatic Drug-Delivery. *Bioconjugate Chem.* **1994**, *5*, 547−556.
(17) Huwyler, J.; Wu, D. F.; Pardridge, W. M. Brain drug delivery of small molecules using immunoliposomes. *Proc. Natl. Acad. Sci. U. S. A.* **1996**, *93*, 14164−14169.
(18) Azzam, T. Hydrophobized Dextran-Spermine Conjugate as potential vector for in vitro gene transfection. *J. Controlled Release* **2004**, *96* (2), 309−323.
(19) Dealler, S.; Rainov, N. G. Pentosan polysulfate as a prophylactic and therapeutic agent against prion disease. *Idrugs* **2003**, *6*, 470−478.
(20) Caughey, B.; Raymond, G. J. Sulfated Polyanion Inhibition of Scrapie-Associated Prp Accumulation in Cultured-Cells. *J. Virol.* **1993**, *67*, 643−650.
(21) Schonberger, O.; Horonchik, L.; Gabizon, R.; Papy-Garcia, D.; Barritault, D.; Taraboulos, A. Novel heparan mimetics potently inhibit the scrapie prion protein and its endocytosis. *Biochem. Biophys. Res. Commun.* **2003**, *312*, 473−479.
(22) Kumar, N.; Azzam, T.; Domb, A. J. Molecular mass distribution of polycations and dextrans by high-performance size exclusion chromatography. *Polym. Adv. Technol.* **2002**, *13*, 1071−1077.
(23) Hosseinkhani, H.; Azzam, T.; Tabata, Y.; Domb, A. J. Dextran-spermine polycation: an efficient nonviral vector for in vitro and in vivo gene transfection. *Gene Ther.* **2004**, *11*, 194−203.
(24) Rogers, M.; Serban, D.; Gyuris, T.; Scott, M.; Torchia, T.; Prusiner, S. B. Epitope Mapping of the Syrian-Hamster Prion Protein Utilizing Chimeric and Mutant-Genes in a Vaccinia Virus Expression System. *J. Immunol.* **1991**, *147*, 3568−3574.
(25) Scott, M. R.; Kohler, R.; Foster, D.; Prusiner, S. B. Chimeric Prion Protein Expression in Cultured-Cells and Transgenic Mice. *Protein Sci.* **1992**, *1*, 986−997.
(26) Naslavsky, N.; Shmeeda, H.; Friedlander, G.; Yanai, A.; Futerman, A. H.; Barenholz, Y.; Taraboulos, A. Sphingolipid depletion increases formation of the scrapie prion protein in neuroblastoma cells infected with prions. *J. Biol. Chem.* **1999**, *274*, 20763−20771.

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