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Differentiating Prion Strains Using Dendrimers

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Dendrimers are synthetic polymers with a highly structured architecture, consisting of a core from which monomers branch out in a symmetrical, structured fashion. They possess numerous biomedical applications and have been investigated as therapeutics for prion diseases.^[1,2] Prion diseases are a group of fatal neurodegenerative disorders that include Creutzfeldt-Jakob disease in humans and BSE in cattle. All prion diseases are invariably fatal and they possess the ominous distinction of being the only known diseases that can be genetic, infectious or sporadic in origin.^[3] The pathogenic agents responsible for these diseases are known as prions and are thought to consist mainly or entirely of PrP^{Sc}, an aberrantly folded isoform of the normal host glycoprotein PrP^C.^[4] PrP^C is predominantly expressed in neurons and its function (if any) remains unknown. Roles in neuro-protection^[5], regulation of oxidative stress^[6] and copper/zinc metabolism^[7] have all been suggested but thus far all studies including those with PrP knockout mice have proved inconclusive.^[8] Distinct “strains” of prions have been described which differ in the incubation times and the lesion profiles they induce in a particular host.^[9-11] Current evidence suggests that these strain specific properties must be encoded in some feature of the pathogenic protein other than its amino acid sequence such as its conformation,^[12,13] its glycosylation pattern,^[14] or in unidentified cofactors.^[15] Different prion strains have distinct interspecies transmission properties and different

pathogenicity patterns, particularly for humans.^[16,17] Distinguishing between different prion strains rapidly is therefore desirable. Various dendrimers have been shown to be capable of interfering with the aggregation of amyloid fibrillar structures associated with Alzheimer's and prion diseases.^[18] Most poignantly, they have demonstrated an *in vitro* ability to eliminate protease resistant PrP^{Sc} (PrP^{res}) in a strain dependant manner.^[2] We explored this strain specific effect and demonstrate that dendrimers of distinct sizes and molecular and chemical properties can distinguish several prion strains against a common genetic background offering a novel mechanism for identifying and investigating these unique infectious agents.

To establish the feasibility of a dendrimer based prion strain typing system, mouse brain homogenates infected with well characterised mouse adapted prion strains were studied. The brain homogenates were treated with increasing concentrations of various dendrimers including: neutral and H-bonding mPPIg5; cationic PPIg4; cationic PAMAMg5; and cationic and H-bonding 0.5mPPIg5 (**Figure 1**). The level of PrP^{res} following treatment with dendrimers was determined by proteinase K (PK) digestion, immunoblotting and densitometry (**Figure 2 and Figure 3, Table S1**).

The immunoblot images and densitometry analysis illustrate that each dendrimer species reduced PrP^{res} in a prion strain dependent manner. For example, 14µM PAMAMg5 was significantly more effective in eliminating protease resistant PrP^{res} from RML and 79A infected brain homogenate than from ME7 and 22A brain homogenate whilst 301C and 301V prion strains were largely resistant to this dendrimer (Figure 2 and 3). This demonstrates that the dendrimer is interacting with the PrP^{res} in a prion strain-specific manner. These strain specific properties are perhaps governed by PrP^{res}'s conformation^[12,13], glycosylation pattern^[14], or unidentified co-factors^[15].

Dendrimers demonstrate different abilities to enhance the susceptibility of prion strains to PK. For example, PrP^{res} from 79A infected brain-homogenate was reduced by PPIg4

concentrations as low as 6 μ M while it was resistant to mPPIg5 (Figure 2A). Dendrimers with cationic surface groups (PPIg4, PAMAMg5 and 0.5mPPIg5) were generally more potent and effective against a wider range of prion strains than mPPIg5, a dendrimer with neutral maltose surface groups (recently demonstrated to possess anti-prion properties due to H-bond driven interactions with PrP^{res}).^[19,20] In fact mPPIg5 only exhibited activity against a single prion strain (RML) and required high concentrations to exhibit this effect (54 and 72 μ M). The potency of the different cationic dendrimers examined varied, indicating structure and size of dendrimer are important features in determining the efficacy of a dendrimer.

While the concentration of brain homogenate employed for each test was constant, the amount of PrP^{res} per brain homogenate varied according to prion strain where: ME7 and 22A > RML > 79A, 301C and 301V. This hierarchy of PrP^{res} content was maintained across all mice examined. The variation in the level of PrP^{res} according to prion strain does not account for the differential effect of the dendrimers as the effect of the dendrimer was assessed relative to an untreated control for each prion strain. In addition there is no correlation between relative PrP^{res} concentration and susceptibility to treatment by dendrimers. For example, RML infected mice produce a relatively high level of PrP^{res}, but this PrP^{res} is very susceptible to PPIg4. On the other hand, 301V infected mice produce a relatively low level of PrP^{res} but this is resistant to PPIg4.

The 301C and 301V prion strains were the most resistant of the prion strains to all dendrimers examined. 301C and 301V are mouse adapted prion strains originally isolated from BSE infected cattle.^[21] The other prion strains examined are all originally derived from scrapie in sheep. This difference in origin may explain the ability of the 301C and 301V prion strains to resist the denaturing effect of the dendrimers examined. For example, PrP^{res} from 301C and 301V infected mice may possess a more compact and stable confirmation in the face of the

various dendrimer species than their scrapie derived counterparts. Differentiation between BSE and scrapie origin prion strains is important for both animal surveillance and protecting human health. In that context, the ability of the dendrimer panel to differentiate between BSE and scrapie derived strains is promising. It suggests the possibility of a simple dendrimer based assay for determining if a prion strain is BSE or scrapie in origin. All prion strains examined in this study were produced in Tg20 mice (transgenic mice that over express wild-type murine PrP^{a/a}).^[22] Interestingly, a previous study has demonstrated that BSE produced in transgenic mice expressing bovine PrP was susceptible to PPI dendrimers whilst a natural scrapie strain produced in the same mice was resistant.^[2] This demonstrates that the susceptibility of scrapie and BSE to various dendrimers may be affected by the host species form of PrP^C. A similar effect has previously been shown for the RML prion strain; it was susceptible to PPI dendrimers when produced in mice (murine PrP) but resistant when produced in hamsters (Hamster PrP).^[2] This ability of PPI to differentiate between a prion strain raised in two different hosts again highlights the discriminatory power of dendrimers in identifying the minute differences that must occur between different forms of prions. The molecular basis for this discriminatory power remains unknown but it is likely that it involves the ability of specific dendrimers to interact with the different structural and biochemical features exposed in different prion strains or perhaps it is dependent on the ability of different prion strains to resist the denaturing effect induced by a particular dendrimer. Future studies with dendrimers with more diverse surface groups should help address this.

Previous studies have shown that prion strains (including RML, ME7, 22L, 301V, BSE and scrapie) which possessed the least stability in Guanidine Hydrochloride (GdnHCL), exhibited the shortest incubation times in mice^[23], and replicated the most rapidly in the protein misfolding cyclic amplification assay (PMCA)^[24]. Our results did not follow this pattern for any of the dendrimer species examined (**Table S2**). For example, 301V proved to be the most

resistant prion strain to all dendrimers examined (Figure 2D and 3) but it was not the most resistant to GdnHCL,^[23] nor did it have the longest incubation time in mice (Table S2). This suggests that the features of a prion strain which govern its susceptibility to the different dendrimers examined, differ from those features of a prion strain which define its incubation time and GdnHCl stability.

Different prion strains have distinct interspecies transmission properties and different pathogenicity patterns, particularly for humans, making it important to be able to discriminate between them.^[17,25] Classically, this has been achieved for mouse-adapted prion strains through the determination of incubation times in at least two mouse lines over 6-10 months.^[26] As this is a slow and costly process, a number of alternatives have been developed including the cell panel assay (CPA)^[27], the conformational stability assay (CSA)^[28] and the differential ELISA system based on Bio-Rad's TSE detection^[29]. However, despite their various advantages, all these methods have their limitations: The CPA takes two weeks to generate a result^[27]. The CSA does not distinguish between certain prion strains such as ME7-H and Syrian hamster Sc237^[28]. The differential ELISA system was designed to distinguish between BSE derived and scrapie origin prion strains and so does not discriminate other prion strains^[29].

In this study, the potential of dendrimers as diagnostic tools in exploring the subtle yet important differences that exist between prion strains is highlighted. We demonstrate that dendrimers of distinct sizes and molecular and chemical properties can distinguish several prion strains against a common genetic background. Individually, the dendrimer species are not sufficient to differentiate between the prion strains. However, when the dendrimer species are combined as a panel, RML, 79A, 301V and 301C prion strains can be distinguished by their unique pattern of susceptibility (Figure 3). 22A and ME7 prion strains are

distinguishable from the other prion strains but not from one another. Enhanced selectivity may be possible through the use of alternative dendrimers with more diverse surface groups. The flexibility to modify surface groups to enhance discrimination is an option that does not exist in other prion strain typing systems. Expansion of this work into naturally occurring prion diseases of economic or medical relevance to humans including BSE, scrapie and CJD will be required to fully exploit its potential. This approach may also be used to examine misfolded proteins associated with other neurodegenerative diseases such as amyloid beta ($A\beta$) from Alzheimer's disease and hyper phosphorylated tau from Parkinson's disease. Studies have demonstrated that dendrimers have a particular affinity for peptides of amyloid structure^[30] and that various dendrimers can inhibit the *in vitro* fibrilization of $A\beta$ ^[31-35] and MAP-Tau protein,^[34] suggesting that dendrimers of different charge, size and structure may serve as useful agents for investigating the pathogenic nature of different proteins involved in a wide range of different diseases. Computational methods including quantum mechanical, molecular dynamics, coarse-grained and continuum approaches could be used as an adjunct to such aggregation studies. The benefit of investigating these amyloidgenic proteins is not limited to the medical field as synthetic amyloid-based nanostructures are increasingly being looked at as highly promising functional materials.^[36]

Experimental section

Reagents: Poly(propylene imine) generation four (PPIg4) and five (PPIg5) dendrimers were obtained from SyMO-Chem (Eindhoven, Netherland). Dendrimer stock compounds were prepared as 20mg/ml solutions in sterile H₂O. The solution was sterilized by filtration through a 0.22µm syringe filter (Millipore). All other reagents including the poly(amido amine) generation five dendrimer (PAMAMg5) were obtained from Sigma-Aldrich unless otherwise stated.

Synthesis of glycodendrimers and charge of dendrimers: The poly(propylene imine) generation five dendrimers modified with an open (0.5mPPIg5) and a dense maltose shell (mPPIg5) were prepared by a reductive amination of PPIg5 in the presence of minor (0.5mPPIg5) and excess (mPPIg5) maltose for 0.5mPPIg5 and mPPIg5 as described previously.^[20, 37] PPIg4 and PAMAMg5 have a cationic surface charge, whilst the surface charge of 0.5mPPIg5 is also cationic, but lower in comparison to PPIg5.^[19,20] In contrast to this, a neutral surface charge is present for the dense shell glycodendrimer mPPIg5.^[19,20] The structure and molecular weights of the dendrimers used in this study are presented in Figure 1.

Preparation and Treatment of Brain Homogenates: The mouse prion strains 22A, ME7, 301V and 301C were obtained from the TSE resource centre and propagated in Tg20 mice (transgenic mice that over express wild-type murine PrP^a).^[22] The RML prion strain was obtained through the intracerebral infection of Tg20 mice with RML infected neuroblastoma cells obtained from Dr. Byron Caughey (Rocky Mountain Laboratories). Brain homogenate was prepared in PBS (pH 7.4) at 10% (w/v) by successive passing through 18, 20, and 23 gauge needles. Nuclei and tissue debris were removed by centrifugation at 8000 g for 5 min at room temperature. The protein concentration of the homogenate was determined by BCA

assay (Pierce). Homogenates were diluted to 1µg/µl in a final concentration of 4% sarkosyl in PBS (pH 7.4) and stored at –80°C.

Treatment of brain homogenates with dendrimers was performed by adding the dendrimer compounds at increasing concentrations to 25µg of brain homogenate. The final volume was made up to 50µl using 4% sarkosyl in PBS. Samples were incubated at 37°C for 3 h shaking at 450rpm. Samples were subsequently subjected to proteolytic digestion with PK at a 1:50 (w/w) ratio. The reaction was stopped with PMSF (5mM). Samples were centrifuged at 16,000g, supernatant removed and pellet resuspended in 1x SDS PAGE loading buffer.

Immunoblot Analysis and densitometry: Protein samples in 1× SDS sample buffer were incubated for 5 min at 95°C prior to electrophoresis on a 12.5% SDS polyacrylamide gel. After transfer (100 mV, 1 h) to a PVDF membrane (Millipore) and rinsing in TBST (TBS-0.5% Tween 20), unspecific binding sites were blocked by incubation in 5% dried milk powder (Marvel) in TBST for 1 h, with shaking (250 rpm). The membrane was washed five times in TBST (5 min each, shaking at 250 rpm), and incubated with SAF83 (SPI Bio, diluted to 20ng/mL in TBST) overnight. The membrane was washed 5 times and exposed to goat anti-mouse IgG-ALP secondary antibody (Promega, diluted to 0.1µg/ml in TBST) for 1.5 h, followed by five additional washing steps in TBST (5 min each, shaking at 250 rpm). Blots were developed by exposure to 10mls 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) for 5 minutes, followed by two washes with dH₂O. Densitometry was performed with the Alphaview Analysis Software. Results were corrected for background by determining the background OD data of two average areas from each gel, and subtracting the average of this from the integral generated for each sample. Brain homogenate from at least two different mice for each prion strain was analysed. A minimum of three repeats was performed. Statistical analysis was performed by running a one-way analysis of variance (ANOVA) with post-hoc analysis by Tukeys test (SPSS version 20.0).

Acknowledgements

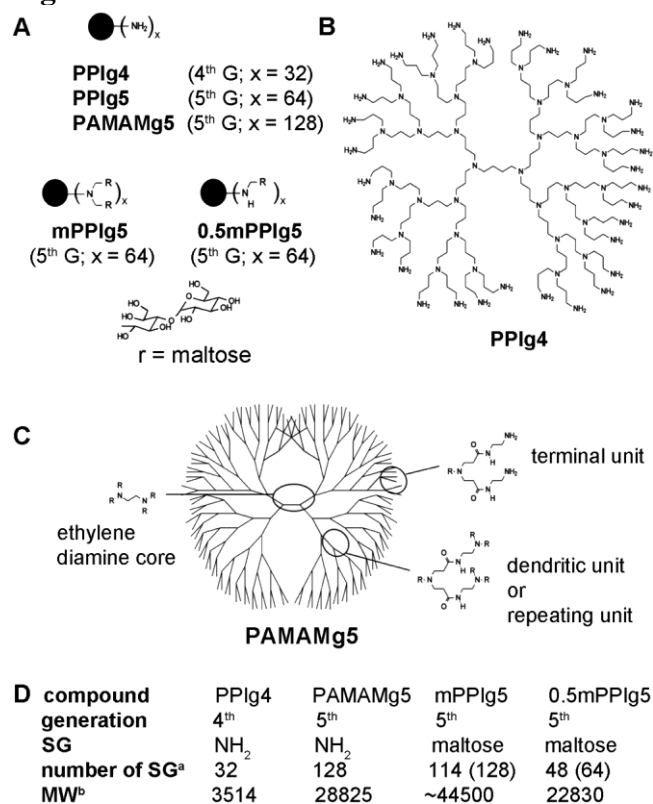
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Figures



^a Number in brackets is the theoretical number of attached maltose units on PPI surface. ^b MW determined by ¹H NMR approach as described in Klajnert *et al* 2008

Figure 1. Schematic description of dendrimers PPIg4, PAMAMg5, 0.5mPPIg5 and mPPIg5 used in this study. mPPIg5 and 0.5mPPIg5 are obtained by reductive amination of PPIg5 [19,20] (A). Molecular structure of cationic 4th generation poly(propylene imine) (PPIg4) with the smallest size used in biological experiments (B). Simplified structure of the 5th generation poly(amido amine) with terminal amino groups (PAMAMg5) (C). Molecular weights for the dendrimers utilised in this study (D).

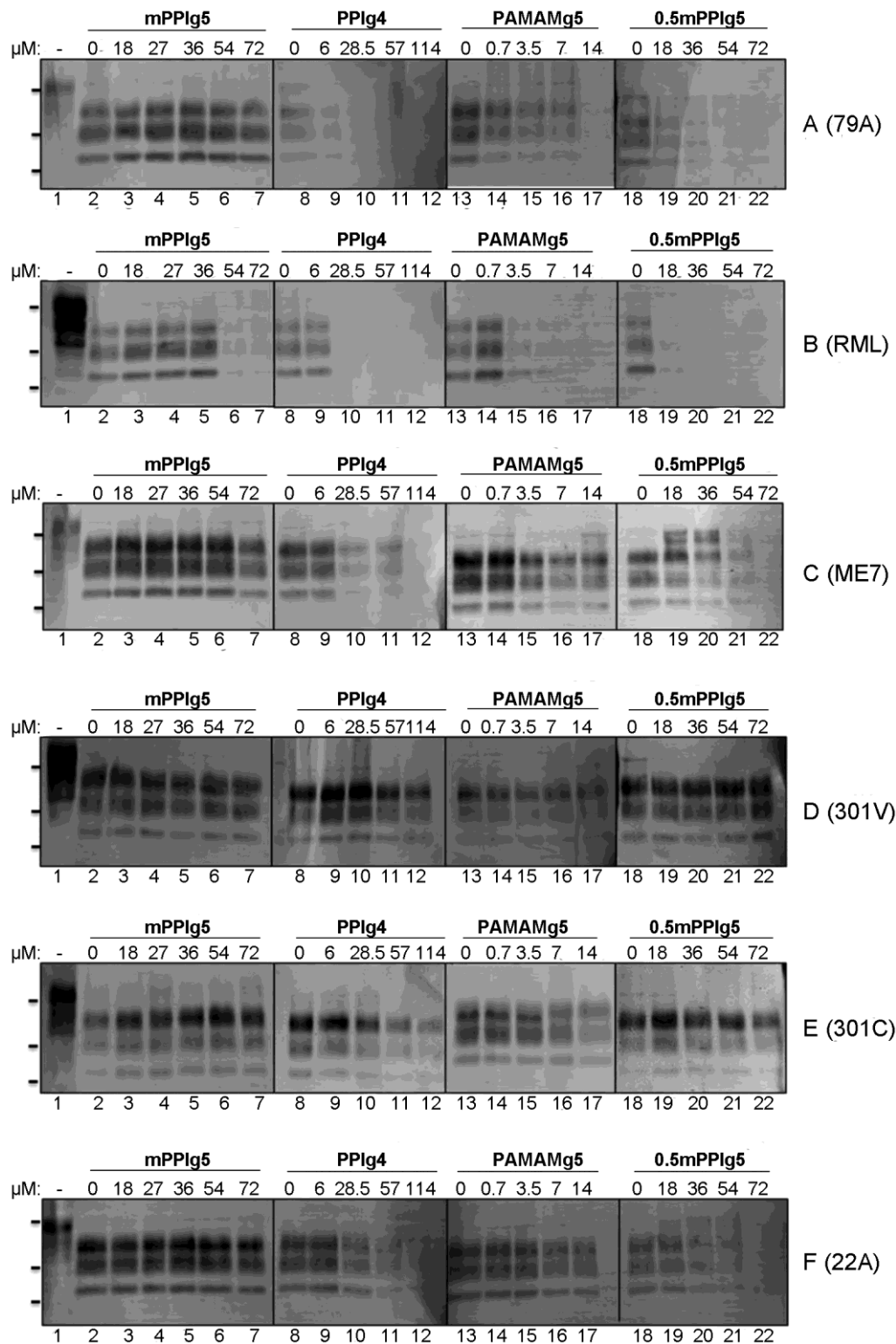


Figure 2. Effect of dendrimer treatment on prion infected brain homogenate. Tg20 mouse brain homogenates (25µg) infected with the prion strains 79A (A), RML (B), ME7 (C), 301V (D), 301C (E) and 22A (F) were incubated with increasing concentrations of the dendrimer species maltose modified PPIg5 (mPPIg5 and 0.5mPPIg5), PPIg4 and PAMAMg5. Treated samples were digested with PK and analysed by western blot for PrP^{res} with the antibody SAF83. Apparent molecular mass based on migration of protein standards is indicated for 17, 25, and 30 kDa

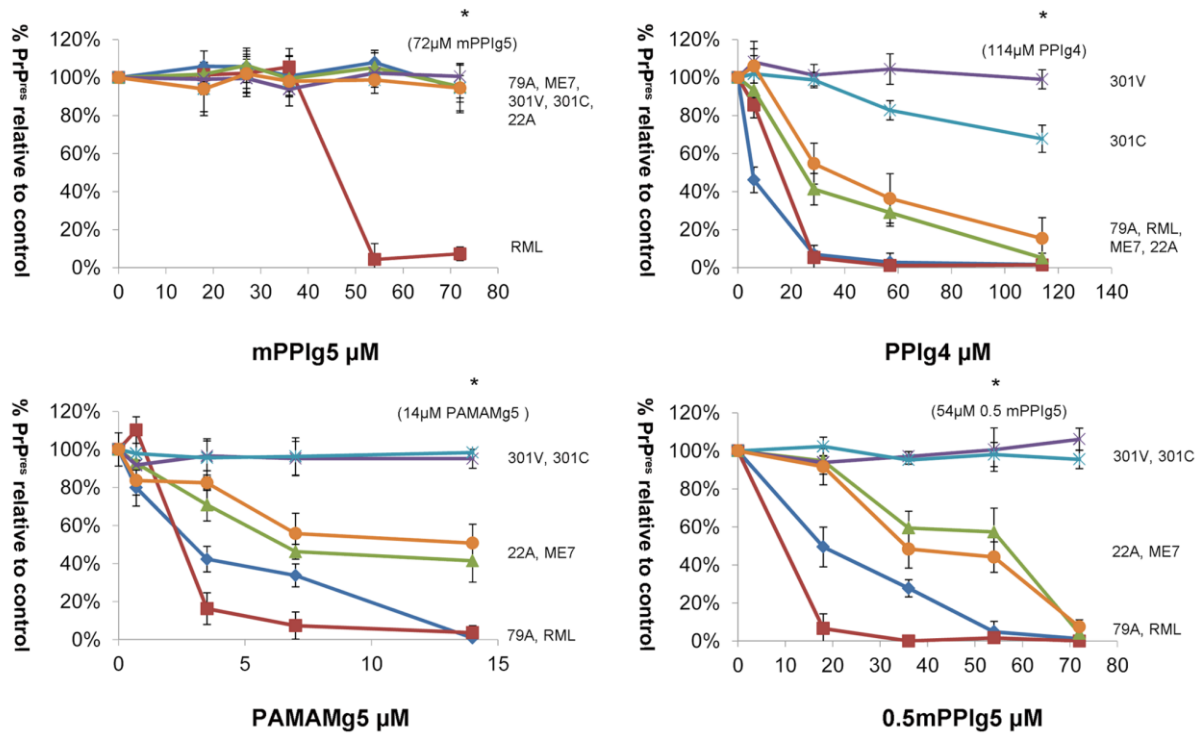


Figure 3. Differentiation of prion strains. Immunoblot images of dendrimer treated brain homogenates were analysed by densitometry for % PrP^{res} levels remaining following dendrimer treatment and protease digestion, calculated relative to a non dendrimer treated control. Legend: —●— 79A; —■— RML; —▲— ME7; —×— 301V; —◆— 301C; —○— 22A. Error bars represent SD; n = 3. * Statistically significant difference between prion strains at the concentrations indicated (p < 0.01). The mean levels of PrP^{res} remaining for each prion strain after treatment with 72μM mPPIg5, 114μM PPIg4, 14μM PAMAMg5 or 54μM 0.5mPPIg5 were compared using a one-way ANOVA. Prion strains 79A, RML, 301V and 301C can be statistically differentiated. 22A and ME7 can be differentiated from the other prion strains but not from one another.

Supporting Information

Differentiating Prion Strains Using Dendrimers

*James M. McCarthy, Beatriz Rasines, Dietmar Appelhans, Mark Rogers**

Table S1 PrP^{res} (%) remaining after dendrimer treatment

Prion Strain	mPPIg5 (μM)					PPIg4 (μM)				PAMAMg5 (μM)				0.5mPPIg5		
	18	27	36	54	72	6	28.5	57	114	0.7	3.5	7	14	18	36	54
79A	106%	106%	101%	108%	94%	46%	7%	3%	2%	80%	42%	34%	1%	49%	28%	1%
+/- SD	8%	5%	11%	5%	7%	7%	2%	5%	3%	10%	7%	6%	1%	10%	5%	5%
RML	101%	102%	106%	4%	7%	86%	5%	1%	1%	110%	16%	7%	4%	7%	0%	2%
+/- SD	6%	10%	10%	8%	4%	7%	6%	2%	3%	7%	8%	7%	4%	8%	3%	3%
ME7	102%	106%	99%	105%	95%	93%	41%	29%	5%	93%	71%	46%	41%	95%	59%	5%
+/- SD	4%	9%	5%	9%	6%	4%	8%	7%	3%	2%	8%	4%	11%	7%	9%	1%
301V	99%	100%	94%	102%	101%	108%	101%	104%	99%	92%	97%	95%	95%	94%	97%	10%
+/- SD	8%	8%	3%	5%	7%	7%	6%	8%	5%	3%	8%	9%	5%	3%	3%	1%
301C	107%	101%	108%	109%	97%	102%	99%	83%	68%	98%	96%	96%	98%	102%	95%	9%
+/- SD	14%	12%	13%	7%	13%	10%	4%	5%	7%	9%	14%	10%	10%	5%	9%	0%
22A	94%	102%	98%	99%	94%	106%	55%	36%	15%	84%	82%	56%	51%	92%	48%	4%
+/- SD	12%	8%	5%	4%	12%	13%	11%	13%	11%	8%	4%	11%	10%	10%	10%	8%





Key to % protease resistant PrP^{res} remaining:  = > 90%;  = 50-90%;  = 10-50%;  = <10%

Table S2 Incubation time of disease in Tg20 mice (days post intracerebral inoculation)

Prion Strain	Incubation Time (Days) +/- SE
79A	63 +/-4
RML	72 +/- 2
ME7	95 +/- 1
301V	158 +/- 5
301C	220 +/- 3
22A	290 +/- 18