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The influence of surface functionality of poly(propylene imine) dendrimers on aggregation and propagation of the scrapie prion protein

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ABSTRACT

Accumulation of PrP^{Sc}, an insoluble and protease-resistant pathogenic isoform of the cellular prion protein (PrP^{C}) is a hallmark in prion diseases. Branched polyamines, including PPI (poly(propylene imine)) dendrimers are able to remove protease resistant PrP^{Sc} and abolish infectivity, offering possible applications for therapy. These dendrimer types are thought to act through their positively charged amino surface groups. In the present study the molecular basis of the anti-prion activity of dendrimers was further investigated employing modified PPI dendrimers, in which the positively charged amino surface groups were substituted with neutral carbohydrate units of maltose (mPPI) or maltotriose (m3PPI). Modification of surface groups greatly reduced the toxicity associated with unmodified PPI, but did not abolish its anti-prion activity, suggesting that the presence of cationic surface groups is not essential for dendrimer action. PPI and mPPI dendrimer of generation 5 were equally effective in reducing levels of protease-resistant PrP^{Sc} (PrP^{res}) in a dose- and time-dependent manner in ScN2a cells, and in preexisting aggregates in homogenates from infected brain. Solubility assays revealed that total levels of PrP^{Sc} in scrapie infected mouse neuroblastoma (ScN2a) cells were reduced by mPPI. Coupled with the known ability of polyamino dendrimers to render protease-resistant PrP^{Sc} in pre-existing aggregates of PrP^{Sc} susceptible to proteolysis, these findings strongly suggest that within infected cells dendrimers reduce total amounts of PrP^{Sc} by mediating its denaturation and subsequent elimination.

KEYWORDS

Prion protein, aggregation, glycodendrimer, polyamino dendrimer

INTRODUCTION

Prion diseases are a group of fatal neurodegenerative diseases that can have sporadic, genetic and infectious origin. They include BSE in cattle, scrapie in sheep, and in humans the familial forms FFI, GSS, and forms of CJD of various aetiologies, such as iatrogenic (iCJD), sporadic (sCJD), familial

(fCJD) and variant (vCJD) which emerged following transmission from BSE to humans[1-2]. Prion diseases are caused by aberrant metabolism of the cellular prion protein, PrP^{C}. PrP^{Sc}, the disease-associated abnormally folded isoform of PrP^{C}, accumulates in the CNS as a hallmark of prion diseases, and is the major constituent in preparations of the infectious agent (termed prion). PrP^{Sc} is derived from PrP^{C} in a posttranslational conversion process[3-4], in which it acquires increased resistance to proteases and reduced solubility in non-ionic detergents[5-6], accompanied by increased beta-sheet content[7-8]. Coding mutations in the human prion protein gene result in hereditary forms of human prion diseases[9-13]. Expression of PrP^{C} is a prerequisite for propagating prions and developing the disease[14-15]. The intriguing characteristics of prion diseases are conveyed in the prevalent hypothesis (protein only hypothesis[16]), defining prions as infectious proteinaceous pathogens[6], which propagate in the absence of nucleic acid by an autocatalytic process, in which PrP^{C} is converted into the disease associated isoform[17]. In agreement with the protein-only hypothesis, spontaneous conversions and hereditary mutations favoring a conversion from PrP^{C} to PrP^{Sc}, account for sporadic and familial cases, respectively, in addition to the infectious nature of prion diseases[18-21].

Accumulation of PrP^{Sc} as amyloid fibrils, often in the form of amyloid plaques[13, 22-24], is accompanied by neuropathological changes primarily in the CNS, spongiform vacuolation in the (cerebral) grey matter of the brain, neuronal loss and astrocytic proliferation (astrocytic gliosis)[9, 25]. Presently, no cure is available for these invariably fatal diseases. The development of therapeutic and/or prophylactic approaches in human prion diseases is problematic, as they are characterized by a long incubation period and short clinical phase, and pre-clinical diagnosis is difficult[23, 25-26]. A potential anti-prion agent was identified in polyamino dendrimers when they were demonstrated to reduce levels of protease resistant PrP^{Sc} (PrP^{res}) in scrapie infected cells and pre-exisiting aggregates of prions, and purging PrP^{Sc} from chronically infected cells by these compounds abolished infectivity in mice-bioassays[27-28].

Dendrimers are synthetic polymers with a highly structured layered architecture, consisting of multiple branched monomers radiating from a core. Layers of monomers are attached stepwise during synthesis, with the number of branch points defining the generation of dendrimer[29-32]. Their wide variety of biological applications include bioimaging, drug, and gene delivery, drugs, carriers for vaccines and scaffold for tissue-repair[33-37]. The various dendrimer compounds for which an anti-prion activity has been demonstrated includes the branched polyamines poly(amido amine) (PAMAM), poly(propylene imine) (PPI), and poly(ethylene imine) (PEI)[27-28] and phosphorous[38] dendrimers. Their anti-prion activity

in reducing PrP^{Sc} in ScN2a cells is dose- and time-dependent, increases with generation number (size), and is considered to depend on a high density of cationic groups on the surface[27, 38-39].

The mechanism by which dendrimers eliminate protease-resistant PrP^{Sc} from the cells or the intracellular site of action remains to be established. However, dendrimers are able to disrupt prion-fibrils, decrease their β-sheet content and render PrP^{Sc} susceptible to proteolytic digestion in pre-formed aggregates of PrP^{Sc}. This ability was dependent on pH, prion strain and presence of urea in these *in vitro* studies, indicating that the denaturation of PrP^{Sc} is involved in this process. These findings suggest that branched polyamines act by removing pre-existing prions. In further agreement, there was a strong correlation between the relative anti-prion activity of various dendrimer-compounds for rendering PrP^{Sc} susceptible to proteolysis in cell culture and *in vitro* studies with brain-homogenates and purified prions[27-28]. An endocytic/lysosomal compartment has been proposed as a potential site of action within the cell. Dendrimers enter cells by endocytosis. Cationic dendrimers can be transported by electrostatic interactions via the cell membrane causing its disruption[40], but also endocytic uptake has been observed[41-43]. Cationic and uncharged dendrimers are probably taken up by non-specific adsorptive (electrostatic) fluid-phase endocytosis. This process was found to be clathrin- and caveolae-independent[44], but clathrin-mediated pathways has also been postulated as an uptake mechanism for cationic dendrimers[42, 45]. The intracellular site of action for dendrimers on PrP^{Sc} is thought to be a lysosomal compartment, as treatment with chloroquine, an agent that alkalinises lysosomes, inhibited the dendrimer-specific reduction of PrP^{Sc} in ScN2a cells, and PrP^{Sc} in brain-homogenates is rendered susceptible to proteases most effectively at acidic pH by polyamino dendrimers[27]. An acidic compartment as the site of action for dendrimers is further supported by the intracellular tracking of a FITC-labelled PPI dendrimer, indicating its accumulation in lysosomes[28].

Cationic dendrimers (PAMAM, PPI and phosphorous dendrimers) have also been shown to have severe effects on the aggregation status of synthetic peptides of the prion protein. PAMAM, PPI and phosphorous dendrimers changed kinetic parameters of aggregation for the prion peptide PrP185-208, such as nucleation and elongation rate and reduced the final amount of amyloid fibrils formed[46-49]. A generation dependent effect on both the inhibition of fibril aggregation and the disruption of fibrils of the PrP185-208 peptide was observed for PAMAM dendrimers[48]. The aggregation process is also influenced by charge of the dendrimer. A high cationic surface charge reduced fibril formation of the

prion peptide PrP106-125 compared to unmodified PPI-dendrimer[50]. Recent studies demonstrated that a modified version of PPI-dendrimers, in which the positive surface charge (amino groups) was abolished on the dendrimer-surface by substitution with maltose, had an inhibitory affect on aggregation of the prion-peptide PrP185-208[49].

The motivation for this study is to extend previous studies on a synthetic prion peptide and compare the biological properties of PPI glycodendrimers and unmodified cationic PPI dendrimers towards endogenous prion protein. For this purpose, 3rd to 5th generation PPI glycodendrimers with a dense maltose shell presenting a multiple H-bonding active glycodendrimer surface as described previously[49] were employed. The molecular structure of the glycodendrimers, presented in Figure 1, is characterized by a dense oligosaccharide shell, in which the formerly peripheral amino groups preferentially possess two chemically coupled maltose[49] or maltotriose[51] units. This specific structural feature in the outer sphere of the glycodendrimers, introduces a neutral charge density in the former cationic PPI dendrimers as determined by polyelectrolyte titration experiments[49].

Furthermore, these glycodendrimers have a non-flexible dendritic PPI scaffold as the dense oligosaccharide shell inhibits the known backfolding properties of terminal units displayed in more flexible dendrimers[49]. Therefore, only H-bonding active OH groups on the glycodendrimer's surface are available to undergo non-covalent interactions with other macromolecules. Comparing the anti-prion activity of PPI polyamino- and glycodendrimers with cationic and neutral surface functionalities, respectively, will provide further insight into the molecular mechanism underlying dendrimer action and allow an evaluation of these compounds as potential anti-prion agents.

EXPERIMENTAL METHODS

Reagents. The poly(propylene imine) (PPI) dendrimers of 3rd, 4th and 5th generation (PPI-g3, PPI-g4 and PPI-g5) were supplied by SyMO-Chem (Eindhoven, Netherland). D(+)-maltose monohydrate, sodium borate (Na borate) and the boran pyridine complex (BH_3*Py, 8 M solution in THF) were used as purchased from Fluka. All other chemical compounds were purchased from Sigma–Aldrich if not stated otherwise. Dendrimer compounds were prepared and stored as 3 mg/ml stocks in sterile H_2O. The solution was sterilized by filtration through a 0.22 μm syringe filter (Millipore).

Synthesis of Glycodendrimers. 3rd – 5th generation PPI dendrimers modified with a dense maltose shell (mPPI) and 5th generation PPI dendrimers modified with dense maltotriose shell (m3PPI) were prepared as described previously[49, 51]. 5th generation PPI dendrimers modified with open maltose shell (0.5mPPI) was prepared according to the same method[49]. For the open maltose shell in 0.5mPPI a conversion ratio of 1:1 was used between each peripheral amino group of the 5th generation PPI dendrimer and maltose. Generally, oligosaccharide units were introduced to the PPI surface based on the reductive amination as one-pot approach[49]. The structural features of dense and open shell PPI dendrimers is presented in Figure 1 with the 5th generation 0.5mPPI with open maltose shell preferably possessing secondary amino groups in the outer shell in comparison to those with preferred peripheral tertiary amino groups in mPPI with dense maltose and maltotriose shell. Compound 0.5mPPI possesses a cationic charge density determined by polyelectrolyte titration experiments (data not shown; experimental procedure as described previously[49]).

The molar masses of the dendrimers, determined by laser-induced liquid bead ionization/desorption mass spectrometry (LILBID-MS[49, 51]), used in this study are: 1687 g/mol for the 3rd generation PPI dendrimer (PPI-g3), 7168 g/mol for the 5th generation PPI dendrimer (PPI-g5), 12128 g/mol for 3rd generation PPI dendrimer with dense maltose shell (mPPI-g3; 32 maltose units attached on PPI surface), 24397 g/mol for 4th generation PPI dendrimer with dense maltose shell (mPPI-g4; 64 maltose units attached on PPI surface), 44500 g/mol for 5th generation PPI dendrimer with dense maltose shell (mPPI-g5; 114 maltose units attached on PPI surface), 22830 g/mol for 5th generation PPI dendrimer with open maltose shell (0.5mPPI-g5; 48 maltose units attached on PPI surface), 58000 g/mol for 5th generation PPI dendrimer with dense maltotriose shell (m3PPI-g5; 104 maltotriose units attached on PPI surface). Further details for attached oligosaccharide units on the surface of PPI are found in previously published data[49, 51].

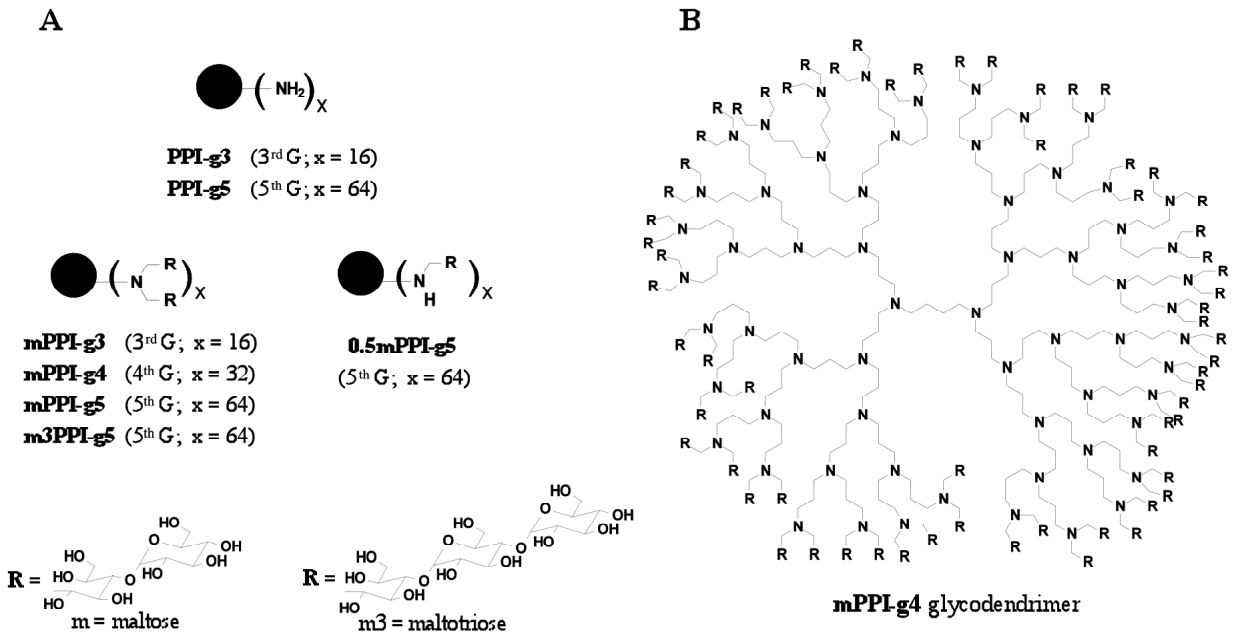

Figure 1. **Structure of PPI dendrimer and surface functionalities.** (A) Identity and number of surface groups of parent PPI dendrimers and PPI glycodendrimers of different generations (G) employed in the present study. (B) Structure of PPI glycodendrimer of generation 4 with dense glucose shell.

Cell culture and dendrimer treatment. A clonal subpopulation (F4) was isolated from the mouse neuroblastoma cell-line N2a (a kind gift from Byron Caughey) and infected with murine-adapted RML scrapie strain by the method of Bosque and Prusiner (Bosque & Prusiner, 2000). Uninfected N2a and infected ScN2a cells were maintained in DMEM (Gibco®) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, from calf, Gibco®), 2 mM L-glutamine, 100 units/ml penicillin and 100 ug/ml streptomycin (Sigma) at 37°C in humidified air with 8% CO_2. Medium was replaced every 3 to 4 days and cells were routinely split for subculture 1:10 every 5 to 7 days after reaching confluence. For exposure of cells to dendrimers, cells were split at a dilution that allowed the cells to reach confluence at the end of the exposure time. One day after plating, cells were exposed to dendrimers at different concentrations and for varying incubation times.

For analysis of intracellular protein content, cells were washed twice with medium prior to addition of fresh cultivation medium and treatment with dendrimer compounds. Dendrimer containing medium was

replaced for exposure times of more than 4 days. At the end of the incubation period of treatment with dendrimers, cells were washed twice in PBS and underwent cold lysis in standard lysis buffer (25 mM Tris (pH 7.4), 10 mM EDTA, 150 mM NaCl, 0.5% sodium deoxycholate, 0.5%Triton X-100) or buffer A (0.5% Triton X, 0.5% DOC in PBS) if subjected to solubility assay. Total cell lysates were separated from nuclei and cellular debris by centrifugation for 5 min at 4000 rpm (~1,300 g, Eppendorf table-top centrifuge 5415 C). Following determination of protein concentration by BCA assay (Pierce), samples not subjected to analysis by detergent solubility assay were prepared for analysis by immunoblot. Samples of 25 µg lysate from N2a cells was mixed with a 4x stock of SDS sample buffer (0.25 M Tris-HCl (pH 6.8), 10% SDS, 50% glycerol, 0.5 mg/ml bromophenol blue, 75.5 g DTT) to yield a final concentration of 1x. Lysates of ScN2a cells were subjected to limited proteolysis, using 250 µg protein and incubation with Proteinase K (PK, Boehringer-Mannheim, prepared as a 1 mg/ml stock-solution in H_20) at a 1:500 ratio for 30 min at 37°C shaking (450 rpm). Reactions were stopped by addition of 5 mM PMSF (from 100 mM stock). Following an additional centrifugation step at 14,000 rpm (16,000 g) for 30 min, the pellets were resuspended in 1x SDS sample buffer.

For analysis of cellular viability, one day after plating cells into 96 well-plates, dendrimers were added directly to the cultivation medium and cells incubated for a further 7 days. Viability of cells was assessed by MTT assay based on the method described by Mosmann[52], applying a sterile solution of MTT in PBS to the cells to a final concentration of 0.5 mg/ml and further incubation at 37°C for 3 h. Supernatant medium was removed from the cells, the remaining formazan crystals were dissolved in 200 ul DMSO (Dimethyl sulfoxide) and absorbance measured at 570 nm in a Wallac 1420 VICTOR 2™ multilabel counter.

Preparation and treatment of brain homogenates. Brain homogenates at 10% (w/v) in PBS were prepared from brains of PrP^C-overexpressing mice (tg20) infected intracerebrally (i.c.) with infective material from ScN2a cells. Brains were homogenized in PBS by successive passing through 18, 20 and 23 gauge needles. After addition of sarcosyl at 2%, nuclei and tissue-debris were removed by centrifugation at 8,000 g for 5 min at room temperature. The protein concentration of the homogenate was determined by BCA assay (Pierce) according to manufacturer's instructions. Treatment of brain homogenates with dendrimers was performed adding compounds at equal molar amounts to 50 μg

brain-homogenates, followed by incubation at 37°C for 2 hours shaking at 450 rpm. Samples were subsequently subjected to proteolytic digestion with PK at a 1:50 ratio. The reaction was stopped with 5 mM PMSF, and samples prepared for immunoblot analysis by addition of 4x SDS sample buffer to a final concentration of 1x.

Immunoblot analysis. Protein samples in 1x 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, shaking (250 rpm). After 2 washing steps in TBST (5 min, shaking at 250 rpm), the membrane was incubated in SAF83 (Cayman Chemical, diluted to 4 x 10^{-2} μg/ml in TBST) over night. The membrane was washed 5x and exposed to the secondary antibody (Santa Cruz Biotechnology Inc., 4 x 10^{-2} μg/ml in TBST) for 1.5 hours in a roller incubator, followed by 5 additional washing steps. Signals on the membrane were detected using the WB ECL detection kit (Pierce) and exposure to an X-ray photography film (Fuji) and subsequent development of the film. Immunoblots were documented using FluorS MultiImager (BioRad).

Detergent solubility assay. Cellular lysates prepared in lysis buffer A were subjected to analysis by detergent solubility assay. Lysates (250 μg) were subjected to proteolytic digestion with PK (1:250) or left untreated (50 μg) prior to separation into soluble and insoluble fractions by centrifugation at 16,000 g for 1 h at RT. The supernatant (soluble fraction) was removed and concentrated by ethanol-precipitation. Precipitation of proteins was performed by adding 9 volumes of ice-cold 100% ethanol to the samples and incubation at -20°C over night. After centrifugation at 15,000 x g for 15 min at 4°C, the pellet was washed with 90% ice-cold ethanol. After an additional centrifugation-step for 5 minutes (at 15,000 g, 4°C), the pellet was allowed to dry before respuspension in 1x SDS-sample-buffer for analysis by SDS-PAGE. The pellet (insoluble fraction) was washed with buffer A followed by an additional centrifugation-step (16,000 g for 1 h at RT) and resuspended in 1x SDS sample-buffer for analysis by immunoblot.

Unfractioned lysates of N2a cells (50 μg) either digested or left undigested by PK served as controls for PK digest and were prepared for immunoblot analysis by mixing with the appropriate volume of 4x SDS sample buffer yielding a concentration of 1x.

RESULTS

Comparative analysis of cytotoxicity by PPI-dendrimers with neutral surface group modification. Amino-terminated dendrimers are reported to have limited potential for application in therapy due to their generation and dose-dependent toxicity profile[53-55]. The functionalisation of their peripheral groups has been reported to improve their toxic properties[56]. It was therefore important to establish the toxicity profiles for different generations of the polyamino dendrimer PPI and PPI compounds with maltose modification of their surface used in this study.

Different types of polyamino dendrimers displayed different effects on cellular viability assessed in N2a cells by MTT assay (Figure 2). Cells were exposed for seven days to various compounds: PPI of generation 3 and 5 and a version of PPI-g5, in which surface groups had been substituted with maltose to 50%, (0.5mPPI-g5). In 0.5mPPI-g5, each amino-surface group is coupled with one maltose unit by substitution of one H-atom, thus converting primary into secondary amino groups. Fully substituted mPPI of generation 3, 4 and 5 and m3PPI was also used in cellular viability assays.

Treatment with PPI resulted in a generation-dependent toxic effect on N2a cells for generation 3 and 5 (Figure 2A). Onset of toxicity was observed at 3.5 to 4 μg/ml for both compounds, but PPI-g5 reduced cellular viability more rapidly than PPI-g3. A concentration of 5 μg/ml (0.7 μM) reduced the viability of cells exposed to PPI-g5 to zero whereas about 60% of cells remained viable following exposure to PPI-g3 at 5 μg/ml (3 μM). Substitution of surface-positions on PPI-g5 to 50% by maltose (0.5mPPI-g5) significantly reduced the toxicity of that compound, and no decrease in cellular-viability was observed for concentrations of up to 20 μg/ml. Elevated levels of absorbance in the MTT assay were observed for 0.5mPPI-g5 applied at low concentrations (5 and 10 μg/ml) compared to untreated cells. As this relative increase became smaller with increasing dendrimer concentration, a stimulating effect on cell-proliferation by the compound was excluded. It is possible that minor inconsistencies in cell plating could result in an accumulated effect over the eight days of cultivation. There was no effect of mPPI generation 3-5 or m3PPI, generation 5 on the viability of N2a cells (Figure 2B). It was concluded that substitution of amino-surface groups of PPI dendrimers by maltose abolishes or reduces toxicity of these compounds, an effect contributed to the abolished surface charge.

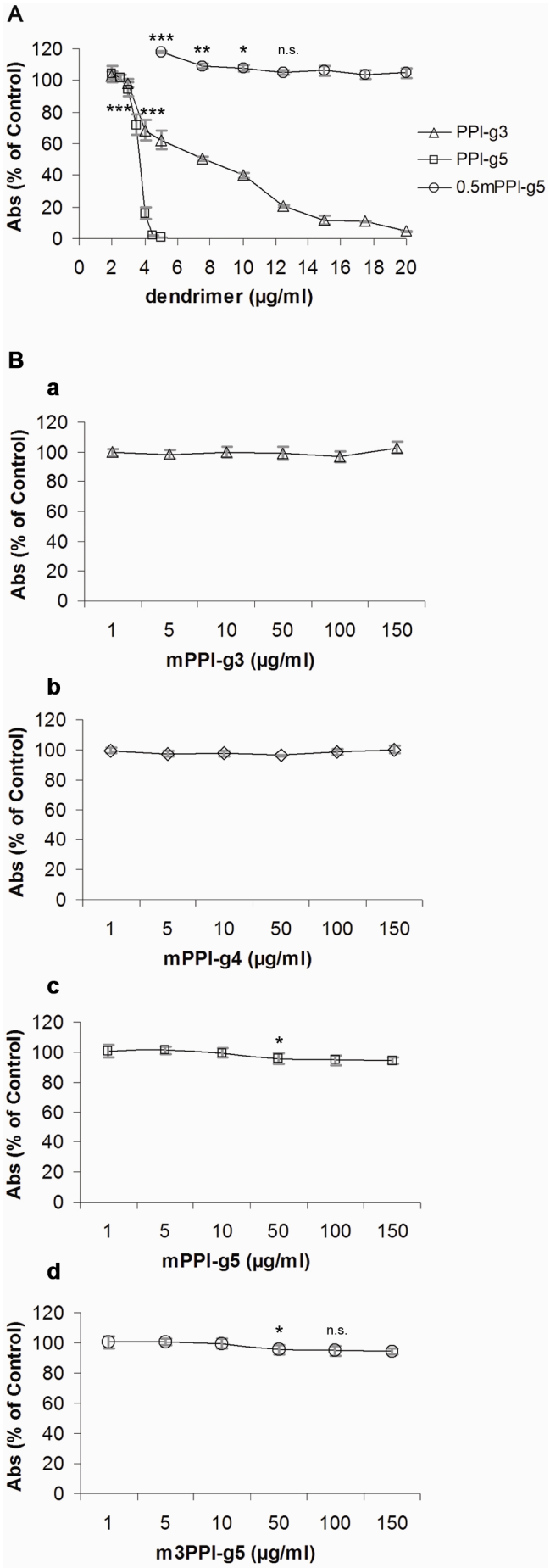
A
Abs (% of Control)
dendrimer (μg/ml)
PPI-g3
PPI-g5
0.5mPPI-g5
***
**
*
n.s.
B
a
mPPI-g3 (μg/ml)
b
mPPI-g4 (μg/ml)
c
mPPI-g5 (μg/ml)
d
m3PPI-g5 (μg/ml)

Figure 2. Toxic effects of PPI and maltose-modified dendrimer compounds. N2a cells were exposed to dendrimers for 7 days at the indicated concentration and cellular viability was assessed by MTT assay (Abs = absorbance). **(A)** Treatment with PPI, generation 3 (–△–), generation 5 (–□–) and generation 5 with 50% of surface charges substituted by maltose, 0.5mPPI-g5 (–○–). **(B)** PPI fully substituted with maltose (mPPI) of generation 3 (panel a), 4 (panel b) and 5 (panel c) and maltotriose of generation 5, m3PPI-g5 (panel d). Data are expressed as percentage from untreated control for n=3 independent experiments (± SEM; n.s.: not significant, * $0.05 \leq p \geq 0.01$, ** $0.01 \leq p \geq 0.001$ and *** $p < 0.001$; for presentation purposes, p-values for statistical significance are indicated for a change in these parameters and therefore apply to successive data points if not indicated otherwise).

Neutrally charged glycodendrimers reduce levels of protease resistant PrP^{Sc} in a time-dependent manner. Following the identification of glyocodendrimers as compounds with an improved toxicity profile compared to their parental dendrimers (Figure 2), the potential of mPPI as an anti-prion compound was assessed (Figure 3). ScN2a cells were exposed to 10 μg/ml of generation 5 (mPPI-g5, the highest generation of mPPI dendrimer available) for different time intervals up to 7 days. Cellular lysates were subjected to limited proteolysis prior to analysis by immunoblot. In comparison to cells mock-treated for 7 days (C, lane 1), exposure to mPPI-g5 caused an efficient reduction in levels of PrP^{res} in ScN2a cells in a time-dependent manner. A reduction in levels of PrP^{res} was apparent after 1 day of treatment (lane 2), and levels declined further over time to undetectable levels after 6 days of treatment (lane 7). A faint signal for PrP^{res} was visible after day 5 but is not reflected in the corresponding figure below (lane 6).

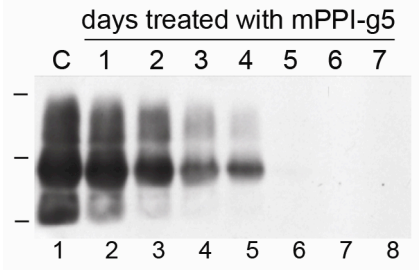

Figure 3. Kinetics of treatment with mPPI, generation 5, on levels of PrP^{res} in ScN2a cells. Cell extracts of mock treated cells (C, lane 1) and after 1-7 days exposure to 10 ug/ml maltose modified PPI,

generation 5 (lanes 2-8) were analysed for PrP^{res} content by digestion with proteinase K prior to immunoblot. Apparent molecular mass based on migration of protein standards is indicated for 17, 25 and 30 kDa.

Generation-dependence of PPI dendrimers. The neutrally charged PPI-macromolecule mPPI effectively eliminated PrP^{res} in ScN2a cells, indicating that the amino groups of PPI dendrimers are not required for this activity (Figure 3). Based on this finding, a systematic approach was undertaken comparing PPI dendrimers with cationic (amino) and neutral (maltose) surface groups regarding their effectiveness in reducing levels of PrP^{res} to gain further insight into their mode of action.

In a previous study, IC_{50} values (the dose of compound required to cause a 50% reduction of PrP^{res} in infected cells) for hyperbranched and dendritic polymers (PAMAM, PEI (polyethyleneimine) and PPI) were presented according to their mass concentration (e.g. μg/ml)[27]. The efficiency of the dendrimers in reducing levels of PrP^{Sc} in ScN2a cells in this study increased with generation number and was related to the density of positively charged amino groups on the surface

For a systematic analysis of the role of surface groups in dendrimer action in this study, comparison between modified and unmodified compounds via their mass concentration was not considered appropriate due to the high impact by the maltose modification on the molecular mass. It was decided instead to use same molar concentrations of PPI and mPPI in experiments. The resulting equal numbers of PPI and mPPI macromolecules thus allow for a direct comparison of the compound efficiency solely based on presence of polar amino groups or uncharged maltose units on the dendrimer's surface.

The influence of type and distribution of surface groups on the dendrimer's ability to reduce levels of PrP^{res} was investigated by dose-response experiments, in which ScN2a cells were exposed to unmodified PPI and maltose-modified PPI of different generations (Figure 4). After a 3 day exposure, extracts from ScN2a cells were prepared and analyzed by immunoblot for relative levels of PrP^{res} after digestion with PK. Control cell lysates (C, lane 1) were prepared from ScN2a cells that had not been treated with dendrimer.

The potential of PPI and mPPI to reduce levels of PrP^{res} were compared directly (Figure 4A) using the highest and lowest generation available for this study, generation 3 (panel a) and 5 (panel b). As outlined in the previous section, PPI (lanes 2-4) and mPPI (lanes 5-7) were applied at equal molarities

for the corresponding generations, respectively, allowing comparisons purely on the identity of their surface groups. To further address the influence of dendrimer's structure on its activity, different generations of the same compound were initially used at equal mass concentrations (e.g. g/L) of the PPI dendrimer backbone, resulting in equal total amounts of monomeric PPI units (and about the same absolute number of surface groups). In a first approach, this did not result in any effect for generation 3 of either PPI or mPPI (data not shown), and concentrations for treatment were increased 2 to 4-fold (panel a) relative to generation 5 (panel b).

A generation-dependent effect on the reduction of PrP^{res} was observed for dose-responses of PPI dendrimers (generation 3 and 5, Figure 4A) and mPPI dendrimers (generation 3, 4 and 5, Figure 4A and B). Generation 3 of PPI and mPPI (Figure 4A, panel a) was less effective in reducing levels of PrP^{res} in ScN2a cells than generation 5 (panel b), even at increased concentrations of generation 3 corresponding to 2-times (lanes 3 and 6) and 4-times (lane 4 and 7) higher amounts of PPI-scaffold. Generation 3 (panel a) of PPI (lanes 2-4) was more efficient than the equivalent maltose-modified version mPPI (lanes 5-7). Levels of PrP^{res} were clearly reduced for 6.4 $x10^{-1}$ μg/ml, the highest dose applied, while PrP^{res} levels remained unaffected following treatment with generation 3 mPPI.

Treatment with generation 5 (Figure 4A, panel b) of PPI (lanes 2-4) and mPPI (lanes 5-7) resulted in a dose-dependent decrease of PrP^{res} levels compared to untreated cells (C, lane 1). Analysis of the generation-dependent effect of PPI (lanes 2-4, panel a and b) shows that generation 5 of PPI efficiently reduced levels of PrP^{res} for all concentrations applied, ranging from 4 $x10^{-2}$ to 1.6 $x10^{-1}$ μg/ml, in a dose-dependent manner, while generation 3 had a small effect between 4 to 6.4 $x10^{-1}$ μg/ml.

Generation 5 of PPI and mPPI reduced levels of PrP^{res} with comparable efficiency to PPI. The signals for PrP^{res} analyzed by immunoblot, were significantly reduced for each concentration applied compared to untreated control. Only small differences in levels of detected PrP^{res} were observed when comparing equal doses for both compounds. Both compounds demonstrated a similar ability to reduce PrP^{res} levels across the range of concentrations applied.

Establishing that generation 3 of mPPI was not effective in reducing levels of PrP^{res} in ScN2a cells while mPPI-g5 had a significant effect (Figure 4A), mPPI-g4 was tested for its anti-prion activity relative to both generations across a range of concentrations (Figure 4B). As dose-responses had already been analysed for generation 3 and 5, here only one concentration each was applied to serve as

comparison with generation 4. Experimental procedures were as described in the previous sections on dose-response studies for different generations of PPI and mPPI, and dendrimer concentrations were adjusted relative to the PPI backbone of the maltose modified compounds. The efficiency of mPPI in reducing PrP^{res} levels increased with generation number, and both generation 4 and generation 5 had an apparent effect. Amounts of PrP^{res} were not affected by mPPI-g3 (lane 2; 1.6 $x10^{-1}$ μg/ml PPI-scaffold), in agreement with a previous study (Figure 4A, panel a). Levels of PrP^{res} were reduced in a dose-dependent manner by treatment with mPPI-g4 (lanes 3-5) and decreased to almost undetectable levels for mPPI-g5 (lane 6). Applied at an equal concentration ($1.6x10^{-1}$ µg/ml PPI-scaffold), mPPI-g4 reduced PrP^{res} to levels between those detected for treatment with mPPI-g3 and mPPI-g5 (lanes 2, 5 and 6).

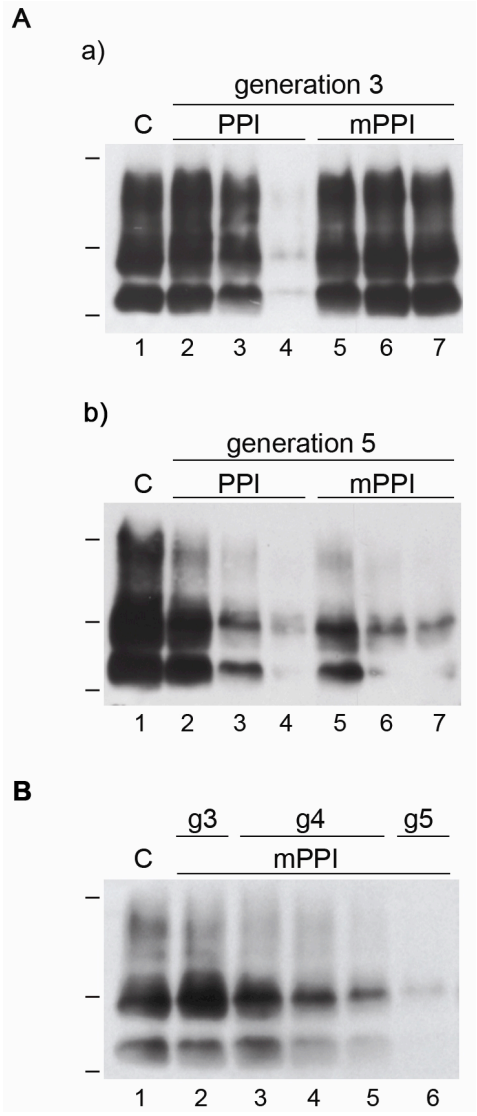

Figure 4. Generation- and dose-dependent reduction of PrP^{res} in ScN2a cells by PPI and mPPI. ScN2a cells were treated for 3 days with various concentrations of dendrimer. Levels of PrP^{res} were

analyzed after PK digestion of cellular lysates on immunoblot. Cells cultivated in the absence of dendrimer served as controls (C, lane 1). Amounts (μg/ml) of maltose-modified PPI dendrimer were adjusted relative to their amount of PPI-scaffold. **(A)** Panel a and b show the effect of different dendrimer types, parental PPI (lanes 2-4) and maltose modified PPI (mPPI, lanes 5-7), used at equal molar amounts for the corresponding generation 3 (0.24, 0.96 and 3.82 $x10^{-1}$ μM, panel a) and generation 5 (0.06, 0.11 and 0.22 $x10^{-1}$ μM, panel b), respectively. **(B)** Different generations of mPPI were compared using an equal amount of 1.6 $x10^{-1}$ μg/ml of their PPI backbone (generation 3 g3, lane 2, generation 4 (g4, lane 5) and generation 5 (g5, lane 6), and additional concentrations of generation 4 (0.4, 0.8 $x10^{-1}$ μg/ml PPI-scaffold, lane 3 and 4) for establishing a dose response. Apparent molecular mass based on migration of protein standards is indicated for 17, 25 and 30 kDa.

Effect of maltose modification on anti-prion activity of PPI (generation 5). In a dose-response study for PPI dendrimers of generation 5, mPPI and PPI were equally effective in reducing levels of PrP^{res} in ScN2a cells (Figure 4A). These two compounds were compared in further detail (Figure 5). Brain homogenates of mice intracerebrally infected with mouse-adapted scrapie prions, were treated with generation 5 of either PPI or mPPI applied at equal molar amounts by shaking for 2 hours at 37°C. Homogenates were digested with PK and analyzed by immunoblot for levels of PrP^{res}, with brain left untreated by dendrimers serving as a control (Figure 5A). Both compounds were equally potent in reducing levels of PrP^{res} in brain homogenates. Treatment with both PPI-g5 and mPPI-g5 resulted in the dose-dependent reduction of PrP^{res} signals, and for corresponding doses (lanes 2+5, 3+6, 4+7) signals were of about equal intensity. The only difference in the experimental setup was the presence (PPI) or absence (mPPI) of positively charged amino groups on the dendrimer's surface as both compounds had been applied at same molar amounts. It was therefore concluded that positively charged amino groups on the surface of PPI were not required for rendering aggregates of PrP^{Sc} susceptible to proteolytic digestion.

Additionally, both compounds were compared in time-course experiments in cultured cells (Figure 5, B). At day one, two and three after the start of treatment, lysates were prepared from ScN2a cells, which had been either treated with 0.022 μM generation 5 of PPI or mPPI or left untreated (control). All cell

extracts were analysed by immunoblot following treatment with PK. Control levels of PrP^{res} (C) remained unchanged during the time-course of the experiment (lanes 1, 4 and 7), whereas PrP^{res} signals decreased in a time-dependent manner for treatment with PPI (P, lanes 2, 5 and 8) and mPPI (M, lanes 3, 6 and 9). PrP^{res} levels were slightly higher for treatment with mPPI (lane 3) than in extracts from cells treated with PPI (lane 2) after one day of treatment. At day 2 and 3, no difference in PrP^{res} levels was observed between application of PPI or mPPI (lanes 5 and 6; lanes 8 and 9, respectively).

Both dose-response and kinetic studies with generation 5 of PPI and mPPI (Figure 4A, panel b and Figure 5, panel A and B) revealed that the replacement of the primary surface amino groups with maltose does not change the potential of PPI to render PrP^{Sc} susceptible to proteolytic digestion. This demonstrates that cationic surface-interactions are not required for this activity. In an additional study it was tested whether the activity of mPPI could be increased further by increasing the number of glucose units in maltose attached. For this purpose, generation 5 of a PPI dendrimer in which the amino groups were substituted with the trisaccharide maltotriose (possessing 3 glucose units), m3PPI-g5, was employed. Generation 5 of both m3PPI and mPPI were compared in their potential to reduce levels of PrP^{res} in ScN2a cells (Figure 5C). As different compounds of the same generation were used, same molar amounts of 3mPPI and mPPI were applied for comparative treatments. ScN2a cells were exposed to three different molarities of each compound for three days. Cellular lysates were subjected to digestion with PK and analysed by immunoblot using lysates of untreated cells as a control (lane 1).

In agreement with previous findings for mPPI-g5 (**Figure 4**A, panel b), a dose-dependent reduction of PrP^{res} levels was reproduced in this study (lanes 2-4). Treatment with maltotriose PPI (m3PPI) equally resulted in a dose-response for reduced PrP^{res} levels (lanes 5-7). No major difference for the dose-dependent reduction of PrP^{res} levels between treatments with either compound was observed for any concentration applied (lanes 2 and 5, 3 and 6, 4 and 7). Therefore, a relative increase in gluocse units on the surface of generation 5 glycodendrimers carrying a maltose or maltotriose modification, respectively, did not confer a higher efficiency in reducing PrP^{res} levels in ScN2a cells.

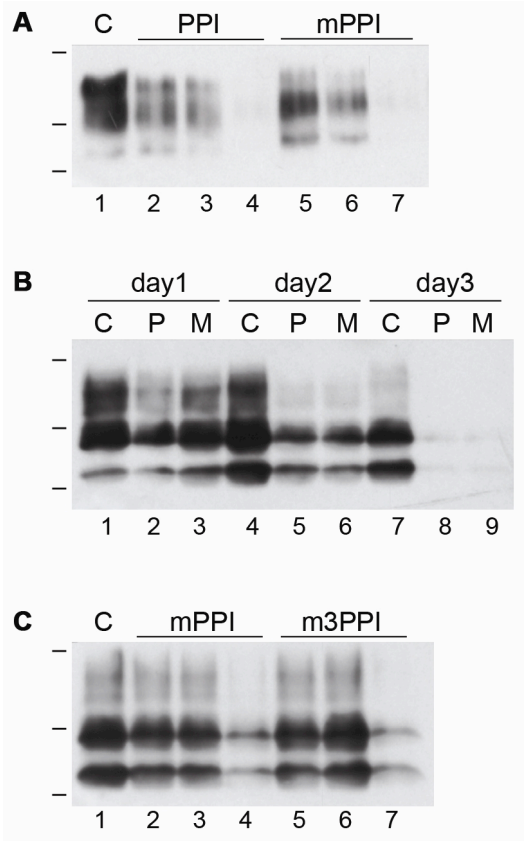

Figure 5. Comparative analysis between generation 5 of PPI and maltose modified PPI. (A) Dose-dependent modulation of protease-resistance of PrP^{Sc} in infected brain homogenates by PPI dendrimers of generation 5. Homogenates from scrapie-infected brain were treated with PPI-g5 (lanes 2-4) and m PPI-g5 (lanes 5-7) at same molar amounts of 1.8 (lane 2 and 5), 3.6 (lane 3 and 6) and 7.2 nmol (lane 4 and 7). Treated samples and untreated control-homogenate (C, lane 1) were analysed for levels of PrP^{res} by immunoblot after PK-digestion and stained with antibody SAF83. **(B)** Time-dependent reduction of PrP^{res} in ScN2a cells by PPI and mPPI of generation 5. ScN2a cells were treated with PPI-g5 (P) and mPPI-g5 (M) at equal molarity (0.022 μM) for 1, 2 and 3 days or left untreated (C). At the end of each incubation-interval, lysates were taken and digested with proteinase K. Samples were analysed by immunoblot with SAF83. Molecular mass is indicated in kDa. **(C)** Dose-response on PrP^{res}-levels for generation 5 PPI dendrimer with varying maltose-modification. ScN2a cells were exposed for three days to equal molar amounts of PPI modified with either maltose (mPPI) or maltotriose (m3PPI) at 0.69 (lane 2 and 5), 2.75 (lane 3 and 6) and 11 nM (lane 4 and 7). Cellular lysates were analyzed for Pres-levels after PK-digestion by immunoblot with SAF83. Extracts from untreated cells (C, lane 1) served as a control. Apparent molecular mass based on migration of protein standards is indicated for 17, 25 and 30 kDa.

Cause of reduction of PrP^{res} levels by dendrimers. In this study, the anti-prion activity of the polyamino dendrimers PPI and mPPI was investigated by analysing levels of PrP^{res} in ScN2a cells or scrapie-infected brain homogenates. Previously, it was shown that a reduction of PrP^{res} levels in brain homogenates and purified prions was caused by disaggregation of prion rods and denaturation of PrP^{Sc} [28], providing a possible explanation for reduced levels of PrP^{res} by dendrimers in cell culture. Here, the potential effects of PPI dendrimers on the expression of PrP^{C} or the solubility of PrP^{C} and/or PrP^{Sc} were investigated (Figure 6). Generation 5 of PPI and mPPI were applied to N2a cells at various concentrations and lysates taken after 3 treatment days to assess the effect of the dendrimers on PrP^{C} expression (Figure 6A). Lysates of treated and untreated cells were subsequently analysed by immunoblot. The concentrations used were equal to or higher than those, for which a clear reduction of PrP^{res}-levels in ScN2a cells had been observed in previous experiments. No reduction of PrP^{C} was observed by treatment with either PPI at 0.25 and 1 μg/ml (lanes 2+3) or mPPI at 1 and 10 μg/ml (lanes 4+5) compared to the untreated control (C, lane 1). It can therefore be excluded that a decrease of PrP^{res}-levels in ScN2a cells is caused by a reduction in the expression of PrP^{C} as the substrate for conversion to PrP^{Sc}.

Detection of protease resistant PrP^{res} occurs following a centrifugation step that concentrates the insoluble PrP^{res} prior to detection by immunoblot. The observed decrease in PrP^{res} levels could be due to a dendrimer dependent shift in the solubility of PrP^{Sc}. To exclude this possibility, a detergent solubility assay was performed (Figure 6B). N2a and ScN2a cells were cultivated over a period of 5 days, with a subset of ScN2a cells exposed to 0.5 μg/ml mPPI-g5 for 4 days, followed by preparation of cell extracts. As a control for efficacy of proteolytic digestion, lysates of N2a cells were treated or left undigested with PK. Finally, an N2a-lysate undigested with PK was separated into soluble and insoluble fraction to serve as a control for the detergent-solubility assay. Lysates from treated and untreated ScN2a cells were split into soluble and insoluble fractions either without or following digestion with PK prior to analysis by immunoblot (**Figure 6**, panel B).

Treatment with mPPI-g5 did not result in changed solubility of PrP^{C} or PrP^{Sc}, but reduced the levels of PrP^{Sc} in the insoluble fraction. The controls for PK-digestion (**Figure 6**, panel B: lanes 1 and 2) confirmed that PrP^{C} was completely degraded by the method applied. Additional control-samples

confirmed that soluble and insoluble fractions were completely separated by the detergent-solubility assay. PrP^{C} from N2a cells was solely present in the soluble fraction (lanes 3+4), while some PrP positive signal was detected in the pellet derived from undigested ScN2a cell extracts, indicating the presence of insoluble PrP^{Sc} (lane 6).

Treatment with mPPI reduced the amounts of PrP^{res} accumulating in ScN2a cells, but did not change its solubility. PrP-specific signals in the soluble fraction remained unaffected by treatment with mPPI (lane 5 and 7), whereas a clear reduction of PrP^{Sc} in the insoluble fraction was observed (lane 6 and 8). It is to note, that ScN2a cells contain an N-terminally truncated version of PrP^{Sc} even without treatment with proteinase K. Treatment with proteinase K completely abolished the PrP signal in the soluble fraction for extracts from both mPPI-treated (lane 11) and untreated (lane 9) cells. Treatment with mPPI decreased levels of PrP^{res} (lanes 10 and 12).

In summary, neither the metabolism of PrP^{C} is significantly affected by treatment with mPPI, nor is PrP^{Sc} rendered soluble, eliminating these possibilities as a reason for the observed reduction of PrP^{res}. A shift in the solubility for PrP^{Sc} promoting its presence in the soluble fraction by the treatment can be excluded based on the observation that levels of prion protein in the soluble fraction remained unaffected. Interestingly, treatment of ScN2a cells with mPPI did not only reduce levels of PrP^{res} in the insoluble fraction, but caused an overall reduction in PrP^{Sc}-levels. The observation that amounts of PrP^{res} in brain homogenates are decreased by dendrimers (Figure 5A) suggests that reduced resistance to proteases of PrP^{Sc} results in elimination of PrP^{Sc} within the cell. It is possible however, that dendrimers can act via more than a single mechanism and could impede formation of PrP^{Sc} in cells generating prions.

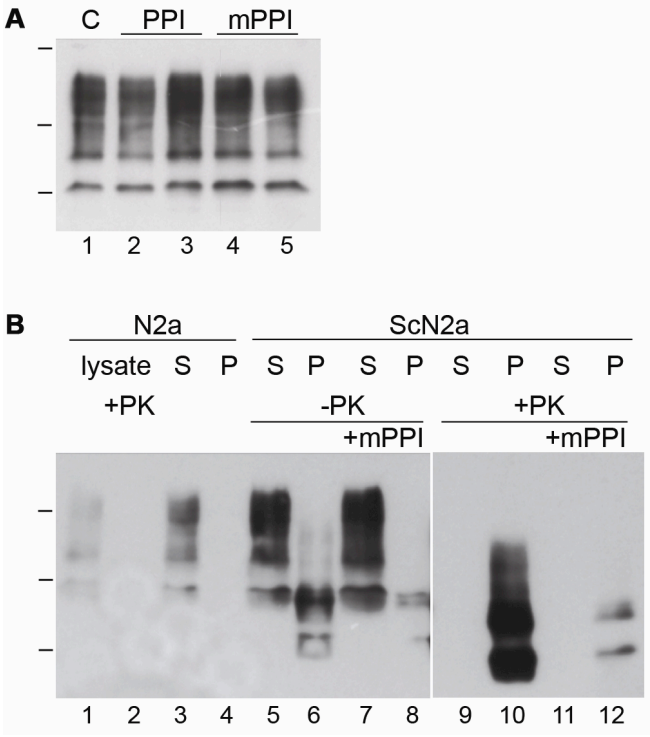

Figure 6. Investigating interference of PPI based dendrimers with the metabolism of the prion protein. **(A)** N2a cells were exposed to high concentrations of generation 5 PPI (0.25 and 1 μg/ml, lanes 2 and 3) and maltose-modified PPI (1 and 10 μg/ml mPPI, lanes 4 and 5) or left untreated (C, lane 1), and PrP^{C} levels were detected in intracellular lysates by immunoblot with SAF83. **(B)** Solubility of PrP^{C} and PrP^{Sc} after treatment with generation 5 mPPI. Distribution of PrP^{C} and PrP^{Sc} in the soluble and insoluble fraction were detected by a solubility assay followed by immunoblot with SAF83 in cellular lysates of N2a (lanes 1-4) and ScN2a cells (lanes 5-12). N2a-lysates were loaded as undigested (lane 1) or PK-digested sample (lane 2) or fractioned into supernatant (S) and pellet (P) (lane 3 and 4). Lysates of ScN2a undigested by PK (lanes 5-8) were fractioned into supernatant and pellet for untreated (lane 5 and 6) and treated sampled (lane 7 and 8). The same set of samples was analyzed after an additional PK-digestion (lanes 9-12). Apparent molecular mass based on migration of protein standards is indicated for 17, 25 and 30 kDa.

DISCUSSION

In this study, we present evidence for the effectiveness of neutrally charged PPI dendrimers on reduction of protease resistant PrP^{Sc} in infected cells and aggregates from infected brain homogenates.

Numerous studies relate the anti-prion activity of polyamino and phosphorous dendrimers to the generation-dependent density of positively charged surface groups[27, 38-39]. Our findings show, that elimination of the positive amino surface groups by substitution with maltose did not generally abolish the compounds anti-prion activity. Generation 4 and 5 mPPI efficiently reduced levels of PrP^{Sc} in ScN2a cells. A direct comparison of mPPI and PPI of generation 5 applied at same molar concentrations revealed that both compounds were equally effective in reducing levels of PrP^{res} with comparable concentration-dependent and time-dependent profiles. This observation may be based on the ability of both compounds to undergo either hydrogen-bonding (mPPI) or electrostatic (PPI) interactions with proteins[49]. The surface-structure of mPPI is predicted to present a "dense-shell dendrimer" with closely organized maltose units[57]. Whilst substitution with only one hydroxyl unit per amino group resulted in abolished activity of a parental PAMAM dendrimer[27] it is possible that the mPPI dendrimers employed in the present study can undergo strong non-electrostatic interactions through the high density of multiple hydroxyl units.

The observed generation-dependent effect of dendrimers' ability to reduce levels of PrP^{res} in cell culture has been reported elsewhere[27, 38-39]. Interestingly, whilst PPI and mPPI of generation 5 were equally competent in decreasing levels of PrP^{res} in ScN2a cells, a difference was observed for the lower generation 3. PPI-g3 was effective in decreasing levels of PrP^{res} in ScN2a cells, whilst mPPI-g3 was not.

For a satisfactory explanation for these observations, the interactions on a molecular level between dendrimers and their target need to be taken into account. Variations in structure and shape of dendrimer are considered to cause generation-dependence. With higher generations the number of surface groups increase exponentially and over-proportionally to the dendrimer's diameter, resulting in a higher density of surface groups.

This explanation is in agreement with findings that hydrogen-bonding interactions of mPPI were found to be generation-dependent with increasing generation numbers toward prion peptide PrP 185-208[49]. Conversely, activity of a polyamino dendrimer was successfully reduced for a linearized version in comparison to its branched counterpart[27]. A polyvalent surface enabling increased binding is an essential feature of dendrimer-interactions and seems of particular importance for relatively weak monovalent binding affinities, such as H-bonding. Carbohydrate-modification (with mannose) of dendrimer surface resulted in a substantial increase of the normally weak interaction between carbohydrates and lectins, and even caused cooperative binding, as the affinity per carbohydrate unit

was increased[58]. The observed discrepancy in anti-prion activity regarding the relative effectiveness between same generations of mPPI and PPI likely originates from their surface and scaffold properties. Lower generations present effectively less dense surface-groups, accounting for the generation-dependence, which differs for parental and modified PPI, with generation 5 being equally effective, but generation 3 mPPI being not and therefore less effective than its parental counterpart. The cationic PPI dendrimer, with its flexible dendritic scaffold, may more efficiently overcome a lower surface-density and undergo stronger interactions than the dense-shell dendrimer mPPI-g3, as its flexibility is reduced by steric inhibitions.

Various amino-terminated dendrimers exhibit a generation-dependent effect in their potential to reduce levels of PrP^{Sc} in cell culture, including generation 2 of PPI and generation 4 of PPI[27]. However, no increased activity was observed between PPI of generation 4 and 5[28]. Generation 4 and 5 PPI are assumed to present dense-shell dendrimers[49] sharing similar characteristics, especially for the glycodendrimers. They display a high surface-density of peripheral groups resulting in reduced flexibility relative to lower generations. These properties may account for the plateau of generation-dependence of PPI, analogously to findings in the present study, that there was no difference between PPI of generation 5 modified with maltose or maltotriose in cell culture experiments.

No data on aggregation-studies with a prion peptide have been reported for maltotriose-modified PPI. However, a variation between effects on prion-peptide aggregation and levels of PrP^{res} in a cell culture system by this compound would not be unusual. Studies employing the same experimental system, reported an inhibitory effect on aggregation of PrP185-208 by generation 3 of mPPI[49], though no effect on PrP^{Sc}-levels in cell culture was observed for this compound in the present study.

An effect by the compound maltose chosen for substitution of amino groups on the surface of PPI-dendrimer other than H-bonding interactions is not likely. A generation dependent decrease of PrP^{res} levels in ScN2a cells was observed for mPPI using equal amounts of PPI dendrimer backbone (thus resulting in about equal numbers of surface groups). Rather, these results support the view that a highly branched architecture is required[27]. In addition, trehalose (a disaccharide of glucose units) caused a decrease in the size of PrP^{Sc} aggregates generated *de novo* in scrapie-infected cells but did not alter levels of protease-resistant PrP^{Sc}[59]. This suggests a different mechanism of action for trehalose and maltose-dendrimers as mPPI rendered PrP^{Sc} susceptible to proteolysis in both brain homogenates and infected cells in this study. In further support of the influence of the dendrimer's structure on its anti-

prion activities are studies showing that cyclic multimers of maltose (*maltose*-cyclodexrins) have a higher potential for reducing levels of PrP^{res} in infected cells compared to their linear counterparts[60]. Both linear and cyclic maltose multimers require elevated levels (500 μM) and prolonged incubation of infected cells (2 weeks) in comparison to maltose in mPPI dendrimers in the present study. This indicates that the effect observed in the present study is not caused by individual units of maltose or maltotriose per se, but rather by their close proximity on the dendrimer's surface and the resulting potential to undergo strong non electrostatic interactions.

In contrast to the data presented in this study for maltose-substituted PPI dendrimers, the anti-prion activity of cyclodextrin did not follow a strict size-dependence. The cyclic maltose heptamer beta cyclodextrin was most efficient in inhibiting replication of PrP^{Sc} in cell culture, compared to cyclodextrins comprising less (six) and more (eight) maltose units[60]. Interestingly, an effect on intracellular PrP^{C} was also observed for the cyclic maltose sextamer.

We have demonstrated that the level of PrP^{C} in N2a cells was not affected by either PPI or mPPI as suggested in previous studies for dendrimers[27, 38-39]. Hence, interference in the expression of PrP^{C}, the template for conversion, does not account for a reduction in PrP^{Sc} by dendrimers.

It has been suggested that polyamino dendrimers render PrP^{Sc} molecules susceptible to proteolysis by acting as chaotropic substances that denature PrP^{Sc}[28]. Detergent-solubility assays employed here demonstrate that treatment with mPPI dendrimer reduced total amounts of PrP^{Sc} in cellular extracts left unexposed to proteolytic digestion. This reduction in PrP^{Sc} levels is likely to be linked to the dendrimer's ability to render PrP^{Sc} susceptible to proteolysis. Destabilizing effects on PrP^{Sc} structure by dendrimers could have profound effects on PrP^{Sc} leading to its enhanced elimination.

Impeding or interfering with the conversion from PrP^{C} to PrP^{Sc} could also be a mechanism of action for dendrimers, and was suggested in a previous study, in which polyamino dendrimers inhibited formation of protease resistant PrP^{Sc} at detectable levels[27]. The established disaggregating properties of dendrimers suggest that destabilization of PrP^{Sc} aggregates is more likely. However, in addition, inhibitory effects during the aggregation-process have been established for dendrimers by *in vitro* studies. Various dendrimers, including compounds with a cationic and neutral surface charge interfered with aggregation of the prion peptide PrP185-208[46-49], and a different prion peptide comprising residues 106-126 (PrP106-126, [50, 61]). The boundaries between a dendrimer's effect on existing and nascent PrP^{Sc}

are not strict, and it remains uncertain, to what extent the denaturing properties of dendrimers influence the conversion process itself. Total amounts of PrP^{Sc} could therefore be reduced by both denaturation of pre-existing PrP^{Sc} and chaotropic effects on formation of nascent PrP^{Sc}.

It remains to be established how decreased resistance to proteolysis results in reduced levels of PrP^{Sc} in ScN2a cells. Enhanced lysosomal degradation of PrP^{Sc} in ScN2a cells has been postulated as the mechanism for clearance of PrP^{Sc} by tyrosine kinase inhibitor STI571[62], and for curing cells from infectious PrP^{res} by branched polyamines in previous studies[27-28]. An acidic compartment, most likely lysosomes was proposed as the site of action for dendrimers as indicated by co-localization-studies and the inhibition of dendrimer-action by alkalinised lysosomes[27-28]. Recently, reductions in levels of PrP^{Sc} and its translocation from late endosomes to lysosomes for PrP^{Sc} by a synthetic fibril-forming peptide has been described[63]. A similar mechanism of action for dendrimers remains to be established.

The therapeutic potential of dendrimers is of significant interest. Clearly, their effectiveness *in vivo* would be a necessary requirement. Phosphorous dendrimers were observed to reduce levels of PrP^{Sc} by more than 80% in the spleen of mice infected with scrapie[38]. Despite an intensive search for therapeutic compounds, their effectiveness is often limited by their restricted access across the blood-brain-barrier (BBB), and no potential candidate has been identified to date that is effective after an infection has established in the CNS[23, 64]. Using an *in vitro* BBB model, polyether-copolyester (PEPE) dendrimers have been shown to cross the BBB. Glycosylation of these dendrimers was found to further increase the permeation[65]. Interestingly, toxicity of uncharged and/or glycosylated dendrimers is lower than cationic dendrimers[38, 53-55]. In agreement with these reports, there was little cytotoxicity observed for PPI glycodendrimers in this study. Toxicity was either abolished or greatly reduced by the maltose modification. The toxicity associated with the most toxic parental dendrimer PPI-g5 used in this study was greatly reduced by full substitution with maltose (mPPI-g5), but also by incomplete substitution by 50% (0.5mPPI-g5). For both compounds, the substitution converts cationic primary amino groups into less charged secondary (0.5mPPI) or tertiary (mPPI) amino groups. Other studies also report low or no toxicity for PPI-dendrimers functionalized with glycine, lactose, phenylalanine, mannose[56] and maltose[49]. The high cationic surface charge of amino dendrimers is considered to bestow good membrane-penetration ability and disruptive properties, leading to their cytotoxicity. Modified non-charged dendrimers appear more suitable therapeutic agents in this respect, as they undergo generally

weaker interaction, for example with negatively charged membranes, than positively charged amino-terminated dendrimers[61].

The results presented in this study show that poly(propylene imine) dendrimers carrying a maltose surface modification show greatly improved toxicity profile, while maintaining their anti-prion activity *in vitro*. These properties make those derivatives promising potential candidates for therapeutics in prion diseases. It will be essential to establish characteristics of glycoamino dendrimers in an *in vivo* model of prion diseases.

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