Guanidino- and Urea-Modified Dendrimers as Potent Solubilizers of Misfolded Prion Protein Aggregates under Non-cytotoxic Conditions. Dependence on Dendrimer Generation and Surface Charge

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Amino-terminated dendrimers are well-defined synthetic hyperbranched polymers and have previously been shown to destabilize aggregates of the misfolded, pathogenic, and partially protease-resistant form of the prion protein (PrP^{Sc}), transforming it into a partially dissociated, protease-sensitive form with strongly reduced infectivity. The mechanism behind this is not known, but a low pH, creating multiple positively charged primary amines on the dendrimer surface, increases the efficiency of the reaction. In the present study, surface amines of the dendrimers were modified to yield either guanidino surface groups (being positively charged at neutral pH) or urea groups (uncharged). The ability of several generations of modified dendrimers and unmodified amino-terminated dendrimers to deplete PrP^{Sc} from persistently PrP^{Sc}-infected cells in culture (SMB cells) was studied. It was found that destabilization correlated with both the generation number of the dendrimer, with higher generations being more efficient, and the charge density of the surface groups. Urea-decorated dendrimers having an uncharged surface were less efficient than positively charged unmodified- (amino) and guanidino-modified dendrimers. The most efficient dendrimers (generation 4 (G4) and G5-unmodified and guanidino dendrimers) cleared PrP^{Sc} completely by incubation for 4 days at less than 50 nM. In contrast to both unmodified and guanidine-modified dendrimers, the uncharged urea dendrimers showed much lower cytotoxicity toward noninfected SMB cells. Therapeutic uses of modified dendrimers are indicated by the low concentrations of dendrimers needed.

Introduction

PrP^{Sc} is the aggregated, protease-resistant, misfolded conformer of the normal prion protein (PrP^{C}) found deposited in neuronal tissue, especially in the brain as a constant feature of all prion diseases (e.g., CJD, BSE, and scrapie). Remarkably, PrP^{Sc} is infectious and pathogenic and, according to the "prion hypothesis" the sole agent responsible for transmission and pathogenesis of the prion diseases.[1]

Although a key feature of PrP^{Sc} is its remarkable stability toward a range of chemical and physical treatments,[2] it does loose infectivity in the presence of high concentrations of protein denaturants like guanidinium and thiocyanate ions.[3] The misfolded prion is also inactivated by SDS at acidic pH.[4] The available evidence suggests that PrP^{Sc} inactivation and dissociation proceed *via* a solubilization of PrP^{Sc}. Therefore, PrP^{Sc} inactivation by such agents may serve as a useful model for protein denaturation and solubilization processes.

A number of compounds, notably peptide β sheet breakers[5] have been shown to reduce PrP^{Sc} infectivity by interfering with the transition of PrP^{C} to PrP^{Sc} and by (partially) unfolding preformed PrP^{Sc}. However, the dendrimers described by Supattapone and co-workers[6,7] are by far the most potent compounds known for the inactivation of pre-existing PrP^{Sc}. Dendrimers of three different types have been shown to have the ability to irreversibly "cure" the PrP^{Sc} expressing cell line ScN2a of PrP^{Sc}.[6,7,9] This was shown to take place without cytotoxicity and at low concentrations of dendrimer. Dendrimers are hyperbranched synthetic polymers characterized by a number of "shells" ("generations") around a central core, and by the chemical entities building up the dendrimer scaffold and the groups localized on the globular surface of the dendrimer.[10,11] In a generation 2 (G2) dendrimer the number of focal points when going from the core to the periphery is 2, and the dendrimer comprises two successive "shells" (Figure 1).

It was previously shown that a G5 polyamido amine (PAMAM) dendrimer (carrying 64 surface primary amino groups) at 1.5 μg/mL (0.1 μM) completely cured ScN2a cells of PrP^{Sc} upon one week of incubation.[7] In addition to dose and incubation time, the process was also shown to depend on the generation number of the dendrimer, higher generations being more efficient than lower generations. Interestingly, the amino-functionalized PAMAM and polypropylene imine (PPI) dendrimers tested were also capable of removing preformed PrP^{Sc} from brain homogenates and purified brain extracts from TSE affected rodents, although much higher concentrations (60 μg/mL (4 μM for a G5 PAMAM dendrimer)) and a pH at or below 4 were needed.[6] At this low pH, the primary surface amines of these dendrimers are positively charged. Certain types (strains) of PrP^{Sc} were clearly more sensitive to dendrimer treatment than others. In contrast, phosphorus dendrimers carrying secondary alkylated amines at their surface had an effect on brain homogenates at neutral pH and furthermore exhibited a broader

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activity with different types of PrP^{Sc} (at 200 μg/mL).[9] *In vivo* activity of the dendrimer was demonstrated by repeated intraperitoneal injection of dendrimer for 30 days substantially inhibiting the development of PrP^{Sc} in the spleen of mice inoculated i.p. with PrP^{Sc} compared to nontreated, PrP^{Sc}-challenged mice.[9]

In all of these studies PrP^{Sc} was mainly defined by its protease resistance, and thus the removal of PrP^{Sc} by the dendrimers simply means that PrP^{Sc} became adequately protease sensitive, or additionally, in the case of cell cultures, the formation of protease-resistant PrP^{Sc} from PrP^{C} could be blocked. As shown by Supattapone and co-workers,[6] dendrimer treatment also resulted in the disappearance of infectivity, another hall-mark of PrP^{Sc}. Finally, recent experiments with synthetic PrP peptides have demonstrated that dendrimers directly destabilise fibril formation, as detected by thioflavin T fluorescence.[12] In this peptide system, a G2 guanidino PPI dendrimer was shown to be more active at neutral pH than unmodified amino terminated G2-PPI.

Supattapone and co-workers proposed that there is a direct interaction between dendrimer and PrP^{Sc} and probably no other cellular or other *in vivo*-derived factors are involved as this ability was unchanged with purified PrP^{Sc} preparations.[6] The interaction was proposed to take place within the segments of PrP delineated by amino acids 89−140 and 222−231, as a deletion mutant PrP^{Sc} lacking the intermediary amino acids was still affected by dendrimers.[8] However, a number of questions remain, including the basic mechanism of the highly efficient actions of dendrimers, and the reason for the subtle differences in susceptibility to dendrimer unfolding of different types of PrP^{Sc}. Here we addressed these questions by investigating the activities of different generations of polypropyleneimine (PPI) dendrimer variants in a sensitive cell culture system. The dendrimer variants included unmodified (amino surface groups), urea-modified, and guanidino-modified. In case of the urea dendrimers, an uncharged but highly polar dendrimer surface is obtained, capable of interacting with the surroundings by strong hydrogen bonding. Both the amino and guanidino dendrimers carry positive surface charges at acidic pH while only guanidino dendrimers are positively charged at the surface at neutral pH.

Experimental Section

Materials and Methods. Synthesis of dendrimer conjugates: All chemicals, including dendrimers, were purchased from Sigma-Aldrich. All solvents were purchased from Labscan and were of HPLC quality. Solvents were dried over molecular sieves (3 Å, Sigma-Aldrich) prior to use. Nuclear magnetic resonance (^{1}H and ^{13}C) measurements were carried out on a Bruker 300 MHz apparatus in d_6-DMSO using TMS as internal standard. Dialysis of dendrimers were performed in dialysis tubes (MWCO: 1 kDa) "Tube-O-lyzer" from Chemicon International.

Synthesis of Dendrimers. *Guanidino-Modified Dendrimers. Typical Procedure. Boc Derivatives*. Dendrimer (50 mg, 0.5 mmol (number of moles dendrimer endgroups)) and *N*,*N*′-bis(*tert*-butoxycarbonyl)-1*H*-pyrrazole-1-carboxamidine (200 mg, 0.65 mmol) were mixed and stirred 48 h in dry NMP (1 mL). Methanol (2 mL) was added, the mixture was transferred to a dialysis tube (MWCO 1 kDa), and the mixture was dialyzed against methanol (3 × 500 mL) for 16 h at 8 °C. After dialysis, the mixture was evaporated to dryness in vacuo. The residue (yellow oil) was dissolved in diethyl ether (3 mL), and the ether was removed carefully in vacuo, giving the product as off-white crystals.

Free Guanidine Dendrimer Hydrochlorides. The Boc-protected guanidine dendrimer (100 mg) was dissolved in 4 M HCl in dioxane (1 mL), resulting in slow gas evolution. The mixture was stirred overnight at room temperature, and diethyl ether (8 mL) was added causing the dendrimer to precipitate. The precipitate was washed with diethyl ether and centrifuged followed by decantation, which gave the product as a white powder.

Urea-Modified Dendrimers. Typical Procedure. Trityl Derivatives (procedure 1). Dendrimer (50 mg, approx 0.5 mmol (number of moles dendrimer endgroups)) and triphenylmethyl isocyanate (177 mg, 0.62 mmol) were dissolved in dry NMP (2 mL). The mixture was stirred overnight at r.t. and added dropwise to a stirred solution of diethyl ether (5 mL), causing precipitation of product. The suspension was centrifuged and the supernatant removed twice. Air-drying gave the desired product as a white powder.

Urea-Modified Dendrimers. Typical Procedure. Trityl Derivatives (procedure 2). Di-*tert*-butyl pyrocarbonate (0.50 g, 2.0 mmol) and DMAP (25 mg, 0.2 mmol) were dissolved in dry dichloromethane (2 mL). Tritylamine (0.500 g, 1.9 mmol) in dry dichloromethane (2 mL) was added at r.t., leading to slow evolution of carbon dioxide. The mixture was stirred for 2 h at r.t. The solvent was removed in vacuo, and a portion of the isocyanate was used directly for further synthesis. Dendrimer (50 mg, approx 0.5 mmol ($n_{endgroups}$)) and triphenylmethyl isocyanate (177 mg, 0.62 mmol) were dissolved in dry NMP (2 mL). The mixture was stirred overnight at r.t. (precipitation) and added dropwise to a stirred solution of diethyl ether (5 mL), causing further precipitation of product. The suspension was centrifuged and the supernatant removed twice. Air-drying gave the desired product as a white powder.Because of poor solubility of the tritylated urea dendrimers, these derivatives could not be analyzed by NMR and were therefore applied directly for further synthesis.

Free Dendrimer Urea Derivatives. Trityl-protected urea dendrimer (100 mg, approximately 0.252 mmol, endgroup concn) was suspended in methanol (0.40 mL), and a solution of 4 M HCl in dioxane (0.313 mL, 1.25 mmol) was added followed by addition of triisopropylsilane (20 μL). The mixture was stirred 16 h at r.t., slowly creating a clear solution. The reaction mixture was filtered and poured into diethyl ether (5 mL), causing precipitation of the product as a sticky solid. The solid was taken up into diethyl ether (5 mL) and stirred vigorously overnight, giving the product as a white powder. Freeze-drying removed residual solvent.

Culturing of Cells. SMB cell lines were purchased from the TSE Resource Centre, England. SMB.s15 cells are infected with Chandler scrapie (isolated from an infected mouse brain and cloned),[13,14] and SMB-PS are SMB.s15 cells cured for scrapie by pentosan polysulfate. Both cell lines were grown in T-80 cell culture flasks (Nunclon, NUNC, Denmark) in Medium 199 with Earle's salts and Glutamax, supplemented with 10% newborn calf serum, 5% fetal calf serum, 1% penicillin (complete medium) (Gibco, InVitrogen, Denmark) at 5% CO_2 and 37 °C in a humidified atmosphere. The cells were detached from the flasks using trypsin and split 1:2 biweekly.

Effect of Dendrimers on SMB-PS Cell Viability (toxicity). As a measure of toxicity, viability of dendrimer-treated SMB-PS cells was evaluated. SMB-PS cells in complete medium were added to each well (5000 cells per well) of a 96-well microtiter plate and were left to attach for 2 h at 5% CO_2 and 37 °C in a humidified atmosphere. Dendrimer stock solutions (1 mg/mL) in water were filter sterilized using a 0.22 μm filter (Millex-GV, Millipore, Denmark) and were diluted in complete medium before addition to the cells. Plates were incubated at 5% CO_2 and 37 °C in a humidified atmosphere for 6 days before cell viability was measured by the WST-1 assay, according to the manufacturer's instructions (Cell Proliferation Reagent, WST-1, Roche, Denmark). Viable cells cleave the WST-1 substrate to a water-soluble formazan product, which was measured at 450 nm with an ELISA reader.

Treatment of Prion-Infected SMB.s15 cells with Dendrimers and Preparation of Lysates. SMB.s15 cells were grown in T-80 cell flasks in complete medium as described above. Dendrimers at varying concentrations were added to the cells, and the cells were incubated at 5% CO_2 and 37 °C in a humidified atmosphere. Cells were split

biweekly into new flasks, and fresh medium and dendrimers were added. After a preset incubation period, the cells were lysed and PrP^{Sc} was concentrated according to a previously described procedure.[15] Briefly, cells were washed with sterile PBS, pH 7.2, and ice-cold lysis buffer (PBS, pH 7.2 containing 0,5% Nonidet P-40, 0.5% sodium deoxycholate, 1 μg/mL pepstatin, 1 μg/mL leupeptin and 2 mM EDTA) (all chemicals from Sigma-Aldrich, Denmark), was added. After incubation at 4 °C for a few minutes, the cell lysate was transferred to a test tube and frozen at −20 °C until further use. For protein determination, cell lysates were thawed and analyzed by the BCA protein assay kit, according to the manufacturer's instructions (BCA-1, Sigma-Aldrich). Cell lysates were adjusted to a protein concentration of 0.6 mg/mL with lysis buffer before analysis.

Western Blotting (WB). WB was done as described in the Prionics Check Western (PCW, Prionics, Switzerland) manual with some modifications. Briefly, 0.6 mg/mL SMB.s15 cell lysates were treated with proteinase K (PK) or mixed directly with SDS-PAGE sample buffer and kept at −20 °C. For PK treatment, PK was incubated with the lysate at 16 μg PK (Sigma-Aldrich) per mg protein for 30 min at 37 °C, and the reaction was stopped with 10 μM PMSF (Sigma-Aldrich). The samples were concentrated by centrifugation for 45 min at 20 000 g at 4 °C before removal of the supernatant, resuspending the pellet with equal amounts of PBS, pH 7.4, and SDS-PAGE sample buffer containing β-mercaptoethanol (chemicals from Sigma-Aldrich).[15] PK- and non-PK-treated samples were run on 12% NuPAGE gels (InVitrogen) and blotted onto PVDF membranes (Hybond-P, Amersham-Pharmacia Biotech, Denmark). The membranes were incubated with the monoclonal antibody 6H4 (from PCW kit, Prionics) diluted 1:5000 in TBST (0.1% Tween 20, 100 mM NaCl, 10 mM Tris-HCl, pH 7.8).

Alkaline phosphatase-conjugated anti-mouse Ig (Tropix) was used for detection and visualized by chemiluminescence (ECL AP Biotech, Denmark). Magic Mark western blot marker (Invitrogen) was used as a molecular weight marker.

Results and Discussion

Synthesis of Dendrimer Derivatives. To elucidate the dependence of dendrimer surface charge on the unfolding ability and the cytotoxicity, guanidine, and urea derivatives of G1-G5 PPI dendrimers were synthesized. Guanidylation of the dendrimer surface primary amines was performed either by di-Boc-*S*-methyl isothiourea or *N*,*N*′-bis(*tert*-butoxycarbonyl)-1*H*-pyrrazole-1-carboxamidine, the latter being the most potent guanidylation reagent at room temperature. The Boc-protected guanidine PPI dendrimers were isolated in good yields, applying dialysis against organic solvents to remove low molecular impurities. Cleavage of the Boc groups was carried out by HCl in dioxane, yielding the guanidine-modified dendrimers as their hydrochloride salts (Figure 2). This deprotection method was preferable to the common method applying 95% aqueous TFA which may otherwise yield the cytotoxic trifluoroacetate salts of the dendrimers. The urea dendrimers were synthesized via their trityl-protected derivatives by a reaction between trityl isocyanate and the dendrimer primary amines. The intermediate isocyanate could also be synthesized *in situ* by reaction with Boc-anhydride in the presence of dimethylaminopyridine (DMAP). The trityl-protected urea dendrimer derivatives were deprotected by HCl in dioxane in the presence of triisopropylsilane as a triphenylmethane scavenger, giving the urea derivatized dendrimer derivatives as hydrochloric acid salts (Figure 2). Both methods afforded the urea-modified dendrimers in good yields.

The dendrimers were hereafter tested for cytotoxicity and PrP^{Sc} clearing ability with the pentosan sulfate-cured SMB cell line (cytotoxicity) and the PrP^{Sc}-expressing SMB.s15 cells,

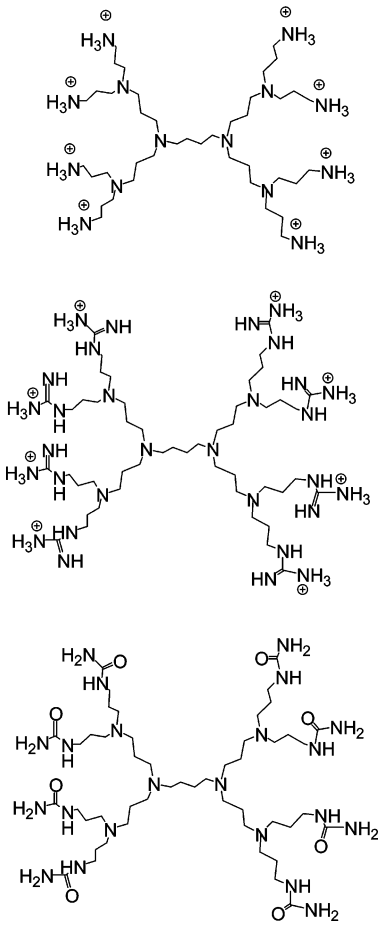

Figure 1. The types of dendrimers used in this study. Top: Unmodified amino-terminated G2-PPI. Middle: Guanidino-modified G2-PPI. Bottom: Urea-modified G2-PPI.

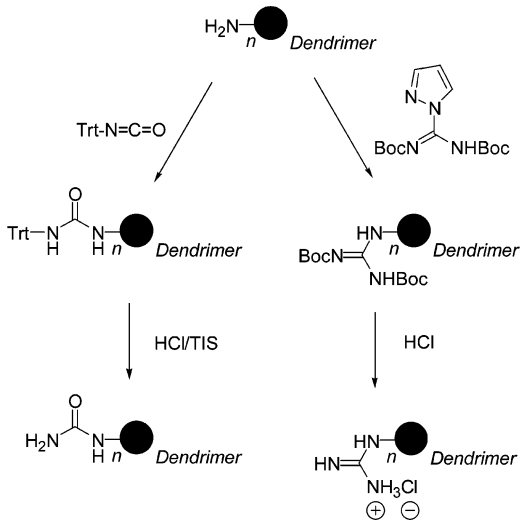

Figure 2. Synthesis of urea- and guanidino-modified PPI dendrimers.

respectively, and correlated with dose, number of generations, type of surface groups, and time of incubation.

In Vitro Cytotoxicity of Dendrimers in SMB-PS Cell Culture. All dendrimers G1−G5 were tested for cytotoxicity by measuring the effect of the compounds on viability of SMB-PS cells (Figure 3). The SMB-PS cell line is SMB.s15 cells cured of PrP^{Sc} and presented the possibility of measuring the direct effect on cell viability, avoiding potential growth-promoting effects of dendrimers interfering with prions (the scrapie-infected cells grow more slowly than the cured cells). In pilot experiments it

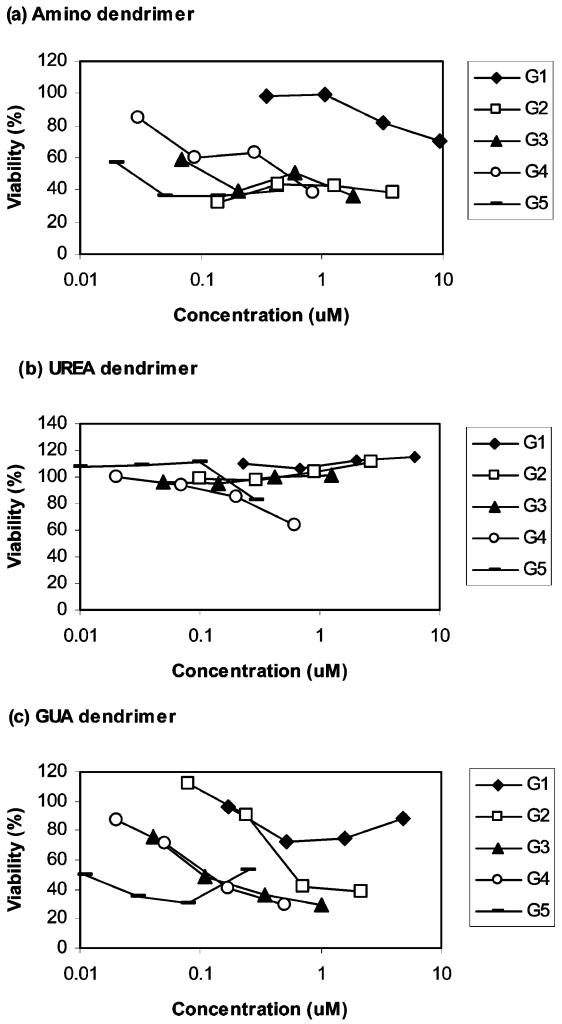

Figure 3. Viability of SMB-PS cells after incubation with dendrimers. Cells were grown in 96-well microtiter plates and incubated for 6 days with varying concentrations (0, 0.11, 0.33, 1.00, 3.00 μg/mL) of compounds: (a) amino dendrimers G1−G5 (0.02−9.49 μM), (b) urea dendrimers G1−G5 (0.01−6.15 μM), or (c) guanidino dendrimers G1−G5 (0.01−4.76 μM), before cell viability was measured by Cell Proliferation Reagent, WST-1 (Roche). The viability is given as percentage of the viability of untreated controls. The experiment is given as the average of two independent measurements and is a representative of at least four independent experiments.

was observed that the G5 dendrimers had pronounced cytotoxic effects at 9 μg/mL (data not shown), and in the following cytotoxicity experiments with SMB-PS cells, lower concentration ranges (3−0.1 μg/mL $\sim$ 9.49−0.01 μM) were used. Cytotoxicity experiments were performed by incubating SMB-PS cells for different time periods (3−8 days) and measuring cell viability. We found that at 6 days of treatment, the effect on cell viability peaked and data from this day is shown in Figure 3. As can be seen from the figure, guanidino dendrimers showed marked dose-dependent cytotoxic effects, as well as a clear generation effect, with generation 5 being more cytotoxic than generation 1 (Figure 3c). Unmodified dendrimers seemed to be more cytotoxic, since they were toxic to cells at all concentrations tested, except for G1 at the lowest concentrations (Figure 3a). Urea dendrimers showed very little cytotoxicity or in some cases even improved cell viability (within the experimental error) with all generations and at all concentrations tested. However, some cytotoxicity was observed with G4 and G5 urea dendrimers at 3 μg/mL, corresponding to molar concentrations of 0.61 and 0.30 μM, respectively (Figure 3b).

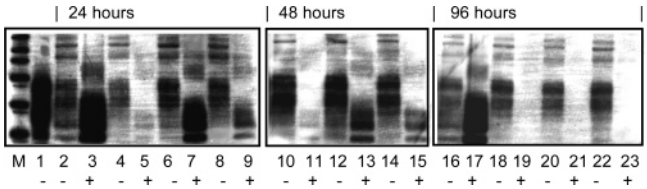

Figure 4. Kinetics of dendrimer action. Western blotting of SMB.s15 cell lysates. Cells were treated for 24 (lanes 2−9), 48 (lanes 10−15), or 96 h (lanes 16−23) with 1 μg/mL amino dendrimer G5 (0.14 μM, lanes 4, 5, 10, 11, 18, 19), urea dendrimer G5 (0.1 μM, lanes 6, 7, 12, 13, 20, 21), or guanidino dendrimer G5 (0.08 μM, lanes 8, 9, 14, 15, 22, 23). Samples in lanes 2, 3 and 16, 17 are untreated controls. Cell lysates were treated (+) or not treated (−) with proteinase K before WB. M indicates molecular weight marker representing molecular weights of 80, 60, 50, 40, 30, and 20 kDa (top to bottom).

Modified G5 Dendrimers Can Eliminate Pre-existing PrP^{Sc} from SMB.s15 Cells, and the Effect Is Dependent on the Surface Modification of the Dendrimer. Initially, the effect of the unmodified G5 and the two modified G5 dendrimers on PrP^{Sc} in SMB.s15 cells was tested, treating the cells over several passages. It was found that PrP^{Sc} was eliminated after a few passages as tested by WB of cell lysates (data not shown). This indicated that the dendrimers could block the cellular formation of new PrP^{Sc}. The next step was to test the effect of the G5 dendrimers on cells that were not split in order to evaluate the effect on existing PrP^{Sc}. SMB.s15 cells were incubated for 24, 48, and 96 h with 1 μg/mL of unmodified and modified G5 dendrimers (0.14 μM (amino); 0.10 μM (urea); 0.08 μM (guanidino)), and the PrP^{Sc} content was analyzed by WB. Unmodified G5 dendrimer had a pronounced effect on the PrP^{Sc} content compared to nontreated control cells (Figure 4, lane 3) already after 24 h, with only very weak PrP^{Sc} bands left after 48 h of treatment, and completely eliminated after 96 h of incubation (Figure 4, lanes 5,11,19). The effect of guanidino G5 dendrimer was not as evident as that of the unmodified dendrimer, but there was a marked difference between the effect of guanidino G5 dendrimer compared to untreated control cells at 24 and 48 h, with complete elimination of the PrP^{Sc} after 96 h of treatment (Figure 4, lanes 9, 15, 23). The effect of the G5 urea dendrimer at 24 h was small but clearly visible after 48 h, with complete elimination of PrP^{Sc} after 96 h (Figure 4, lanes 7, 13, 21). It should be taken into account, however, that the molecular weights of the modified dendrimers are almost twice that of the unmodified dendrimers, thus resulting in a molar concentration being approximately half of the unmodified dendrimer, e.g., MW of G5 guanidino and urea dendrimers are 12193 and 9922, respectively, compared to 7168 for the G5-unmodified dendrimer resulting in a 1 μg/mL concentration of 0.08 μM (guanidino dendrimer), 0.1 μM (urea dendrimer), and finally 0.14 μM of the unmodified dendrimer. Interestingly, the amount of protease-sensitive PrP was virtually unchanged over time and identical for the three different treatments, indicating that the treatments did not affect the amount of PrP^{C} to total cellular protein. We have observed this in preliminary experiments as well, and it seems to be a constant feature. For this reason, protease-sensitive PrP is not shown in the following experiments.

Elimination of PrP^{Sc} from SMB.s15 Cells with Modified Dendrimers Is Dose-Dependent. As shown above, the effect of the G5 dendrimers on PrP^{Sc} content in SMB.s15 cells was dependent on the surface modification of the dendrimers with guanidino and unmodified dendrimers working faster than urea dendrimers (Figure 4). In order to evaluate whether the PrP^{Sc} clearing effect was concentration dependent, we treated SMB.s15

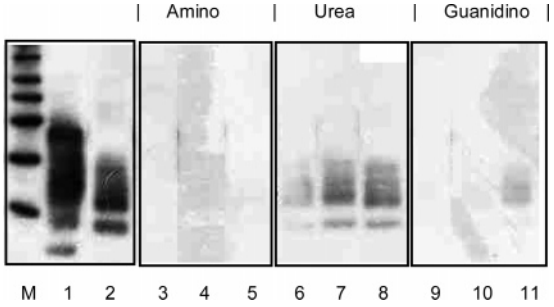

Figure 5. Titration of dendrimers. Western blotting of SMB.s15 cell lysates. Cells were treated for 96 h with amino dendrimer G5 (lanes 3−5), urea dendrimer G5 (lanes 6−8), or guanidino dendrimer G5 (lanes 9−11) at the following concentrations: 1 μg/mL amino- (0.14 μM, lane 3), urea- (0.10 μM, lane 6), or guanidino-dendrimer (0.08 μM, lane 9); 0.5 μg/mL amino- (0.07 μM, lane 4), urea- (0.05 μM, lane 7), or guanidino-dendrimer (0.04 μM, lane 10); 0.25 μg/mL amino- (0.03 μM, lane 5), urea- (0.03 μM, lane 8), or guanidino-dendrimer (0.02 μM, lane 11). Lane 1 represents a positive control from the WB kit. Sample in lane 2 is a lysate of untreated control cells. All cell lysates were treated with proteinase K, except for lanes 1 and 2. M indicates molecular weight marker representing molecular weights of 80, 60, 50, 40, 30, and 20 kDa (top to bottom).

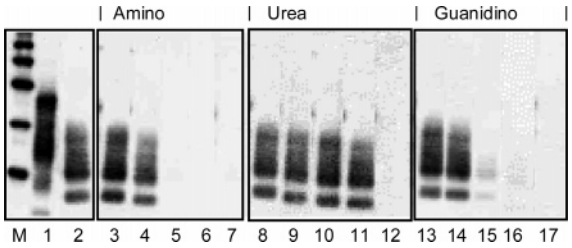

Figure 6. Effect of dendrimer generation. Western blotting of SMB.s15 cell lysates. Cells were treated for 96 h with 0.5 μg/mL of amino dendrimers (1.58, 0.65, 0.30, 0.14, 0.07 μM for G1−G5 dendrimers respectively in lanes 3−7), urea dendrimers (1.02, 0.45, 0.21, 0.10, 0.05 μM for G1−G5 dendrimers respectively in lanes 8−12), or guanidino dendrimers (0.79, 0.36, 0.17, 0.08, 0.04 μM for G1−G5 dendrimers respectively in lanes 13−17) with increasing generation number: G1 (lanes 3, 8, 13), G2 (lanes 4, 9, 14), G3 (lanes 5, 10, 15), G4 (lanes 6, 11, 16), or G5 (lanes 7, 12, 17). In lane 2 is a lysate of untreated control cells. In lane 1 is a positive control from the WB kit. All cell lysates were treated with proteinase K, except for lanes 1 and 2. M indicates molecular weight marker representing molecular weights of 80, 60, 50, 40, 30, and 20 kDa (top to bottom).

cells for 96 h with varying concentrations (1, 0.5, and 0.25 μg/mL; for molar concentrations see legend to Figure 5) of the G5 dendrimers and tested the content of PrP^{Sc} in the cell lysates by WB (Figure 5). Unmodified G5 dendrimer completely eliminated PrP^{Sc} at 1, 0.5, and 0.25 μg/mL (Figure 5, lanes 3, 4, 5). With the modified dendrimers, concentration effects can be seen. The guanidino-modified dendrimer completely cleared the cells at 1 and 0.50 μg/mL, but not at 0.25 μg/mL (Figure 5, lanes 9, 10, 11), whereas visible bands can be seen at all three concentrations tested with the urea-modified dendrimer (Figure 5, lanes 6, 7, 8). The guanidino dendrimer is clearly more efficient than the urea dendrimer and has an effect on protease-resistant PrP that is comparable to the unmodified dendrimer at similar molar concentrations.

Elimination of PrP^{Sc} from SMB.s15 Cells with Dendrimers Is Dependent on the Generation Number. Surface-modified dendrimers (G1 to G5) were tested at 0.5 μg/mL (for molar concentrations, see legend to Figure 6) for their efficacy in eliminating PrP^{Sc} from SMB.s15 cells after 96 h of treatment. For both unmodified dendrimers and surface-modified dendrimers, a clear generation effect was observed (Figure 6). For the unmodified dendrimers G5, G4 and G3 were able to completely eliminate PrP^{Sc} (Figure 6, lanes 5, 6, 7), whereas G2 had a weak effect and G1 had no effect on the PrP^{Sc} (Figure 6, lanes 3,4) content compared to the untreated control (Figure 6, lane 2). Guanidino-modified dendrimers were almost as effective as the unmodified dendrimers in eliminating PrP^{Sc} with complete elimination at G5 and G4, very weak bands at G3 and no effect of G2 and G1 at the concentrations tested (Figure 5 lanes 13−17). Urea dendrimers, on the other hand were not as effective, with only G5 dendrimer having an effect (Figure 6 lanes 8−12).

In the present study the ability of surface-modified dendrimers to eradicate protease-resistant PrP^{Sc} from scrapie-infected cells was studied. Three different surface functionalities were investigated; first, unmodified dendrimers having an amine-functionalized surface, creating a positively charged surface at low pH. Second, the guanidine-surfaced dendrimers, capable of maintaining a positive charge over a broad pH range, which should render these dendrimers active (i.e., positively charged) regardless of the pH conditions. Third, the urea dendrimers with an uncharged but highly polar surface can interact with the surroundings by hydrogen bonding.

We found that the potency of the dendrimers to remove PrP^{Sc} from chronically infected SMB.s15 cells correlated with their surface-modification, the guanidino and amino-surfaced dendrimers being more potent than urea-modified dendrimers at physiological pH. Furthermore the potency correlated with the dendrimer generation number, the higher generation dendrimers being the most potent. Generation 3, 4, and 5 amino and guanidino dendrimers were highly efficient after 4 days of treatment, whereas only generation 5 of the urea-modified compounds was able to remove PrP^{Sc}. In addition, it should be taken into account that the molecular weight of the modified dendrimers is approximately two times that of the unmodified dendrimers. Comparison of molar amounts of the compounds will result in relatively higher potency of the gunidinio- and urea-surfaced dendrimers.

Several factors may influence the lower potency of the urea-modified dendrimers to clear cells of PrP^{Sc}. First, the noncharged surface of these dendrimers may interact more weakly with PrP^{sc} than the guanidino- and amino-surfaced dendrimers in which long distance Coulomb interactions may play a key role by the direct interaction with PrP^{Sc}. Furthermore, high cationic charge density is important for the attraction, adherence, and interaction with the negatively charged cell membrane that is a prerequisite for the uptake of the dendrimers into the cells. Therefore, in a cellular system, a dendrimer with a noncharged urea surface may show reduced activity in comparison to its positively charged counterparts. The noncharged surface may as well account for the nontoxicity properties of the urea dendrimers, as compared to the positively charged guanidino and amino-surfaced dendrimers, which were clearly more cytotoxic. These findings agree well with the general finding that by minimizing the cationic charge density on the surface of dendrimers or other polymers cytotoxicity is greatly reduced.[16] The observed cytotoxicity of the positively charged dendrimers may be due to disruption of the cell membranes by interaction with the negatively charged groups and hydrophobic parts ('tenside effect').[16,17]

It has been suggested that the PrP^{Sc} eliminating action of dendrimers can be localized to acidic compartments of the cells.[7] At low pH the guanidino- and amino-surfaced dendrimers are positively charged, whereas the urea dendrimers have a neutral surface also at low pH. This may also contribute to observed lower potency for elimination of PrP^{Sc} of the urea dendrimers.

We found that the effect on cell viability peaked at 6 days of treatment. This could be interpreted to mean that, after day 6, cells became adapted to the dendrimers, as suggested by Fuchs and co-workers.[17]

An interesting observation is the effect of the dendrimers on pre-existing PrP^{Sc}, observed by analyzing the effect on nonsplit cells, indicating that dendrimers act directly on prion aggregates. This was also observed by Supattapone et al.,[7] who suggested that dendrimers interact with prion fibrils, resulting in unfolding of PrP^{Sc}. In addition to the studies on dendrimer effects on prion peptide 106−126 fibrillation by our group,[12] Klajnert and co-workers[18] investigated the effect of PAMAM dendrimers on the formation of prion peptide 185−208 amyloid fibrils. They found a correlation between generation number of the dendrimers and the degree of inhibition of fibril formation. The authors concluded that interference with amyloid formation might occur by a combination of two mechanisms: blocking of fibril growth and breaking of existing fibrils. It is plausible that unfolding of PrP^{Sc} and breakage of fibrils by dendrimers would make the prion protein more vulnerable to proteases in the endosome, causing elimination of PrP^{Sc} from the cells.

It could be interesting to investigate whether an extended exposure to non-cytotoxic concentrations of these dendrimers could completely cure the cells of PrP^{Sc}, without affecting the viability of the cells. Dendrimers that have PrP^{Sc}-removing effects at non-cytotoxic concentrations upon long incubation might be interesting from a medical point of view as drugs for postexposure prophylaxis of transmitted prion diseases and even, provided favorable blood−brain barrier characteristics can be obtained, for treatment of prion diseases.

Conclusions

Two sets (generations 1−5) of new dendrimers have been synthesized having guanidine- and urea-functionalized surfaces, respectively. The guanidino-functionalized dendrimers have a positively charged surface over a large pH range whereas the urea-modified dendrimers have no charge at their surfaces. The PrP^{Sc}-solubilizing effect of the new sets of dendrimers was investigated on a SMB cellular system infected with the prion disease Chandler scrapie and compared to unmodified amino-surfaced PPI-dendrimers. The new dendrimers and the unmodified dendrimers had comparable effect in clearing the cells from aggregates of the misfolded isoform of PrP^{C}. Both unmodified and modified dendrimers showed a generation dependent effect in solubilizing the prion aggregates, the high generation dendrimers being the most efficient. The same generation dependence was observed in the cytotoxcity of the dendrimers, the high generation dendrimers being the most toxic. In contrast, the urea dendrimers had a very low cytotoxicity compared to both unmodified and guanidine-modified dendrimers, although still being effective in clearing the prion aggregates. These beneficial properties make these adducts interesting as potential therapeutics, and further investigation of these derivatives is currently under way.

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Supporting Information Available. Yields and analytical data on the modified dendrimers. This information is available free of charge via the Internet at http://pubs.acs.org.

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