The inhibition of prions through blocking prion conversion by permanently charged branched polyamines of low cytotoxicity

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**Abstract**

Branched polyamines are effective in inhibiting prions in a cationic surface charge density dependent manner. However, toxicity associated with branched polyamines, in general, often hampers the successful application of the compounds to treat prion diseases. Here, we report that constitutively maintained cationic properties in branched polyamines reduced the intrinsic toxicity of the compounds while retaining the anti-prion activities. In prion-infected neuroblastoma cells, quaternization of amines in polyethyleneimine (PEI) and polyamidoamine (PAMAM) dendrimers markedly increased the nontoxic concentration ranges of the compounds and still supported, albeit reduced, an appreciable level of anti-prion activity in clearing prions from the infected cells. Furthermore, quaternized PEI was able to degrade prions at acidic pH conditions and inhibit the in vitro prion propagation facilitated by conversion of the normal prion protein isoform to its misfolded counterpart, although such activities were decreased by quaternization. Quaternized PAMAM was least effective in degrading prions but efficiently inhibited prion conversion with the same efficacy as unmodified PAMAM. Our results suggest that quaternization represents an effective strategy for developing nontoxic branched polyamines with potent anti-prion activity. This study highlights the importance of polyamine structural control for developing polyamine-based anti-prion agents and understanding of an action mechanism of quaternized branched polyamines.

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1. Introduction

Prion diseases are fatal neurological disorders in humans and animals caused by infectious, proteinaceous pathogens termed prions [1]. Progressive neurodegenerative conditions such as spongiform degeneration, gliosis, and accumulation of prions in the brain accompany the disease progression [1]. Although prion diseases pose a potential risk for public health [2], therapy for these diseases is not available.

Prions are composed of misfolded prion proteins (PrP) which are designated scrapie-associated PrP (PrPSc) [1, 3]. Prion replication appears to be facilitated by conformational conversion of cellular PrP (PrPc) to PrPSc [4]. Although the covalent structure of PrPc and PrPSc is identical, these PrP isoforms exhibiting different conformational states are distinguished by their characteristic biochemical properties. PrPc is sensitive to hydrolysis by proteases, soluble in non-denaturing detergents, and rich in α helices, whereas PrPSc is partially resistant to proteolysis, insoluble in such detergents, and rich in β-sheets [1]. Proteinase K (PK)-resistant PrPSc referred to as PrP27-30 tends to form amyloid fibrils under proper detergent conditions and serves as a surrogate marker for prions [1].

Many protein-denaturing agents can increase solubility of PrPSc and decrease resistance to proteolysis [5]. Among numerous chemicals exhibiting anti-prion activity, branched polyamines are reported to exert their effect through degrading PrPSc [6, 7]. Supattapone et al. discovered that treatment of a prion-infected neuroblastoma cell line (ScN2a) with the SuperFect transfection reagent (Qiagen, Valencia, CA), a mixture of branched polyamines derived from polyamidoamine (PAMAM) dendrimers, resulted in clearance of pre-existing PrPSc [6]. Furthermore, intact PAMAM, polypropyleneimine, and other polyamines such as linear polyethyleneimine (PEI) showed similar activities [6]. The branched polyamine-treated cells were completely cured [6] and not infectious in mice [7]. A structure–activity relationship study using branched polyamines of various generations revealed that anti-prion efficacy was proportional to the charge density increased by molecular masses and numbers of amine groups at the surface of the compounds [6]. Branched polyamines favorably degraded PrPSc...
under acidic conditions [6,7]. PrPSc became PK-sensitive after incubation with branched polyamines at pH 4 or less but remained unaltered if incubated at neutral and basic pH. Also, PrP27-30 aggregates were dissociated and lost β-sheet conformations by branched polyamines in similar acidic conditions. Thus, together with the evidence that branched polyamines localized at the endosomes and lysosomes of the cells [6], these compounds are considered to be useful in the acidic subcellular compartments in which prion conversion takes places and PrPSc accumulates. In support of these studies, cationic dendrimers modified to contain phosphorus, guanidine, or urea groups reduced the level of prions in the infected cells and animals, improved toxicity associated with parental compounds, and increased bioavailability [8,9].

Recently, polyamine dendrimers were shown to interfere with fibril formation of PrP peptides [10–12]. An inhibitory effect of branched polyamines on formation of PrP peptide fibrils was dependent on the surface charge density and structure of dendrimers [10–14] as well as the acidic pH conditions [15]. Phosphorus- and maltose-modified dendrimers also exhibited similar inhibition on PrP peptide fibril assembly [16,17].

Because branched polyamines contain a large number of secondary and/or tertiary amines that exhibit pKₐ values between physiological and lysosomal pH [18,19], these compounds become more positively charged in acidic pH due to increased protonation of amine groups. Thus, it is possible that anti-prion activity of branched polyamines obtained at acidic pH can be realized at neutral pH if such reversible, positive charges of the compounds are modified to be permanent. We hypothesized that an irreversible increase of charge density in cationic branched polyamines by quaternization would secure pH-independent enhancement in anti-prion activity.

Additionally, although branched polyamines possess promising potentials as anti-prion agents, their cytotoxicity profiles still need to be improved because polycationic polyamines generally display substantial cytotoxicity. Previously, several reports have shown that the quaternization of amines can be a strategy to decrease the cytotoxicity of polyamines [20–22]. Based on this finding, we questioned whether the quaternization could reduce the cytotoxicity of branched polyamines while maintaining their anti-prion activity.

In this study, we synthesized quaternized (q) PAMAM G4.0 and branched PEI 25 kDa of varying degrees of quaternization and examined their cytotoxicity and anti-prion activity by performing several assays such as the clearance of PrPSc from prion-infected neuroblastoma cells, the degradation of PrPSc in vitro, and the inhibition of in vitro prion conversion.

2. Materials and methods

Hazardous chemicals and mouse-adapted prion materials from cells and animals were handled according to the protocols approved by the Biological Safety Office and the Institutional Animal Care and Use Committee at the University of Kentucky. All experiments using prions were performed in biosafety level-2 facility.

2.1. Synthesis of qPAMAM G4.0 and qPEI

qPAMAM was synthesized as described previously with slight modification [21]. Briefly, the solvent (methanol) was removed from manufacturer’s PAMAM G4.0 (Sigma–Aldrich, St. Louis, MO) solution by a rotary evaporator prior to reaction. The dried PAMAM G4.0 (40 mg) was redissolved in 0.4 ml dimethylformamide and 0.5 ml (110 times molar excess over the nitrogen content in PAMAM G4.0) of methyl iodide (Sigma–Aldrich) was slowly added. The reaction continued with stirring for 15 h at 37°C. qPAMAM G4.0 was precipitated from the reaction mixture by adding an excess of tetrahydrofuran (Sigma–Aldrich). For further purification, the precipitate was redissolved in water/methanol and reprecipitated with tetrahydrofuran (3×). From the 1H NMR spectra, the degree of quaternization was determined by dividing the experimental proton numbers of the N–methyl groups (3.16 ppm) with the total proton numbers.

Branch PEI (average molecular weight of 25 kDa) was purchased from Sigma–Aldrich. PEIs with various degrees of quaternization were synthesized as described above. After the completion of reaction, qPEI precipitated from the reaction mixture by adding a large excess of acetonitrile (Sigma–Aldrich). For further purification, the precipitate was redissolved in water and reprecipitated with acetonitrile (3×). The degree of quaternization was calculated by dividing the experimental proton numbers of N–methylene groups (3.11 ppm) with the total proton numbers.

2.2. Cell culture and treatments with compounds

ScN2a (scrapie-infected N2a) [23] neuroblastoma cells were cultured as described previously [24]. Cells were maintained in Dulbecco’s Modified Eagle’s Medium (high glucose; Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum, 1% penicillin–streptomycin, 1% glutamax with 5% CO₂ at 37°C. For treatments, ScN2a cells were plated to about 2–3×10⁶ confluency. Treatment of ScN2a cells with PAMAM G4.0, PEI, and their quaternized compounds was carried out as described previously [6]. These compounds were dissolved in phosphate buffered saline (PBS) and stored at 4°C during the treatment. Treatment lasted for six days with various concentrations of the compounds added directly to culture media. Culture media including the compounds were changed every three days.

2.3. Cytotoxicity assay

Cytotoxicity of quaternized polyamines was measured by the MTT [3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay described previously [25]. After treatment with the compounds, the cells were further incubated in the fresh media with final concentration of 0.5 mg/ml MTT for 1.5 h. The insoluble precipitate was redissolved by adding phenylmethylsulfonylfluoride to the final concentration of 2 μl, the lysate was centrifuged at 16,000 g for 1 h, PK-resistant pellet representing PrPSc was subjected to Western blotting. To detect total PrP including both PrPSc and PrPα, ~20–40 μg of lysate was directly analyzed by Western blotting.

2.4. Measurement of PrP from cultured cells

After treatment with quaternized PEI and PAMAM G4.0, cell lysate was prepared in lysis buffer (20 mM Tris, pH 8.0; 150 mM NaCl, 0.5% NP-40, 0.5% deoxycholate, sodium salt). Following quantification of the cell lysates using BCA protein assay kit (Pierce, Rockford, IL), 0.5 mg of cell lysate was digested with 20 μg/mg PK for 1 h at 37°C following PK inactivation by adding phenylmethylsulfonylfluoride. The reaction was neutralized with an equal volume of 0.2 M HEPES, pH 7.5 containing 0.3 M NaCl. The proteins were recovered by methanol–chloroform precipitation [26] and redissolved in 25 μl PBS with 2% Sarkosyl. Twenty μl aliquots digested with 20 μg/ml PK and 5 μl undigested samples were analyzed by Western blotting.

2.5. In vitro PrPres degradation assay

In vitro degradation of PrPres by PEI, PAMAM G4.0, and their quaternized forms was assayed as described previously with minor modifications [6,7]. Ten μl of brain homogenate (10%, w/v) obtained from RML prion-infected CD-1 mice at the terminal stage was mixed with 100 μl of 50 mM sodium acetate buffer (pH 3–7) containing 1% NP-40. One hundred μg of PEI and its quaternized forms or 20 μg of PAMAM G4.0 and its quaternized forms were added prior to incubation at 37°C. The incubation lasted 3 h with vigorous shaking. The reaction was neutralized with an equal volume of 0.2 M HEPES, pH 7.5 containing 0.3 M NaCl. The proteins were recovered by methanol–chloroform precipitation [26] and redissolved in 25 μl PBS with 2% Sarkosyl. Twenty μl aliquots digested with 20 μg/ml PK and 5 μl undigested samples were analyzed by Western blotting.

2.6. Protein misfolding cyclic amplification (PMCA)

PMCA [27], a cell-free assay mimicking conformational conversion of PrPSc to PrPres, was performed according to methods published previously [28]. Prions (RML strain) in 10% brain homogenate of CD-1 mice at the terminal stage of disease were diluted 2500-fold in 10% brain homogenate of healthy CD-1 mice prepared in PMCA buffer (PBS, pH 7.2 including 150 μM NaCl, 1% Triton X-100, 4 μM EDTA) with protease inhibitors (CompleteMini, Roche, Basel, Switzerland). Various concentrations (0–1000 nM) of PEI, PAMAM G4.0, and their quaternized forms were added prior to incubation. A 20 μl pre-amplification aliquot was saved at 0°C until used for Western blotting. The remaining 100 μl underwent 94 cycles of 40 s sonication pulsed every 30 min at a power of ~300 W in a Misonix Model 3000 microsonicator maintained at 37°C. Pre- and post-PMCA samples (20 μl each) were digested with 100 μg/ml PK at 37°C for 1 h and analyzed by Western blotting.

2.7. Western blotting

Samples were separated in 12% Tris–Glycine SDS-PAGE gels and transferred to PVDF membrane (Immobilon-F, Millipore, Billerica, MA). After blocking with 5% non-fat milk, PrPSc and PrPres were detected by incubation with monoclonal anti-PrP antibodies.
3. Results

3.1. Synthesis of quaternized polyamine dendrimers and their features

Quaternization of PEI and PAMAM G4.0 was carried out as described previously and their structures are shown in Fig. 1. Analysis of quaternized compounds by $^1$H NMR revealed spectra with following peaks (Fig. 2). PEI: $^1$H NMR (250 MHz, D$_2$O) $\delta$ 2.3–2.9 (br m, CH$_2$N), 3.0–3.9 (br m, CH$_2$N$^+$). PAMAM G4.0: $^1$H NMR (250 MHz, D$_2$O) $\delta$ 2.90 (br m, CH$_2$CONH), 3.16 (br, CH$_3$), 3.51 (br m, CONHCH$_2$CH$_2$N$^+$), 3.67 (br m, N$^+$(CH$_2$)$_2$N$^+$). The degree of quaternization was determined by comparing the integrated peak area of newly incorporated methyl groups to that of parental compounds. The degrees of quaternization of two different types of qPEIs synthesized in this study were either 8% or 93%, which were designated as qPEI(8) and qPEI(93). qPAMAM G4.0 was completely quaternized to a degree of 100%. Positive charges in qPEI(8), qPEI(93) and qPAMAM G4.0 at both pH 5 and 7.4 were compared to those in unmodified compounds. As a result of quaternization, we obtained polyamine dendrimers containing increased charge densities at pH 7.4 (Table 1).

3.2. Cytotoxicity of quaternized polyamine dendrimers

Quaternization of compounds is a useful method to decrease toxicity associated with parental compounds [20–22]. To test whether qPEI(8), qPEI(93), and qPAMAM G4.0 improve cytotoxicity of PEI and PAMAM G4.0 in prion-infected cells, ScN2a cells were treated with increasing concentrations of these compounds. Cell viability of branched polyamines improved as the degree of quaternization increased. qPEI(8) and PEI showed similar cytotoxicity, but qPEI(93) exhibited much less cytotoxicity than PEI (Fig. 3A). Surprisingly, qPAMAM G4.0 did not show any sign of cytotoxicity even at the high concentration tested in this study (Fig. 3B). As shown in an earlier study [29], PEI was more toxic to ScN2a cells than PAMAM G4.0 (Fig. 3). Quaternization did not alter
this trend. qPEI was more toxic than qPAMAM G4.0 (Fig. 3) as shown in their parental compounds. Our results suggest that quaternization is effective in decreasing toxicity caused by unmodified branched polyamines.

Table 1

<table>
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<th>Compounds</th>
<th>Mol. wt. (kDa)</th>
<th>Quaternion (%)</th>
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<th>pH 7.4</th>
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<td>116</td>
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<tr>
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<td>8</td>
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<tr>
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<td>32.4</td>
<td>93</td>
<td>290</td>
<td>270–290b</td>
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<td>0</td>
<td>190</td>
<td>64</td>
</tr>
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<td>100</td>
<td>190</td>
<td>190b</td>
</tr>
</tbody>
</table>

a adopted from [35].
b Positive charges of quaternized compounds are approximate values based on the differential amine contents.

Fig. 2. $^1$H NMR spectra of quaternized PEI and PAMAM G4.0. A. PEI, B. qPEI(8), C. qPEI(93), D. PAMAM G4.0, E. qPAMAM G4.0.

Fig. 3. Cytotoxicity of quaternized PEI and PAMAM G4.0. Cell viability of Scn2a cells was measured by MTT assays. Tested concentrations were between 0 and 3000 nM. Percent cell viability of treated cells was obtained by normalization using viability of untreated control. Data points and bars represent the mean and standard deviation from three independent measurements. A. Changes in cell viability by PEI and its quaternized compounds. Circle, PEI. Triangle, qPEI(8). Square, qPEI(93). B. Changes in cell viability by PAMAM G4.0 and its quaternized compounds. Circle, PAMAM G4.0. Triangle, qPAMAM G4.0.
3.3. Clearance of PrPSc from prion-infected cells by quaternized polyamine dendrimers

Earlier studies have shown that enhancement of PrPSc clearance in prion-infected cells correlated with the increased number of amine groups on the surface of branched polyamines [6,7], suggesting that an increase of cationic density would enhance the efficacy. To test whether positive charges of branched polyamines permanently maintained at a high level increases prion clearance, the levels of PrPSc in ScN2a cells treated with quaternized branched polyamines were measured. The results showed that qPEIs and qPAMAM G4.0 could efficiently eradicate PrPSc from ScN2a cells. Treatment did not change the levels of β-actin and high molecular weight PrP species that mainly represent PrPC (Fig. 4A and B). Overall, the levels of anti-prion activity were higher with PEI and PAMAM G4.0 than with qPEIs and qPAMAM G4.0. Disappearance of PK-resistant PrPSc was achieved more efficiently with PEI than with qPEIs (Fig. 4A and C). Similarly, higher concentrations of qPAMAM G4.0 were required to achieve the same level of PrPSc clearance resulting from PAMAM G4.0 (Fig. 4B and D). The difference in anti-prion activity between quaternized and unquaternized PEI was rather small, whereas the difference was more pronounced in PAMAM. These results indicate that anti-prion activity of modified polyamines was inversely correlated to quaternization. Nevertheless, qPEIs and qPAMAM G4.0 retained potent anti-prion activity with inhibitory concentration 50 values determined under low nM range: 1–3 nM for qPEIs and 30 nM for qPAMAM G4.0. These results suggest that the anti-prion activity is dependent on the polyamine structure as well as the degree of quaternization.

3.4. Degradation of PrPSc by quaternized polyamine dendrimers

Degradation of PrPSc by the compounds was proposed as a mechanism responsible for branched polyamines-mediated PrPSc clearance [6,7]. To assess how efficiently the quaternized compounds degrade PrPSc, brain homogenate of prion-infected mice at the terminal stage of disease was incubated with PEI, PAMAM G4.0, and their quaternized compounds in the buffer adjusted to acidic and neutral pHs. In support of the results obtained from the studies in ScN2a cells (Fig. 4), qPEIs were active in destroying PrPSc in vitro, although the levels of activity were rather small compared to their parental compound (Fig. 5A). No PK-resistant PrPSc remained if PEI was incubated in the buffer adjusted to pH 3, whereas incubation with qPEI(8) and qPEI(93) was not able to degrade all PrPSc. Similarly, at pH 4 and 5, more...
PrPSc was undegraded in the reactions with qPEI(8) and qPEI(93) than with PEI. Although the ability of these compounds to degrade PrPSc commonly depends on pH, the most quaternized PEI was least responsive to pH. Conversely, qPAMAM G4.0 was not able to degrade PrPSc even at pH 3 (Fig. 5B). PrPSc reacted with qPAMAM G4.0 at pH 3–7 remained at an almost equivalent level. In contrast, PAMAM G4.0 degraded PrPSc more efficiently in the buffer adjusted to pH 3 than in the buffers at pH 4–7 (Fig. 5B). However, degradation of PrPSc by both quaternized and unmodified compounds at pH 4–7 was not dramatically different (Fig. 5B). These results suggest that the activity to degrade PrPSc at acidic pH is negatively affected by quaternization of branched polyamines.

### 3.5. Effect of quaternized and unmodified polyamine dendrimers in prion conversion

Quaternization of PAMAM G4.0 resulted in loss of the ability to degrade PrPSc (Fig. 5B), while qPAMAM G4.0 was able to clear PrPSc from prion-infected cells (Fig. 4B). These results suggest that qPAMAM G4.0 exerts its anti-prion activity through a certain mechanism other than PrPSc degradation. Therefore, we hypothesize that quaternized branched polyamines block conversion of PrPc to PrPSc that facilitates propagation of prions because clearance of PrPSc can result from either eliminating pre-existing PrPSc or inhibiting nascent PrPSc formation. To determine the effect of quaternized branched polyamines on PrPSc formation, PMCA, an in vitro assay facilitated by conversion of PrPc to PrPSc, was performed with varying concentrations of qPEIs, qPAMAM G4.0, and their parental branched polyamines. qPAMAM G4.0 inhibited prion conversion in PMCA (Fig. 6A). The level of PrPSc generated in the absence of qPAMAM G4.0 was maintained if the concentrations of the compound in PMCA were below 100 nM, but became undetectable at 1000 nM. Interestingly, PAMAM G4.0 exhibited an identical inhibitory effect in PMCA in response to the increasing concentrations of compound (Fig. 6A and C), suggesting that the inhibitory effect of PAMAM G4.0 is not reduced by quaternization. Similar to PAMAM G4.0 and its quaternized form, PEI, qPEI(8), and qPEI(93) also showed an inhibitory effect on prion conversion (Fig. 6B). At the concentrations higher than 10 nM, these compounds completely abrogated generation of nascent PrPSc in PMCA. The abrupt disappearance of PrPSc generation by PAMAM G4.0 and its quaternized form was repeated only with qPEI(93). In contrast, PEI and qPEI(8) gradually reduced the level of newly formed PrPSc in a concentration-dependent fashion (Fig. 6B and D), which is reminiscent of the activity of PEI and qPEIs in the cleavage of conformationally changing PrPSc. Overall, our results demonstrate that, in addition to the ability to degrade pre-existing PrPSc, both quaternized and parental branched polyamines effectively inhibit PrPSc propagation facilitated by conversion of PrPc to PrPSc. This activity is maintained regardless of quaternization of amines in PAMAM G4.0, but slightly reduced by quaternization of PEI, suggesting that the effect of quaternization in prion conversion is dependent on the structure of branched polyamines.

### 4. Discussion

Any effort toward treatment of prion disease is valuable due to the lack of therapy for these diseases. In particular, development of potent agents targeting pre-existing PrPSc is important because natural clearance mechanisms for these disease-associated aggregates, which have not been clearly elucidated, are thought to operate quite slowly [30]. Therefore, supplementation of agents that destroy pre-formed PrPSc would be a beneficial approach to primarily relieve cells from PrPSc burden and simultaneously block de novo generation of nascent PrPSc in which pre-existing PrPSc participates as templates. In addition, elimination of pre-existing PrPSc implicates a potential opportunity to inactivate contaminated prions [31]. Previously, we showed that RNA interference [32] and a stereoisomer of small tricyclic compound quinacrine [25] are effective in inhibiting prions. However, these approaches target either expression of PrP or formation of PrPSc, but not pre-existing PrPSc. In this context, we looked for an approach to obtain efficacious but less toxic anti-prion agents exerting their effects on PrPSc.

In our current studies, we attempted to measure the effect of quaternized branched polyamines on cytotoxicity. Previously, several studies demonstrated quaternization is effective in decreasing intrinsic toxicity of branched polyamines [20–22]. Our results correspond to these studies by showing that cytotoxicity of PEI and PAMAM G4.0 decreased as the degree of quaternization increased. Thus, cytotoxicity does not appear to be proportionally correlated with charged states of the compounds created by quaternization. Rather, cytotoxicity is more dependent on the chemical nature of the polycations [33] as shown in the comparison of cytotoxicity of PEI, PAMAM G4.0, and their quaternized derivatives in this study. Reduction of intrinsic toxicity of branched polyamines is potentially important for the treatment of prion diseases in vivo.
Branched polyamines are known to block accumulation of PrP<sub>Sc</sub> in prion-infected cells and eliminate pre-existing PrP<sub>Sc</sub> [6–9]. Several lines of evidence indicate that the cellular site of action of branched polyamines is secondary lysosomes [6,7] where the pH is about 4.5. Branched polyamines such as PAMAM and PEI are partially charged at physiological pH (7.4) as the pK<sub>a</sub> values of secondary and tertiary amines are less than 7.4 [18,19,34]. When PEI and PAMAM are put under acidic pH conditions, the charge density of the compounds is increased. For example, PEI shows a level of protonation of 20% at pH 7.4 compared to about 45% at pH 5 due to the protonation of secondary and tertiary amines [35]. Together with the fact that the optimum pH for PrP<sub>Sc</sub> denaturation is <5.0, it can be speculated that the increased overall charge density in branched polyamines by the protonation of secondary and tertiary amines within the lysosome might be responsible for the heightened anti-prion activity at low pH.

When PEI and PAMAM G4.0 are quaternized, they would be fully charged positive and maintain a high charge density state at all ranges of pH. The quaternary ammonium cations are permanently charged, independent of the pH of their solution unlike the primary, secondary, or tertiary amines. Thus, we expected that qPEIs and qPAMAM G4.0 would be superior to their unmodified counterparts in eradicating PrP<sub>Sc</sub>. This strategy was thought to be advantageous because quaternized compounds could be active in any physiological compartment or location of the cells, not just limited to the acidic subcellular organelles. Although this approach represented a useful strategy and improved cytotoxicity, quaternization was not effective in enhancing anti-prion activity of branched PEI and PAMAM G4.0. These results suggest that reversible protonation on amine groups leads to greater anti-prion activity than irreversible amine quaternization.

In fact, our observation described in this report appears to be attributed to decreased interaction of qPEI and qPAMAM G4.0 with PrP<sub>Sc</sub> due to the lack of hydrogen bonding formation during protein–polyamine interaction. Amine groups are more likely to form hydrogen bonds, but quaternized ammonium groups are not. Recent studies proposed that protein–dendrimer interaction and consequent destabilization of proteins are modulated by electrostatic/hydrogen bonding [17,36]. Prevention of hydrogen bonding to PrP<sub>Sc</sub> through quaternization of branched polyamines appears to reduce the activity against prions. Alternatively, it is possible that the accessibility of qPEI and qPAMAM G4.0 can be reduced due to

![Fig. 6. Inhibitory effect of quaternized PEI and PAMAM G4.0 in PMCA. Brain material of terminally sick CD-1 mice inoculated with RML prions was diluted in 10% brain homogenate of healthy uninfected wild type CD-1 mice for PMCA of PrP<sub>Sc</sub>. PMCA were performed with 0–1000 nM PAMAM G4.0 and qPAMAM G4.0 (A), or with 0–10 nM PEI, qPEI(8), and qPAMAM G4.0 (B). Both pre- (−) and post-PMCA (+) samples were digested with 100 µg/ml PK and analyzed by Western blotting. Symbols denote PK digested material used for PrP<sub>Sc</sub> seeds (RML-infected brain homogenate, J) and PrP<sub>C</sub> sources (wild type brain homogenate, ▲). Molecular weight markers (kDa) are shown to the left of each blot. The level of PK-resistant PrP<sub>Sc</sub> was measured by densitometry. The levels of newly generated PrP<sub>Sc</sub> was normalized by untreated controls and plotted as a function of the concentrations of compounds. C. PAMAM G4.0 (circle) and qPAMAM G4.0 (triangle). D. PEI (circle), qPEI(8) (triangle), and qPEI(93) (square).]
the steric hindrance created by the additional three methyl groups in quaternary amines. Furthermore, our results provide insight into the action mechanism of branched polyamines in inhibiting prions. Earlier studies suggested that polyamine dendrimers act on PrPSc and anti-prion activity of branched polyamines is facilitated by degradation of pre-existing PrPSc. As shown in these studies, we also confirmed that pre-existing PrPSc becomes sensitive to PK treatment at acidic pH, but remains resistant at neutral pH, confirming the activity of polyamine dendrimers and their quaternized compounds in destabilizing PrPSc at acidic pH. Thus, it is reasonable to speculate that PrPSc is removed in the acidic cellular compartments, which reduces the possibility of PrPSc available for conversion of PrPSc to PrPSc and inhibits de novo generation of PrPSc in the cells. However, this mechanism was insufficient to fulfill the gap shown in activity of qPAMAM G4.0 in clearing PrPSc from ScN2a cells and its inability to degrade PrPSc at both acidic and neutral pH. Interestingly, during the assessment of quaternized branched polyamine effects on PMCA, we found that permanently charged polyamines inhibited PrPSc propagation at neutral pH, in which both quaternized and parental polyamine dendrimers are limited in degrading PrPSc aggregates and/or blocking PrPSc fibril formation. Therefore, our results provide evidence that polyamine dendrimers directly disturb conversion of PrPSc to PrPSc. This suggests a potential action mechanism of polyamine dendrimers at neural pH in addition to the proposed mechanism under acidic pH conditions. Together, it appears that branched polyamines include at least two different mechanisms to inhibit prions, which are differentially affected by quaternization depending on structural nature of branched polyamines.

5. Conclusions

Quaternization of amine groups found in PEI and PAMAM G4.0 was carried out to reduce the intrinsic cytotoxicity of polyamines and to achieve an increase of charge density in the compounds based on the previous studies showing positive correlation of anti-prion activity with charge density. As the study revealed, the quaternization of amines was an effective strategy to improve the cytotoxicity profile of branched polyamine. Although quaternization of amines was an effective strategy to increase of charge density in the compounds and to achieve an increase of charge density in the compounds, it was carried out to reduce the intrinsic cytotoxicity of polyamines. The study revealed, the quaternization of amines was an effective strategy to improve the cytotoxicity profile of branched polyamine. However, quaternization of PEI and PAMAM G4.0 had somewhat reduced activity compared to parental compounds, the quaternized branched polyamines still displayed appreciable anti-prion activity in clearing and degrading PrPSc. Moreover, quaternized branched polyamines are able to inhibit prion propagation facilitated by conversion of PrPSc to PrPSc without dramatic loss of activity displayed by parental compounds. PEI has randomly branched structure while PAMAM has perfectly branched structure. In addition to quaternization, the structural control of branched polyamines can provide additional room for improvement since the cytotoxicity profile and anti-prion activity were also critically dependent on the structure of branched polyamines.

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