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The suppression of prion propagation using poly-L-lysine by targeting plasminogen that stimulates prion protein conversion

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Abstract

Poly-L-lysine (PLL), a homopolymer of amino acid L-lysine (LL), has been frequently used for drug delivery. Here, we report that PLL is an effective agent to inhibit propagation of prions that cause fatal and incurable neurologic disorders in humans and animals, termed prion diseases. In our recent investigation on prion propagation facilitated by conversion of the cellular prion protein (PrP) to the misfolded, disease-associated PrP (PrP^{Sc}), we demonstrated that plasminogen stimulates PrP conversion as a cellular cofactor. In the current study, we targeted plasminogen using PLL and assessed its anti-prion efficacy. The results showed that PLL strongly inhibited PrP^{Sc} propagation in the cell-free, cell culture, and mouse models of prion disease. These results confirm the role of plasminogen in PrP^{Sc} propagation, validates plasminogen as a therapeutic target to combat prion disease, and suggests PLL as a potential anti-prion agent. Therefore, our study represents a proof-of-concept that targeting plasminogen, a cofactor for PrP conversion, using PLL results in suppression of prion propagation, which represents a successful translation of our understanding on details of prion propagation into a potential therapeutic strategy for prion diseases.

Keywords

Poly-L-lysine; prion; PrP conversion; cofactor; plasminogen; therapeutic target; translational research

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1. Introduction

L-lysine (LL) is an essential amino acid necessary as a building block of proteins in the animal body and plays an important role in many biochemical events that occur during physiological processes [1]. Synthetic polymers of LL, termed poly-L-lysine (PLL), are the homopolymers composed of a few to hundreds of LL monomers (Fig. 1). PLL exhibits a number of biological properties such as anti-viral, anti-bacterial, and anti-tumor activities [2–5]. In addition, PLL enhances the cellular uptake of macromolecules [6]. Thus, PLL has been successfully used to develop a non-viral vehicle for delivery of pharmacologically active chemical, nucleotide, and peptide drugs [7–13].

Prions are infectious proteins that cause fatal neurologic disorders such as sporadic Creutzfeldt-Jakob disease (CJD), familial CJD, variant CJD, iatrogenic CJD, Gerstmann–Sträussler–Scheinker syndrome, and fatal familial insomnia in humans, as well as other transmissible encephalopathies in animals [14,15]. Prions invade the central nervous system and cause progressive degeneration of neurons [15]. Prions are devoid of nucleic acids and composed predominantly, if not exclusively, of abnormally folded prion proteins (PrP), designated PrP^{Sc} [15]. Conformational misfolding of the host-encoded normal, cellular PrP (PrP^C) to PrP^{Sc} is the central biochemical process for propagation of prions [16].

Currently, effective therapy for prion diseases is unavailable. In the previous attempts to develop anti-prion therapy, a number of agents and methods have shown a certain degree of efficacy in cell culture and animal models of prion disease [17]. However, none has been proven to be clinically useful in human patients. While the major target of these approaches was either PrP^C or PrP^{Sc}, no other molecular target to inhibit prion propagation has been identified.

Recently, we discovered a potential molecular target other than PrP^C or PrP^{Sc} to suppress prion propagation. We identified plasminogen as a cofactor to stimulate PrP misfolding by accelerating the conversion of PrP^C to PrP^{Sc} [18]. One of us (CR) and others previously reported that plasminogen interacts with PrP through lysine-binding motifs of its kringle domains [19,20]. Furthermore, LL abrogates activity of plasminogen that enhances PrP misfolding [18] and selectively inhibits the PrP-plasminogen interaction in a concentration dependent manner [19,20]. These studies suggested that plasminogen is a potential therapeutic target and LL can be used as a therapeutic agent for the treatment of PrP misfolding diseases [18,21]. Nevertheless, the therapeutic application of LL *in vivo* appears to be impossible because the concentration of LL to effectively inhibit PrP conversion is in the millimolar range [18] and there is no method to achieve such a high local LL concentration in the body.

The objective of the study is to provide a proof-of-concept that plasminogen is a valid therapeutic target to suppress prion propagation using PLL, the synthetic polymers of LL. We hypothesized that PLL is efficacious to inhibit prion propagation through interference with PrP conversion stimulated by plasminogen. In this study, we measured efficacy of PLL in the *in vitro* and *in vivo* models of prion disease.

2. Materials and Methods

2.1. PLL and LL

PLL and LL (Fig. 1) were purchased from Sigma-Aldrich (St. Louis, MO). PLLs with various molecular weights used in this study include PLL1, PLL3, PLL10, PLL23, PLL50, PLL110, PLL225, and PLL300, where the number indicates the average molecular weight (Table 1). LL (m.w.=146) was used as a control.

2.2. Cell culture

ScN2a cells [22], the Neuro2a neuroblastoma cell line (ATCC CCL-131) permanently infected by RML (Rocky Mountain Laboratory) prions, were cultured as described previously [23]. Under 5% CO₂ and saturated humidity conditions at 37°C, ScN2a cells were cultured with Dulbecco's Modified Eagle's Medium (DMEM, high glucose; Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum, 1% penicillin-streptomycin, and 1% glutamax. Cell culture medium was replaced every three days.

2.3. Cell-based anti-prion activity assay

Anti-prion activity of PLL was measured in ScN2a cells by the method published previously [24]. ScN2a cells passed 1:50 dilution were incubated with PLL and vehicle (PBS) for six days. Culture media containing PLL, LL, or PBS were changed once on Day 4 during the incubation. If necessary, treated cells were further cultured up to 60 days in the absence of the compounds. 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) and briefly centrifuged for 30 s at 7,000 × *g*. Assays to quantify total proteins were conducted using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL) as recommended by the manufacturer. Briefly, the cell lysate obtained after six-day treatment was reacted with the mixture of BCA and cupric sulfate. The colorimetric detection was performed at 562 nm. Following protein quantification, cell lysate was digested with 20 µg/ml proteinase K (PK) for 1 h at 37°C, and centrifuged at 16,000 × *g* for 1 h at 4°C. PK-resistant pellet containing PrP^{Sc} was analyzed by Western blotting to compare the level of PrP^{Sc} eliminated as a function of treatment with compounds. As controls, β-actin and total PrP including both PrP^C and PrP^{Sc} were detected from ~30 µg of undigested lysate. Western blotting was performed as described elsewhere [23]. Monoclonal anti-PrP antibody, 6H4 (Prionics, Zurich, Switzerland) and anti-β-actin antibody ACTN05 (Neomark, Oviedo, FL) were used for Western blotting. Western blots were developed using ECL Plus™ Detection Reagents (Amersham Biosciences, Piscataway, NJ) and visualized after scanning in Fuji Film FLA 5000 image reader (Fuji Film, Edison, NJ). Doc-It Image Analysis Software (UVP, Upland, CA) was used for image analysis and densitometry.

2.4. Cytotoxicity assay

Cytotoxicity of PLL was measured by two independent methods: MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) and protein assays. MTT assay was performed as described previously [24]. ScN2a cells were passed and incubated as described above. After treatment for six days, the cells were further incubated in the fresh DMEM media with the final concentration of 0.5 mg/ml MTT for 2 h. The formazan products dissolved in acidic alcohol (0.05 N HCl-isopropanol) were measured at 570 nm. Measurement of total protein quantity using bicinchoninic acid protein assay kit (Pierce, Rockford, IL) was performed as recommended by the manufacturer (See also Section 2.3).

2.5. PrP conversion assay

The cell-free PrP conversion assay was carried out using protein misfolding cyclic amplification (PMCA) that mimics conformational conversion of PrP^C to PrP^{Sc} [25]. The PMCA procedure was performed as described previously [26] with minor modifications. For PMCA, brain homogenate (10% w/v) of RML prion-infected CD-1 mice at the terminal stage was diluted 250 fold in brain homogenate (10% w/v) of healthy CD-1 mice prepared in PMCA buffer (PBS pH 7.2 including 150 mM NaCl, 1% Triton X-100, and 4 mM EDTA) with protease inhibitors (CompleteMini, Roche, Basel, Switzerland). Additionally, 0.5 µM human plasminogen (HCPG-0130, Haematologic Technologies Inc, Essex Junction, VT) and various concentrations (0–4 µM) of PLL50 were supplemented to the reaction. PMCA

underwent 94 cycles of 40 s sonication pulsed every 30 min at 37°C in a microsonicator (300 W, Misonix Model 3000, Farmingdale, NY). A pre-amplification aliquot was stored at -20°C until used for Western blotting. 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.

2.6. Animals and *in vivo* efficacy test

Experiments associated with animals were conducted according to the protocol approved by the Institutional Animal Care and Use Committee and the Biological Safety Office at the University of Kentucky. Groups of five week old Tg(MoPrP)⁴¹¹²^{+/-} mice (hereafter referred to as Tg4112, n=3–8/group), which overexpress PrP^C (~5 X) in the brain [26], were used to assess the efficacy of PLL against prion disease. Tg4112 mice were maintained as hemizygotes for the transgene by the screening methods described previously [26]. The mice were intracerebrally inoculated with 30 µl RML prion inocula using a 26-gauge needle inserted in the left parietal lobe to a depth of 2–3 mm as described previously [27]. The inocula were prepared by diluting pooled brain homogenate (10%, w/v) of FVB mice terminally ill by RML prions in PBS lacking calcium and magnesium ions to 1% (w/v) final. PLL50 was dissolved in PBS and the first dose was administered 3 h after prion inoculation through either intravenous or intraperitoneal routes. Mice in the control (n=8) group received no PLL. Mice in group I (n=6) received a weekly dose of 8 mg/kg PLL50 directly into the tail vein for four weeks. Both groups II (n=5) and III (n=3) received intraperitoneal doses of 40 mg/kg PLL50 three times a week for either four or eight weeks, respectively. During the administration period, drinking/eating pattern and weight changes of mice were monitored whether PLL50 exhibits side effects. After completion of treatment, animals were closely observed for clinical signs (ataxia, piloerection, kyphosis, cachexia, rigid tail, etc) of prion disease. The incubation time (a period from prion inoculation until appearance of a characteristic set of clinical signs) was measured from each group to compare the efficacy of treatment.

2.7. Measurement of PrP^C and PrP^{Sc} in the brains of mice

Once mice were identified to exhibit multiple neurological signs consistent with prion infection, mice were humanely sacrificed and the brains were harvested. A 20 µl aliquot of brain homogenate (10% w/v) prepared in PBS was diluted in 100 µl Sarkosyl (2% final)-PBS. Five microliters of this brain material was used for analysis of the PrP^C levels without PK treatment. The remainder was used to detect the level of PrP^{Sc} after treatment with 20 µg/ml PK. The samples were analyzed by Western blotting.

2.8. *Ex vivo* imaging of PLL

Labeling of PLL50 with the fluorophore Alexa Fluor 750 (AF750) was performed by the amine-mediated conjugation methodology. Briefly, PLL50 dissolved at 10 mg/ml in 0.1 M sodium bicarbonate buffer, pH 8.3 was mixed with a reactive form of AF750 carboxylic acid succinimidyl ester dissolved ~10 mg/ml in high-quality, anhydrous dimethyl sulfoxide by slowly adding the latter. The reaction lasted for 1 h at room temperature and stopped by the addition of 0.75 M hydroxylamine, pH 8.5. Unbound dye was removed by dialysis. After freeze drying, AF750-conjugated PLL50 (PLL50-AF750) was redissolved in PBS and utilized for injection to mice. A single injection of 10 mg/kg PLL50-AF750 was given to six week old wild type FVB mice (Harlan, Indianapolis, IN) either intravenously or intraperitoneally. The brains were extracted at 24 h post-injection and the biofluorescence was detected by the IVIS Spectrum (Caliper Life Sciences, Alameda, CA) with the near-infrared filter for AF750 (excitation and emission wavelengths at 745 and 820 nm). Regions of interest from displayed images were designated around the brain and fluorescence was quantified by total photon counting using Living Image ver3.1 software (Caliper Life Sciences).

2.9. Statistical analysis

The log-rank test was used to validate a statistically significant difference in incubation times between control and PLL-treated groups of mice. Analysis of variance (ANOVA) was used for statistical analysis of difference in the PrP^{Sc} levels of mouse brains. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Decrease of PrP^{Sc} in ScN2a cells incubated with LL

LL was tested first as a control to confirm its anti-prion activity. The level of PrP^{Sc} in ScN2a cells treated with LL was reduced in a concentration-dependent manner (Fig. 2A). The half-maximal concentration to inhibit PrP^{Sc} (IC₅₀) was ~ 7.63 mM. However, complete elimination of PrP^{Sc} was not achieved by treatment with LL because incubation of ScN2a cells with >20 mM LL resulted in a decrease of cell numbers in the culture. Treatment with ≤ 10 mM LL did not alter the levels of actin and total PrP including PrP^C. Cytotoxicity studies in ScN2a cells confirmed that higher concentrations (20 and 50 mM) of LL were toxic (Fig. 2B and C). The half-maximal lethal concentration (LC₅₀) of LL was 17.8 – 18.1 mM, depending on the assay used.

3.2. Elimination of PrP^{Sc} from ScN2a cells by PLLs

We evaluated anti-prion activity of various PLLs ranging from 1.25 to >300 kDa in the average molecular weight (Table 1) at a fixed concentration (2.5 µg/ml). This created a condition that the absolute numbers of LL monomer equivalents and the charge density supplied by PLLs were identical across the treatments, although the molar concentrations differed (Table 1). PLL showed differential efficacy in eliminating PrP^{Sc} from ScN2a cells depending on its molecular weight but not total charges provided by PLL (Fig. 3A and B). PLLs with average molecular weights > 10 kDa were effective (Fig. 3A, lanes 4–9) in eliminating PrP^{Sc}, whereas PLLs with average molecular weights ≤ 3 kDa were not able to alter the PrP^{Sc} level in ScN2a cells (Fig. 3A, lanes 2 and 3). This suggests that there is a threshold of molecular weights for PLL to exhibit anti-prion activity. The level of actin or cell viability was unaffected by treatments with various PLLs at 2.5 µg/ml (Fig. 3A and C). Cytotoxicity of PLL was proportional to its concentrations and molecular weights, suggesting that cytotoxicity is determined by the charge density of PLL (Fig. 3D and E). The LC₅₀ of PLLs with the molecular weight above the threshold to exhibit anti-prion activity was ~6.27 µM for PLL10, 0.54–1.16 µM for PLL23, and 0.18–0.22 µM for PLL50.

3.3. *In vitro* anti-prion activity of PLL50

We further determined anti-prion activity of PLL50 because our results indicate that PLL50 is the optimal PLL composition as it showed the lowest cytotoxicity among PLLs that completely eliminated PrP^{Sc} from ScN2a cells (Fig. 3). On the basis of cytotoxicity studies of PLL50, we focused on concentrations below the LC₅₀. ScN2a cells were treated with 0–0.1 µM PLL50. PrP^{Sc} in the culture disappeared in a concentration-dependent manner (Fig. 4A). The IC₅₀ of PLL50 was ~0.015 µM. We also assessed whether PLL could permanently cure cells of a chronic prion infection. ScN2a cells pre-incubated with 0.05 µM PLL50 for six days were further cultured for additional 30 or 60 days in the absence of PLL50. The undetectable level of PrP^{Sc} in pre-treated cells remained the same even after the extended cell culture under PLL-free conditions (Fig. 4B), suggesting a complete extinction of PrP^{Sc} in the cells by PLL50.

3.4. PrP conversion blocked by PLL50

Because LL competes with PrP for kringle domains of plasminogen that include the lysine binding motifs [20] and interferes with PrP conversion in PMCA supplemented with plasminogen [18], we investigated whether PLL inhibits prion propagation by the same mechanism. PLL50 inhibited PrP conversion during plasminogen-supplemented PMCA in a concentration-responsive manner (Fig. 5). This indicates that anti-prion activity of PLL is attributed to the plasminogen-targeted inhibition of PrP conversion as shown by LL.

3.5. Efficacy of PLL50 in a mouse model of prion disease

We then assessed *in vivo* anti-prion efficacy of PLL50. We administered PLL50 for four to eight weeks either intraperitoneally (40 mg/kg) or intravenously (8 mg/kg) to Tg4112 mice inoculated with prion. These PLL50 doses are below the sub-half-maximal lethal doses (LD₅₀) [5,28] and was tolerated in Tg4112 mice. The mice received PLL50 showed temporary hypoactivity immediately following injection, but recovered within 30 min and behaved as usual. Compared to that of control group, the mean incubation times were prolonged ~40% in three separate PLL-treated groups of mice (Table 2 and Fig. 6). Mice treated with PLL50 succumbed to disease at ~80–82 days, while the control group developed clinical signs at ~58 days post-inoculation. The log-rank test demonstrated that the prolongation of incubation periods in the PLL-treated groups were statistically significant ($P = 0.0005 - 0.008$). These results demonstrate that PLL is effective in delaying the onset of disease in prion-infected mice.

3.6. Reduction of PrP^{Sc} accumulation in the brains of PLL50-treated mice

We assessed whether administration of PLL reduces the PrP^{Sc} level *in vivo*. The brains of mice from each group were collected at the terminal stage of disease when multiple clinical signs were presented. Western blotting to measure the PrP^{Sc} level in brain homogenate showed that the accumulation of PrP^{Sc} was reduced in the brains of mice treated with PLL50 compared to controls (Fig. 7A, $P = 0.0004 - 0.002$), while the level of PrP^C remained the same (Fig. 7B). These results suggest that the PrP^{Sc} levels decreased in the brains of PLL-treated mice were the result of abrogated PrP^{Sc} generation, but not altered PrP^C expression, by PLL.

3.7. Distribution of PLL50 to the brain

We examined whether injected PLL50 was distributed to the brain where prion replication takes place and the PrP^{Sc} level was decreased by PLL. We assessed the accumulation of fluorophore AF750-conjugated PLL50 (PLL50-AF750) in the brain by *ex vivo* biofluorescence imaging. The brain extracted at 24 h post-injection from mice that intravenously or intraperitoneally received PLL50-AF750 showed that PLL was available to the brain (Fig. 8A). The photon count analysis revealed that the intravenous injection resulted in greater accumulation of PLL50-AF750 in brain (Fig. 8B). These results suggest that PLL targets the brain.

4. Discussion

The current study demonstrates that PLL is effective in inhibiting prions in the cell culture and animal models of prion diseases. Since therapy for prion disease does not exist, development of effective agents and/or methodologies to prevent or cure prion diseases has been a high priority in prion research. In particular, an approach to an unexplored target to hinder the progression of prion disease is of great importance. Unlike a number of previous strategies that target the isoforms of PrP [17], we targeted a cellular protein cofactor for PrP conversion in this study. Such approach has not been possible presumably due to the lack of

information on the identity of the cofactor. We previously showed that plasminogen interacts with PrP and stimulates PrP conversion, while LL prevents their interaction and inhibits PrP^{Sc} generation [18,20]. In the current study, as a logical extension from our recent understanding, we performed translational investigations using PLL to target the interaction between PrP and plasminogen, a functional cofactor for PrP conversion. As a result, we validated our hypothesis that PLL interfering with plasminogen-enhanced PrP conversion eliminates PrP^{Sc} from prion-infected cultured cells, delays the onset of disease in prion-inoculated mice, and reduces the level of PrP^{Sc} in the mouse brains. Our study not only confirms that a cofactor (plasminogen) for PrP conversion is an excellent target to inhibit prion propagation but also provides the evidence that PLL interfering with the function of plasminogen could be the potential lead compounds for future development of anti-prion agents.

Comparison of the IC₅₀ values of LL (~7.36 mM) and PLL50 (~0.015 μM) revealed that anti-prion efficacy was tremendously improved by multimerization of LL. Also, the switch from LL to PLL improved the ratio of toxicity to efficacy (LC₅₀/IC₅₀) from 2.3 (LL) to 13.3 (PLL50). These results suggest that PLL is more preferable to inhibit prions than LL, although an immediate clinical application of PLL does not appear to be feasible. Furthermore, compared to LL, PLL50 improved anti-prion activity ~ 1.6×10³ and 5×10⁵ folds in PMCA and ScN2a cells, respectively (Table 3). These results suggest that application of the multimerization strategy is effective in enhancing anti-prion activity of LL as previously observed in other biologically or pharmacologically active peptides and chemicals [29,30]. We postulate that the increased anti-prion potency of PLL is due to the enhanced interaction between the linear chain structure of PLL and kringle domains of plasminogen. The polymer chain might not only bind to the kringle domains but also wrap entire plasminogen more tightly than small molecule LL, preventing plasminogen from interacting with PrP more effectively to inhibit PrP conversion. This hypothesis is supported by the observation that the anti-prion activity is dependent on the molecular weight of PLL rather than the charge density available under the treatment conditions. Therefore, the interaction between PLL and plasminogen may not be attributed to simple neutralization of charges.

Our data provide evidence that the molecular basis of anti-prion activity of PLL relies on the blockade of the conversion process. The underlying mechanism of anti-prion activity exhibited by both LL and PLL appears to be identical. LL and PLL are considered to be bound to the lysine-binding sites of the kringle domains of plasminogen, which prevents plasminogen from interacting with PrP and results in inhibition of PrP conversion. Alternatively, PLL may directly interact with and destroy PrP^{Sc} as previously proposed by a group of synthetic cationic dendrimers and their derivatives that degrade PrP^{Sc} at acidic pH [31,32]. Since PLLs are also cationic polymers, degradation of PrP^{Sc} by PLLs at acidic pH may be feasible, although this possibility remains to be explored. Interestingly, it is worthwhile to note that cationic branched polyamines and their derivatives with permanent positive charges variably degrade PrP^{Sc} at acidic pH, but uniformly block PrP conversion at neutral pH [33]. Thus, additional investigations are necessary to advance our comprehension on the specific action mechanism of structurally different individual cationic polymers.

The results of our *in vivo* efficacy and *ex vivo* distribution studies suggest that intravenous administration was more efficient than intraperitoneal administration in treating the prion diseases and delivering PLL to the brain. Toxicity of PLL, however, still needs to be carefully considered for further development of our PLL-mediated anti-prion therapy because intravenous administration of PLL can cause severe side-effects [28], which limited us to apply regimens with a higher dose and more frequent administration for our current studies. In this respect, further development of the PLL-based anti-prion strategy that can

avoid PLL toxicity would be necessary. Although our study demonstrated that PLL exhibits potent anti-prion activity, it is not clear whether PLL is universally effective against different prion strains and in various animal species. Additional evaluations are required to determine whether PLL will be useful in treating various types of prion diseases.

Our study offers a unique opportunity to develop PLL-based multifunctional therapeutics for prion disease. The advantage of PLL is on its versatile applications unlimited to the inhibition of PrP conversion. PLLs can be used for non-viral delivery of protein and nucleotide drugs. Therefore, we envision a PLL-based combination therapy that can, for instance, concurrently inhibit PrP conversion, suppress PrP^C expression [34], and destabilize PrP^{Sc} [35].

5. Conclusions

We demonstrate here that PLLs are effective inhibitors of prion propagation by targeting plasminogen that stimulates PrP conversion. The study represents an example of successful translation of the knowledge on the cofactor-assisted PrP misfolding mechanism into the development of a potential therapeutic intervention for prion diseases, providing a proof-of-concept that interference with plasminogen using PLL is effective to inhibit PrP conversion. We also explore a new therapeutic target that has hitherto not been recognized to hinder the progression of prion disease, and suggest that PLL can both inhibit prion propagation and deliver other anti-prion agents.

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Fig. 1.
Structure of LL (L-lysine) and PLL (poly(L-lysine)). n, numbers of LL monomer.

Fig. 2.

Elimination of PrP^{Sc} and cytotoxicity in ScN2a cells incubated with LL. **A.** Western blots of β -actin, total PrP (-PK), and PK-resistant PrP^{Sc} (+PK). Densitometry analysis of the PrP^{Sc} levels shows the means of relative PrP^{Sc} levels with standard deviations (error bars, n=5). **B.** Cell viability measured by MTT assays (n=6). **C.** Total protein contents measured by BCA protein assays (n=3). The effect of LL was presented as relative values after normalization with the level of untreated control.

Fig. 3.

Anti-prion activity and cytotoxicity of PLLs in ScN2a cells. **A.** Inhibition of PrP^{Sc} propagation in ScN2a cells incubated with 2.5 μ g/ml PLLs of various molecular weights (lanes 2–9). Numbers following PLL, the average molecular weights (kDa) of each PLL; Ctrl, incubated with no PLL (lane 1); –PK, total PrP; +PK, PK-resistant PrP^{Sc}. PrP^{Sc} was analyzed from 0.5 and 2 mg of cell lysates. **B.** The composite plot of the PrP^{Sc} levels measured by densitometry (bars), total charge density available from added PLLs (●), and the molecular weights of each PLL (▲). The means of relative PrP^{Sc} levels and standard deviations (error bars) were determined from the analyses with 0.5 (filled bar, n=3) and 2 (open bar, n=6) mg cell lysates. The numbers of LL unit equivalent represent the charge density of PLL. **C.** Cytotoxicity of various PLLs at 2.5 μ g/ml measured by MTT assays (n=3). Cell viability was presented as % normalized with the untreated control (Ctrl) value. For **D** and **E**, LC₅₀ of PLL10 (▲), PLL23 (■), and PLL50 (●) in ScN2a cells. **D.** Cytotoxicity of PLLs measured by MTT assays (n=3). **E.** Total protein contents in PLL-treated cells measured by BCA protein assays (single measurement of triplicates).

Figure 4.

Prion inhibitory effect of PLL50. **A.** The IC₅₀ of PLL50. ScN2a cells were incubated with no (lane 1), 0.02 μM (lane 2), 0.04 μM (lane 3), 0.06 μM (lane 4), 0.08 μM (lane 5), and 0.1 μM (lane 6) PLL50. Two mg of cell lysates were used for the analysis of PrP^{Sc} in Western blots. -PK, total PrP; +PK, PK-resistant PrP^{Sc}. The IC₅₀ of PLL50 was obtained from densitometry data that present the means of relative PrP^{Sc} levels and standard deviations (error bars, n=3). **B.** Extinction of PrP^{Sc} from ScN2a cells treated with PLL50. ScN2a cells incubated with 0.05 μM PLL50 for six days were further cultured in the absence of PLL50 for 30–60 days. Untreated (–) control (lane 1); after six day treatment with (w/) PLL50 (lane 2); 30 day (lane 3) and 60 day (lane 4) culture post-treatment without (w/o) PLL50.

Figure 5.

Inhibition of PrP conversion by PLL50. PMCA supplemented with (0.5 μ M, lanes 3–14) or without (lanes 1–2) plasminogen (Plg) was performed in the presence of 0.1 – 4 μ M PLL50 (lanes 5–14). Odd number lanes (–), the PrP^{Sc} levels of initial seeds before PMCA; even number lanes (+), the levels of newly generated PrP^{Sc} after PMCA.

Figure 6.

Clinical onset of prion disease in Tg 4112 mice treated with PLL50. The percent of mice remained asymptomatic was plotted over the course of disease. PBS, vehicle; IV, intravenous delivery once a week for 4 weeks (wks); IP, intraperitoneal delivery three times a week for 4 and 8 wks. The *P*-values were obtained by the statistical analysis using the log-rank test.

Figure 7. The PrP^c and PrP^{Sc} levels in the brains of PLL50-treated mice. **A.** The level of PrP^{Sc} accumulated in the brains of mice treated with PLL50. Individual mouse brains from sparse groups: the PBS-treated control group (Lanes 1–3 and 10–12, *n*=3 each); the group received IV 8 mg/kg weekly for 4 wks (lanes 4–9, *n*=6); the group received IP 40 mg/kg three times a week for 4 wks (lane 13–15, *n*=3); and the group received IP 40 mg/kg three times a week for 8 wks (lanes 16–18, *n*=3). The PrP^{Sc} levels of individual brains were plotted with the averages (bars). The *P*-values were obtained by the statistical analysis using ANOVA. **B.** The levels of PrP^c in the brains of mice treated with PLL50. Individual mouse brains from the PBS-treated control group (lanes 1–3); the group received IV 8 mg/kg weekly for 4 wks

(lanes 4–8); and the group received IP 40 mg/kg three times a week for 8 wks (lane 9–11) and 4 wks (lane 12).

Figure 8.

Distribution of PLL to brain. Wild type FVB mice received PLL50-AF750 either IV or IP. The brain extracted at 24 h post-injection was subjected to detect biofluorescence using the IVIS Spectrum imaging system. **A.** *Ex vivo* fluorescence image of brains. **B.** Total photon counts of fluorescence. Ctrl, vehicle (PBS) injected.

Table 1

PLLs and LL used for anti-prion assays.

Compound	m.w. range (kDa)	Average m.w (kDa)	n	Conversion of concentrations		N in 2.5 µg/ml LL and PLL
				µg/ml	Molar concentrations	
LL	0.146	0.146	1	2.5	17.1 µM	1.0 × 10 ¹⁹
PLL1	0.5–2	1.25	8.6	2.5	2 µM	1.0 × 10 ¹⁹
PLL3	1–4	2.5	17.1	2.5	1 µM	1.0 × 10 ¹⁹
PLL10	4–15	10	68.5	2.5	0.25 µM	1.0 × 10 ¹⁹
PLL23	15–30	22.5	154.1	2.5	0.11 µM	1.0 × 10 ¹⁹
PLL50	30–70	50	342.5	2.5	0.05 µM	1.0 × 10 ¹⁹
PLL110	70–150	110	753.4	2.5	22.7 nM	1.0 × 10 ¹⁹
PLL225	150–300	225	1541.1	2.5	11.1 nM	1.0 × 10 ¹⁹
PLL300	> 300	>300	2054.8	2.5	8.35 nM	1.0 × 10 ¹⁹

m.w., molecular weights; Da, Dalton; LL, L-lysine; PLL, poly(L-lysine); n, average numbers of LL monomers per molecule (multimerization degree); N, numbers of LL monomer equivalents, which represent charge densities at a given concentration.

Table 2

Incubation time of prion-infected Tg 4112 mice that received treatment with PLL50.

Group	Inoculated prion strain (route)	Treatment		Mean incubation time (days $\pm$ S.E.M.)	n/n ₀	P-value
		Dose	Frequency (duration)			
control	RML (IC)			58.4 $\pm$ 0.9 ^a	8/8	
I	RML (IC)	8 mg/kg	Once/week (4 weeks)	81.5 $\pm$ 1.3	6/6	0.0005
II	RML (IC)	40 mg/kg	3 times/week (4 weeks)	80.2 $\pm$ 1.2	5/5	0.001
III	RML (IC)	40 mg/kg	3 times/week (8 weeks)	80.6 $\pm$ 4.6	3/3	0.008

S.E.M., standard error of the mean; n, number of mice diagnosed ill with prion diseases; n₀, number of mice inoculated; IC, intracranial; IV, intravenous; IP, intraperitoneal.

^a Adopted from [26]. The P-values were calculated using the log-rank test.

Table 3

Improved anti-prion efficacy of PLL.

	Anti-prion activity	
	ScN2a cells ^a	PMCA ^b
LL	7.36 mM	1 mM ^c
PLL50	15 nM	0.6 μ M
Efficacy improved	5×10^5 fold	1.6×10^3 fold

^aThe IC₅₀ of LL and PLL50 represent the anti-prion activity in ScN2a cells.

^bThe concentrations of LL and PLL50 that abrogated plasminogen-mediated PrP conversion represent the anti-prion activity in PMCA.

^cAdopted from [18].