Orally administered amyloidophilic compound is effective in prolonging the incubation periods of cerebrally infected prion disease animals in a prion strain-dependent manner.

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ABSTRACT

Establishment of effective therapeutic interventions for prion diseases has been demanded. We report a newly developed amyloidophilic compound that displays therapeutic efficacy when administered orally. This compound inhibited abnormal prion protein formation in prion-infected neuroblastoma cells in a prion-strain dependent manner: effectively for RML prion and marginally for 22L prion and Fukuoka-1 prion. When the highest dose (0.2% (w/w) in feed) was given orally into cerebrally RML-prion inoculated mice from inoculation to the disease terminal, it extended the incubation periods 2.3 times longer than the control. The compound exerted therapeutic efficacy in such a prion-strain dependent manner as observed in the cell culture study: most effective for RML prion, less effective for 22L prion or Fukuoka-1 prion, and marginally effective for 263K prion. Its effectiveness depended on the earlier start of administration. The glycoform pattern of the abnormal prion protein in the treated mice was modified and showed di-glycosylated form predominance, which resembled that of 263K prion, suggesting that di-glycosylated forms of abnormal prion protein might be least sensitive or resistant to the compound. The mechanism of the prion strain-dependent effectiveness needs to be elucidated and managed. Nevertheless, the identification of an orally available amyloidophilic chemical encourages pursuit of chemotherapy for prion
diseases.
Transmissible spongiform encephalopathies, or prion diseases, are a group of fatal neurodegenerative disorders that include Creutzfeldt-Jakob disease (CJD) and Gerstmann-Sträussler-Scheinker syndrome (GSS) in humans, and scrapie, bovine spongiform encephalopathy and chronic wasting disease in animals. These disorders are characterized by accumulation in the brain of an abnormal isoform of prion protein (PrP), which is a main component of the pathogen, prion, or a pathogen itself, and which is rich in beta-sheet structure and resistant to digestion with proteinase K (24). Recent outbreaks of variant CJD and iatrogenic CJD through use of cadaveric growth hormone or dura grafts in younger people have necessitated the development of suitable therapies.

Dr. Caughey and his colleagues first found Congo red and sulfated glycans to inhibit abnormal PrP formation \textit{in vitro} (5, 6), although Congo red was much earlier described as a staining device for prion amyloid rods (23). Since the discovery of therapeutic activity of Congo red, amyloidophilic compounds such as amyloid dye derivatives and glucoseaminoglycan mimetics have been noted as one class of possible therapeutic candidates for prion diseases (4, 32). Recently, the most advanced progress in amyloidophilic compounds, which bear excellent permeability through the blood-brain barrier, has been made in the field of diagnosis of Alzheimer’s disease. Some
amyloidophilic compounds are developed as imaging probes to visualize amyloid deposits in the brain of Alzheimer’s disease patients using positron emission tomography or single photon emission computed tomography technology (3). Some of these chemicals are also useful to visualize abnormal PrP amyloids of some types of prion diseases in the brain (2, 14, 15, 28, 30).

We previously reported that some of these amyloid-imaging probes are effective as anti-prion compounds and prolong the incubation periods of cerebrally infected prion disease animals (14). We also reported that a new class of amyloidophilic chemicals, styrylbenzoazole derivatives, which have better penetration through the blood-brain barrier and which have more discrete labeling activity of amyloid deposition in brain tissues affected by either Alzheimer’s disease or prion diseases, are effective as anti-prion chemicals (15, 19). However, the efficacy of these weekly intravenously administered amyloidophilic compounds was not remarkable, but rather limited. In addition, their effectiveness was suggested to be in a prion-strain dependent fashion, but this was not fully evaluated because of the limited availability of the compounds in quantity and dosing route. It can be assumed that elevated brain chemical levels are necessary for a compound’s efficacy. Therefore, a multiple dosing regimen, which causes more sustained elevation in brain chemical levels, might be preferable to a single weekly dosing. In this
paper, to ascertain undefined benefits and limitations of amyloidophilic compounds as therapeutic drug candidates for prion diseases, a new class of amyloidophilic compounds which have no similarity in chemical structure with previously reported anti-prion compounds was synthesized and tested for either anti-prion activity \textit{in vitro} or therapeutic efficacy \textit{in vivo} when administered orally as a mixture with feed.
MATERIALS AND METHODS

Chemicals and experimental models

Test compounds were synthesized at the Tokyo R & D Center of Daiichi Pharmaceutical Co., Ltd. (Tokyo, Japan). The compounds’ structure is shown in Table 1. The compounds were dissolved in 100% dimethylsulfoxide (DMSO) using ultrasonication and stored at -30°C until use.

Cultured cells were grown in Opti-MEM (Invitrogen Corp., Carlsbad, CA) supplemented with 10% fetal calf serum. As cellular models for the screening of anti-prion compounds, either mouse neuroblastoma cells (N2a) or N2a with five-fold PrP overexpression (N2a#58), which are persistently infected with a distinct prion strain, were used: N2a infected with RML scrapie prion (ScN2a) (25), N2a with 22L scrapie prion (N167), N2a#58 with RML scrapie prion (N002 or Ch2), or N2a#58 with Fukuoka-1 GSS prion (F3) (15). The Ch2 cells are a subclone of N002 cells.

Five-week-old Tga20 mice overexpressing murine PrP (11) or Tg7 mice overexpressing hamster PrP (26) were used as disease animal models after intracerebral infection with 20 µl of 1% (w/v) brain homogenate of RML prion, 22L prion, or Fukuoka-1 prion for Tga20 mice, or of 263K scrapie prion for Tg7 mice. Five-week old ICR mice and Syrian hamsters were also used after they were infected intracerebrally.
with 20 µl of 1% (w/v) brain homogenate of RML prion, or 40 µl of 1% (w/v) brain homogenate of 263K prion, respectively. Each animal was maintained under deep ether anesthesia for minimum distress during intracerebral inoculation. Permission for the animal study was obtained from the Animal Experiment Committee of Tohoku University, Japan.

**In vitro PrP imaging**

Autopsy-diagnosed brain samples from the cases of GSS, which were kindly provided by Dr. Toru Iwaki from the Department of Neuropathology, Kyushu University, Japan, were used. After fixation in 10% buffered formalin for 2 weeks, the sample was immersed in 98% formic acid for reduction of prion infectivity, embedded in paraffin, and cut into 7-mm-thick sections. For neuropathological staining, deparaffinized sections were immersed in 1% Sudan black solution to quench tissue autofluorescence. They were then incubated for 30 min in 1 mM solution of compound B (cpd-B), rinsed with distilled water and examined under a fluorescence microscope (DMRXA; Leica Microsystems GmbH, Wetzlar, Germany) using an FITC filter set.

For comparison, each section was subsequently immunostained for PrP as described in a previous study (7). Briefly, the sections were treated with a hydrolytic autoclave and
incubated with a rabbit primary antibody, anti-PrP-C, which was raised against a mouse PrP fragment, amino acids 214-228 (1:200; Immuno-Biological Laboratories Co. Ltd., Gunma, Japan), followed by incubation with EnVision+System-HRP labeling polymer (Dako, Glostrup, Denmark). The reaction product was developed with 3,3’-diaminobenzidine tetrahydrochloride solution and counterstained with hematoxylin.

In vitro treatment in cell cultures

Anti-prion activity was evaluated by assaying the content of protease-resistant PrP (PrPres) in the cellular models, as described in earlier studies (6, 8, 18). Briefly, test compounds were added at the designated concentrations when cells were passaged at 10% confluence while maintaining the final concentration of DMSO in the medium at less than 0.5%. The cells were allowed to grow to confluence and were lysed with lysis buffer (0.5% sodium deoxycholate, 0.5% Nonidet P-40, phosphate-buffered saline (PBS)). For analysis of PrPres, samples were digested using 10 µg/ml proteinase K for 30 min at 37°C; the digestion was stopped using 1 mM phenylmethylsulfonyl fluoride. The samples were centrifuged at 100,000 × g for 30 min; then pellets were resuspended in 1× sample loading buffer and boiled for 5 min. For analysis of the total level of cellular PrP in N2a cells treated with a test compound, cell lysates were mixed directly with a quarter
volume of 5× sample loading buffer and boiled for 5 min.

The samples were analyzed by immunoblotting. They were separated using electrophoresis on a 15% Tris-glycine-sodium dodecyl sulfate polyacrylamide gel and electrophorized onto a polyvinylidene difluoride filter. The PrP was detected using a monoclonal antibody, SAF83 (1:5000; SPI-Bio, Massy, France), followed by an alkaline phosphatase-conjugated goat anti-mouse antibody (1:20,000; Promega Corp., Madison, WI). Immunoreactivity was visualized using a CDP-Star detection reagent (Amersham, Piscataway, NJ). More than three independent assays were performed in each experiment.

The cell surface level of cellular PrP was assayed using flow cytometry, as described previously (10). Briefly, N2a cells dispersed by treatment with 0.1% collagenase (Wako Pure Chemical Industries Ltd., Osaka, Japan) were washed with ice-cold 0.5% fetal calf serum in PBS (FCS/PBS) and incubated with SAF83 (1:500) or isotype-matched control IgG1 for 20 min on ice. Cells were washed with FCS/PBS and incubated with goat F(ab’)2 fragment anti-mouse IgG(H+L)-PE (1:100) (Beckman Coulter Inc., CA) for 20 min on ice. After washing, cells were analyzed using an EPICS XL-ADC flow cytometer (Beckman Coulter Inc., CA).

Pharmacokinetic studies
Brain cpd-B levels in the animals were assayed as described (20) after one-week feeding with 0.2% cpd-B *ad libitum*. All animals were sacrificed at 9:00 am of day eight by excision of the carotid artery under deep ether anesthesia to remove the blood as much as possible, and the brain was collected, rinsed with saline and weighed. Because preliminary studies found no significant difference in the data between perfused brains and non-perfused brains, the brain was not perfused with saline to remove residual blood. The brain was homogenized with 2 ml of 100% methanol for mouse brain or 4 ml for hamster brain. After centrifugation of the homogenate at 800 × g for 10 min, the supernatant was diluted with nine volumes of 20 mM phosphate buffer, pH 6.5 (PB) and then filtered to obtain the sample for analysis. Then the sample was applied to a conditioned C18 solid-phase extraction cartridge. The compound was eluted with methanol and was diluted with an equal volume of PB. Then, the compound was separated by high-performance liquid chromatography using a reversed phase column (C₄, 4.6 × 150 mm; Phenomenex Inc., Torrance, CA). The compound was detected using a UV detector at 285 nm; the dose of cpd-B per gram of brain tissue was determined.

The kinetics of brain uptake and washout of cpd-B were also investigated as described (20). The compound was solubilized in 5% Tween-80 in ethanol. Then its 0.2 mg/ml solution containing 5% Tween-80 and 5% ethanol in saline was prepared. The
compound at a dose of 1.0 mg/kg body weight was administered intravenously to ICR
mice under ether anesthesia. Both Tween-80 and ethanol are FDA approved solubilizers
of lipophilic medicinal chemicals. The dose of neither solubilizer used in the study has
been reported to cause any toxicity or to affect the pharmacokinetics. At 2 min or 30 min
after injection, the blood was collected from the heart using heparin; then the brain was
obtained as described. The blood plasma was mixed with three volumes of acetonitrile
and centrifuged at 10,000 × g for 5 min. The supernatant was mixed with the same
volume of PB and subsequently filtered to obtain the plasma sample for analysis. Either
the preparation for the brain sample for analysis or the assay of the samples was
performed as described. The percentage of the injected dose per gram of tissue or fluid
(%ID/g) was used as a measure of the brain or plasma level of the compound.

**In vivo** treatment in animal models

In experimental animals that had been infected intracerebrally with a prion pathogen,
cpd-B was given orally *ad libitum* as a mixture with powder feed in the following dose:
0.1%, 0.13%, 0.2%, and 0.33% weight in the feed corresponding respectively to *ca.* 150
mg/kg body weight/day, *ca.* 225 mg/kg body weight/day, *ca.* 300 mg/kg body weight/day,
and *ca.* 500 mg/kg body weight/day in Tga20 mice, as each mouse consumed an average
of 3.75 g of the feed per day. The animals were monitored every day until the disease
terminal: the incubation period, which was defined in the present study as the length from
the inoculation to the disease terminal, was assayed.

Pathological and infectivity assays

The right brain hemisphere of the mice was fixed using 10% buffered formalin, then
embedded in paraffin. Five-millimicron-thick sections of the coronal slice sited around
one-third of the distance from the interaural line to the bregma line were dewaxed and
immunostained using an anti-PrP-C antibody, as described above, or an antibody against
glial fibrillary acidic protein (GFAP) (1:5,000; Dako, Glostrup, Denmark), as described
in a previous study (9).

For detection of PrPres by immunoblotting, the left brain hemisphere was
homogenized with nine volumes of lysis buffer; after low-speed centrifugation, the
supernatant was treated with 50 µg/ml proteinase K for 30 min at 37°C. An aliquot
corresponding to 0.13 mg of brain tissue for PrPres assay or 0.83 µg of brain tissue for
total PrP assay was electrophoresed on a 13.5% Tris-glycine-sodium dodecyl sulfate
polyacrylamide gel and analyzed by immunoblotting as described above.

For the infectivity assay, the left brain hemisphere was homogenized with PBS to
produce 10% brain homogenate. Serially diluted homogenate samples for assay were produced by diluting the brain homogenate serially with 10% brain homogenate of non-infected mice fed with 0.2% cpd-B for one month. A 20 µl aliquot of each sample was then inoculated intracerebrally into each of the Tga20 mice. Incubation times were assayed as described above.

Statistical analysis
Statistical significance was analyzed using the Kruskal-Wallis test followed by Scheffè's $F$-test for multiple comparisons. Correlation analysis was performed using the rank correlation coefficient method by Spearman. The regression coefficient was determined using simple linear regression analysis. The survival rate was calculated using the Kaplan-Meier method; its significance was evaluated using the log-rank method.
RESULTS

Anti-prion activity in vitro

The anti-prion activity of newly synthesized compounds was investigated using ScN2a cells, which are N2a cells that are persistently infected with RML scrapie prion and are commonly used for drug screening. At a half-maximum effective dosage (EC$_{50}$ value) of about 60 pM, cpd-B inhibited PrPres formation (Table 1 and Fig. 1A). Other related compounds were also potent within a non-toxic dose range up to 10 µM.

To investigate whether the efficacy of the compounds depends on the pathogen strains, cpd-B was tested in four other cell lines that had been infected individually with distinct prion strains. As shown in Fig. 1A, cpd-B was only effective in N002 (EC$_{50}$: 320 nM) and Ch2 (EC$_{50}$: 300 nM), both of which are N2a#58 cells infected with RML prion. However, the inhibitory activity in these cells was not as strong as in ScN2a cells, which are derived from N2a cells expressing one-fifth of the normal PrP molecules of N2a#58 cells. In contrast, cpd-B was ineffective in both N167 cells (N2a cells infected with 22L scrapie prion) and F3 cells (N2a#58 cells infected with Fukuoka-1 GSS prion) at a dose less than 10 µM. However, at a dose of 10 µM, marginal reduction of the PrPres signals was observed in both cells. At a dose greater than 10 µM, cell toxicity was observed. The results suggest that cpd-B exerts an inhibitory activity on PrPres formation in a prion
strain-dependent manner: more effectively for RML prion, and marginally for 22L prion
or Fukuoka-1 prion.

The inhibition mechanism included no alteration of either the total or the cell surface level of normal PrP, as demonstrated in non-infected N2a cells treated with 1 µM cpd-B, using either the immunoblot analysis of the cell lysate without protease digestion or flow cytometry analysis of the cell surface PrP immunodetection (Figs. 1B and 1C). In addition, cpd-B did not facilitate the digestion of abnormal PrP by proteinase K, nor did it interfere in immunodetection because PrPres signals were not modified after cpd-B was mixed and incubated with a cell lysate of non-treated ScN2a cells before proteinase K-digestion (data not shown).

Pharmacological properties

Abnormal PrP amyloid imaging by cpd-B was performed in brain sections of GSS cases to examine amyloidophilic properties of cpd-B. The compound bound to and fluorescently labeled most of the PrP plaques in cerebellar cortices of GSS cases (Fig. 1D). Background staining was barely observed after rinsing off the excess compound. Immunohistochemical analysis of PrP revealed that the compound achieved high-specificity labeling. The compound displayed no signal in control sections without
amyloid lesions (data not shown).

Next, to examine the brain accessibility of cpd-B when administered orally, brain levels of cpd-B in the experimental animals used in the study were assayed after the animals were fed ad libitum with 0.2% cpd-B-containing feed for one week. The brain level of cpd-B was 39.16±22.15 nmol/g brain tissue in Tga20 mice \( (n=4) \), 26.04±12.50 nmol/g brain tissue in ICR mice \( (n=4) \), and 22.94±7.64 nmol/g brain tissue in Tg7 mice \( (n=4) \). Syrian hamsters, however, showed a lower level: 7.26±2.47 nmol/g brain tissue \( (n=4) \). A considerable amount of cpd-B was detected in the brains of all experimental animals; no significant difference in the brain cpd-B levels was found among the types of the mice.

Further study of the pharmacokinetics of cpd-B in the blood and the brain was performed in ICR mice after cpd-B was injected into the tail vein. The %ID/g value was determined: the percentage of injected dose per gram of tissue or fluid. The brain uptake level of cpd-B at 2 min after intravenous injection was 8.01±1.27%ID/g tissue, whereas the blood plasma level was 2.92±1.00%ID/g fluid. Consequently, the ratio of cpd-B concentration in the brain vs. that in the blood plasma is 2.7:1, indicating that cpd-B is equal to the best brain-entering amyloidophilic chemicals previously identified (15). On the other hand, both the brain level and the blood plasma level of cpd-B at 30 min after the
intravenous injection were below the measurable level of 50 pM, which indicates that
cpd-B is very rapidly washed out from the brain and blood.

Regarding toxicity of cpd-B, about 16% body weight loss in Tga20 mice, and about 5% body weight loss in Tg7 mice, was observed after cpd-B was given orally *ad libitum* for one week at a dose of 0.33% weight in feed, which corresponds to ca. 500 mg/kg body weight/day. Other doses of cpd-B tested in this study produced no apparent toxic effects in the experimental animals used.

Therapeutic efficacy *in vivo*

The therapeutic activity of cpd-B *in vivo* was assayed in murine PrP-overexpressing Tga20 mice that had been cerebrally infected with RML scrapie prion. The non-treated infected mice started exhibiting abnormal clinical signs such as staggering, rotating, irritation, and motionlessness about two months after the infection; then the mice wasted into the disease terminal in a week. Treatment by feeding cpd-B-containing feed *ad libitum* was initiated immediately after the infection and continued until the disease terminal. The cpd-B treated mice did not exhibit such abnormal signs as described and wasted gradually into the disease terminal. As shown in Fig. 2A, oral cpd-B treatment significantly prolonged the incubation periods of infected Tga20 mice in a
dose-dependent manner: 68.5±5.9 days in the non-treated control mice, 108.0±2.8 days in the mice treated with 0.1% cpd-B feed, 120.5±10.7 days in the mice with 0.13% cpd-B feed, and 154.3±19.9 days in the mice with 0.2% cpd-B feed. Therefore, oral cpd-B treatment at the highest dose produced a 2.3-fold extension in the incubation periods of the mice. Statistical analyses demonstrated a significant linear correlation between the incubation periods and the cpd-B doses ($r=0.95$, $p<0.01$); the correlation equation was:

$$Y=426.37X+ 66.93$$

($Y$, incubation period (days); $X$, cpd-B dose (% in feed)) and the correlation coefficient was 0.89 ($p<0.01$).

In our previous studies, the effectiveness of amyloidophilic chemicals in the extension of incubation periods of infected animals was only observed in the Tga20 mice infected with RML prion (14, 15). Then, ICR mice were examined for the therapeutic efficacy of oral cpd-B treatment to investigate whether effectiveness of amyloidophilic compounds is restricted only to Tga20 mice. Non-treated control ICR mice that had been cerebrally infected with RML prion exhibited disease terminal at 154.2 ±18.4 days post-infection, whereas the mice treated with 0.2% cpd-B feed lived significantly longer ($p<0.01$). Even though the oral cdp-B treatment was discontinued at day 187 post-infection when the last of the non-treated animals became the disease terminal, more than half of the treated-mice survived to day 270 post-infection (Fig. 2B).
Next, the therapeutic efficacy of oral cpd-B treatment was investigated against other prion strains. The cpd-B treatment significantly prolonged the incubation periods of Tg20 mice that had been cerebrally infected with 22L scrapie prion ($p<0.01$); 96.3±5.9 days in the non-treated control mice, 126.3±10.3 days in the mice treated with 0.2% cpd-B feed, indicating 1.3-fold extension of the incubation periods (Fig. 2C). Control mice started exhibiting distinguished opisthotonus with head rotating a week before the disease terminal, whereas cpd-B treated mice showed no such clinical sign, even in the terminal.

The cpd-B was also effective against Fukuoka-1 GSS prion; cerebrally infected Tg20 mice lived significantly longer by oral cpd-B treatment ($p<0.05$): 101.6±12.1 days in the non-treated control mice and 142.2±21.0 days in the mice treated with 0.2% cpd-B feed, indicating 1.4-fold extension of the incubation periods (Fig. 2D). Staggering was observed as an initial clinical sign in the control mice more than one week before the disease terminal, although this clinical sign was not recognized in the cpd-B treated mice.

In contrast to these prion strains, the efficacy of oral cpd-B treatment was very marginal for 263K scrapie prion, when Tg7 mice expressing hamster PrP were used as the host (Fig. 2E). The incubation periods of the cpd-B-treated mice (52.7±2.8 days) were significantly but very marginally prolonged from those of the non-treated mice (48.0±3.0 days).
days) \((p<0.05)\). This prion is a hamster-adapted scrapie prion strain: Syrian hamsters were used as the host to examine whether the marginal efficacy of oral cpd-B treatment is chiefly attributable to the host Tg7 mouse or to the pathogen strain 263K prion. As observed in Tg7 mice, hamsters treated with 0.2% cpd-B feed also exhibited a marginal increase in the incubation period compared to that of the non-treated control hamsters that had been cerebrally infected with 263K prion \((p<0.05)\): 107.0±2.5 days in the cpd-B treated hamsters and 97.4±6.9 days in the non-treated hamsters (Fig. 2F). These results indicate that oral cpd-B treatment is not so effective for 263K prion.

Timing and duration of dosing

The effectiveness of cpd-B throughout various timings and durations of oral administration was analyzed in Tga20 mice that had been cerebrally infected with RML prion (Fig. 3). The incubation periods of the non-treated mice were 63.0±1.8 days, whereas the incubation periods of the mice treated with 0.2% cpd-B feed were reversely correlated with the post-infection durations to the commencement of cpd-B treatment \((r=-0.79, p<0.01)\): 174.5±7.6 days when started at day 0 post-infection, 117.2±7.0 days at day 35 post-infection, 88.7±17.3 days at day 49 post-infection. On the other hand, the incubation periods of the mice treated with 0.2% cpd-B feed were also correlated with the
durations of cpd-B treatment, which started immediately after cerebral infection ($r=0.95$, $p<0.01$): 102.1±2.9 days when treated for 14 days from the infection, 120.2±5.2 days for 35 days from the infection, 142.5±7.8 days for 70 days from the infection. In addition, when the cpd-B treatment was discontinued during early disease stages, the remaining incubation times were longer than that of the control mice.

Pathological evaluation

The PrPres content in the brain of cpd-B-treated mice was analyzed sequentially by immunoblotting and compared with that of the non-treated control mice (Fig. 4A). The PrPres signals in the non-treated mice were very strong at the disease terminal (day 63 post-infection). In contrast, in the mice treated with 0.1% cpd-B feed from the infection, PrPres signals were faint at day 63 post-infection and distinct at the terminal (day 108 post-infection). However, the PrPres signals at the terminal did not reach the high level shown by the non-treated control mice at the terminal. Comparison of the signal intensity of the di-glycosylated PrPres form showed that six-fold to fifteen-fold diluted samples of the non-treated terminal mice exhibited similar signal intensities as undiluted or two-fold diluted samples of the 0.1% cpd-B-treated terminal mice (Fig. 4B). Similarly, in the mice treated with 0.2% cpd-B feed from the infection, PrPres signals gradually increased
according to the time course after infection: no signals detected at day 63 post-infection, distinct signals at day 120, similar or more distinct signals at the terminal (day 154 post-infection). The PrPres signal levels of the 0.2% cpd-B-treated mice at the terminal were indistinguishable from those of the 0.1% cpd-treated mice at the terminal.

Glycoform patterns of PrPres differed completely. As shown in Fig. 4B, when the samples were diluted and re-assayed so that the signal intensities of di-glycosylated PrPres forms were equalized as much as possible, the difference was much more distinct. The glycoform patterns in the non-treated mice, which were uniform in analyzed samples, were predominantly mono-glycosylated, whereas the glycoform patterns in the cpd-B-treated mice were not necessarily uniform but were always predominantly di-glycosylated. This di-glycosylated PrPres predominance was also observed for 263K prion (Fig. 4C), but not for other prion strains used in this study (data not shown).

Modification in the pathology of the brain of cpd-B-treated mice was analyzed (Fig. 4D). For non-treated control mice with an incubation period of 63 days, the brain showed prominent pathological changes consisting of abnormal PrP deposition and glial cell reaction in the thalamus, although the brains of the mice treated with 0.2% cpd-B feed showed no such pathological changes at day 63 post-infection and milder levels of abnormal PrP deposition at the terminal (day 154 post-infection). No difference was
apparent in the pattern or distribution of abnormal PrP deposition in the brains between
the non-treated mice and the cpd-B-treated mice.

Infectivity analysis

Infectivity levels are reversely correlated with the incubation periods (24). Therefore, infectivity levels of the brain of the mice treated with 0.2% cpd-B feed were evaluated by assaying the incubation periods of animals that had been cerebrally inoculated with the brain homogenate (Table 2). The 10^2-fold diluted brain homogenates from the cpd-B-treated mice at day 63 post-infection exhibited similar incubation periods as the 10^5-fold or greater diluted brain homogenates from the non-treated mice; the 10^2-fold diluted brain homogenates from the cpd-B-treated mice at the terminal (day 154 post-infection) showed similar incubation periods as the 10^4-fold or 10^5-fold diluted brain homogenates from the non-treated mice. The data indicate that the brains of mice treated with 0.2% cpd-B feed had much lower infectivity levels than that of the non-treated mice at the same time point after infection, and even at the terminal. A 100-fold to 1,000-fold difference in infectivity levels was apparent between the non-treated terminal mice and the cpd-B-treated terminal mice, although a less than 100-fold difference in PrPres levels between the two mouse groups was estimated from the immunoblot data shown in Fig. 4B.
On the other hand, no inconsistency was apparent in the gaps of the infectivity levels and the PrPres levels between the cpd-B-treated mice at day 63 post-infection and those at the terminal. The gap in infectivity levels between these two groups was around ten fold; ten-fold dilution of the samples of the cpd-B-treated terminal mice similarly produced no signals on the immunoblot, as observed in the samples of the cpd-B-treated day 63 post-infection mice (data not shown).
DISCUSSION

In this study, newly synthesized chemical cpd-B was discovered as an orally available anti-prion compound that is effective for prolonging the incubation periods of cerebrally infected prion disease animals. This compound has no similarity in chemical structure to previously reported anti-prion compounds, although the compound shares the following properties with anti-prion amyloidophilic chemicals we previously reported such as BSB and styrylbenzoazole chemicals: binding to PrP amyloid plaques in the brain tissue; inhibiting abnormal PrP formation in prion-infected cells without any effect on either normal PrP expression level or protease sensitivity of abnormal PrPres; preferential anti-prion effects in RML prion-infected cells rather than 22L prion-infected or Fukuoka-1 prion-infected cells; prolonging the incubation period in RML prion-infected Tga20 mouse model but never or only marginally in 263K prion-infected Tg7 mouse model. Discovery of orally available cpd-B effectiveness reinforces the idea that amyloidophilic chemicals can serve as one class of anti-prion drug candidates.

This study has unveiled that prion strains are definitely influential to the outcome of the treatment with anti-prion compounds. Treatment with cpd-B was effective against all tested prion strains, but both its anti-prion effectiveness in vitro and its therapeutic efficacy in vivo were consistently dependent on the prion strain. In fact, cpd-B was most
effective against RML prion, but less effective against 22L prion and Fukuoka-1 prion either in vitro or in vivo. In addition, its lowest effectiveness in therapeutic efficacy was demonstrated identically in either 263K prion-infected Tg7 mouse model or 263K prion-infected hamster model, although its effectiveness against 263K prion could not be evaluated on the same host background as that used for the other prion strains. It is unlikely that differences of the hosts used in this study are influential on the therapeutic efficacy of cpd-B treatment because brain chemical levels of all types of mice fed with 0.2% cpd-B for one week were not significantly different.

Amyloidophilic chemicals are not the only class of anti-prion compound that exhibits the therapeutic efficacy in a prion strain-dependent manner. Polyene antibiotic amphotericin B is another example, but it is opposite to amyloidophilic chemicals and is specifically effective against 263K prion (1). Either variation in strain-specific PrP conformational structures or variation in microenvironments facilitating PrP conformational changes might be involved in the mechanism of prion strain-dependent efficacy. Results of this study showed that prions producing di-glycosylated molecule predominant PrPres were least sensitive or resistant to cpd-B treatment, which suggests that either the conformational structure responsible for such PrPres or the di-glycosylation moieties might affect the interaction of the compound with abnormal PrP
molecules, although this inference must be examined further. The findings indicate that each class of anti-prion compounds must be examined using various prion strains to learn more about prion strain-dependency.

Even in the disease terminal, both abnormal PrP deposition levels in the brain and infectivity levels in the brain were reduced in the mice treated with cpd-B, compared to the non-treated control mice. It remains unclear why this gap occurs. One possibility is that the treated mice prematurely fell into the terminal because of accumulated cpd-B toxicity. This inference, however, does not seem to be correct because the non-infected mice treated with 0.2% cpd-B feed for more than one year showed no clinical signs and appeared healthy. Another possibility is that neuronal cells in the brain might be more vulnerable to lower levels of abnormal PrP in the presence of cpd-B, or that abnormal PrP bound with cpd-B might be more toxic to the neuronal cells in the brain. However, these inferences also seem to be unlikely because the toxicity of PrP106-126 peptide amyloid, which is reminiscent of abnormal PrP, in primary neuronal cell cultures is attenuated by the presence of cpd-B (unpublished data). Another possibility is that prion strains modified or selected from the original by the compound might multiply in the animals and cause the disease; this inference is supported by data showing that PrPres molecules with different glycoform patterns were detected in the treated mice. Further study,
however, must provide evidence to support this inference. The findings indicate that life-threatening levels of either infectivity or abnormal PrP in the brain are not necessarily the same between treated animals and non-treated animals.

A large quantity of cpd-B was needed for the efficacy in vivo; disease progression was not halted even though the treatment commenced immediately after the infection and continued to the disease terminal. This limited effectiveness of cpd-B might be partly attributable to the pharmacological properties of its rapid washout from either the brain or the blood because it is assumed that the compounds with better brain permeability and longer retention in the brain might produce more beneficial results in prion infected animals. In addition, some metabolic instability of the compound might be responsible for its limited effectiveness, especially the efficacy loss during long-term administration. In fact, cpd-B is easily metabolized in the presence of mouse liver microsome extracts (unpublished data). Therefore, the pharmacokinetic parameters of this compound must be improved for better efficacy.

The effectiveness of cpd-B is dependent upon the timing and duration of administration; the earlier start of administration is necessary to maximize beneficial results. Therefore, diagnostic measures in much earlier disease stages, especially presymptomatic stages, are vital to produce more beneficial outcomes. In addition,
multi-drug combination chemotherapy using several anti-prion compounds with different actions might produce more beneficial results. This study suggests that cpd-B inhibits new formation of abnormal PrP but does not facilitate the degradation of already formed abnormal PrP because a mixture of cpd-B with abnormal PrP did not modify the protease-resistant property of abnormal PrP. In addition, cpd-B itself has no activity to protect neuronal cells from neurotoxic insults aside from PrP amyloid (unpublished data), suggesting that cpd-B does not protect neuronal cells from neurodegenerative insults that are induced secondarily by abnormal PrP. Combinations of cpd-B with other compounds such as doxycycline, flupirtine and simvastatin might be examples, but their efficacy must be evaluated. Doxycycline is a tetracycline antibiotic known to destabilize abnormal PrP (12). Flupirtine is a centrally acting nonopioid analgesic and protects neuronal cells from apoptotic cell death induced by toxic PrP106-126 peptide amyloid (29). It was used in clinical trials, where beneficial effects on cognitive functions in patients with CJD were proved (21). Simvastatin is one of cholesterol-lowering drugs known to prevent abnormal PrP formation in prion-infected cells presumably by redistribution of normal PrP away from cholesterol-rich lipid rafts (13, 31). It prolongs survival times in prion-infected animals (16, 17).

Recently, long-term cerebroventricular administration of pentosan polysulfate
(PPS), a clinical approach based on our preclinical study in rodent models of prion
diseases (9), has been carried out in 26 patients with various types of diseases (27).
Although its therapeutic efficacy remains to be confirmed, preliminary clinical
experience indicates prolonged survival in some patients receiving long-term PPS (22,
27). Further prospective investigation of PPS administration is necessary to obtain
high-quality evidence for its clinical benefits. However, this treatment has some
weaknesses. One is the requirement for surgical implantation of a continuous infusion
pump and an intraventricular catheter, which could become an obstacle to extension of
clinical trials because of the potential risks of prion contamination in operating rooms and
of operation instruments, although most developed countries now possess clearly defined
and well established guidelines for safe surgical and anesthetic management of patients
with prion diseases. Compared to such treatments, the treatments using orally available
anti-prion compounds are absolutely preferable and practical.

The compounds tested in the study were originally designed as therapeutic lead
chemicals are very effective in vitro in either inhibiting beta-amyloid formation or
protecting neuronal cells from beta-amyloid toxicity; in addition, cpd-B has therapeutic
efficacy in an Alzheimer’s disease mouse model (unpublished data). Therefore, cpd-B is
a therapeutic candidate not only for prion diseases but also for Alzheimer’s disease. Drug
search and development for prion diseases reportedly do not interest pharmaceutical
companies because of the limited number of patients, but amyloidophilic chemicals as
drug candidates for both prion diseases and Alzheimer’s disease might attract and
accelerate the development of therapeutic drugs for prion diseases.

In conclusion, our findings related to a newly synthesized amyloidophilic chemical
cpd-B are encouraging, but further improvement of its safety profiles and
pharmacokinetic properties is necessary before clinical application can be considered.
Moreover, additional problems exist with its prion strain-dependent effectiveness and
with its reduced effectiveness if administered at later disease stages.
Acknowledgments

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References


Figure Legends

Fig. 1  Cpd-B effects on prion-infected or non-infected cells, and its amyloidophilic property.

(A) Immunoblot analyses of PrPres formation in various prion-infected cells treated with designated concentrations of cpd-B. N2a#58 is a stable transformant of N2a and expresses five times higher levels of PrP than N2a. Ch2 is a subclone of N002. Small bars on the left are molecular size markers for 41, 32 and 18 kDa.

(B) Immunoblot analysis of total normal PrP in non-infected N2a cells treated with cpd-B. Molecular size markers on the left are 47, 32, and 25 kDa.

(C) Flow cytometric analysis of cell-surface-normal PrP in non-infected N2a cells treated with 1 µM cpd-B. The solid line and broken line respectively indicate cpd-B-treated cells and non-treated cells. Gray line peaks on the left show their respective controls using isotype immunoglobulin as a first antibody.

(D) Imaging of abnormal PrP plaques in the brain tissue by cpd-B. Abnormal PrP deposition in a cerebellar tissue from a case of GSS was labeled fluorescently with cpd-B (cpd-B) and subsequently immunostained with an anti-PrP antibody (PrP). The scale bar shows 50 µm.
Orally administered cpd-B effects on cerebrally infected prion disease animals.

Cpd-B was given orally in a mixed form with powder feed *ad libitum* throughout the incubation periods in all disease animal models except the ICR-RML model, in which oral cpd-B treatment was discontinued at the time when the last of the non-treated animals became the disease terminal. In this study, the incubation periods are defined as the periods from the cerebral infection to the disease terminal. Survival rates were calculated using the incubation periods and are plotted using the Kaplan-Meier method.

Fig. 3 Cpd-B effects throughout various timings and durations of oral administration.

The Tga20 mice cerebrally infected with RML prion were treated with 0.2% cpd-B feed at different timings and durations; their incubation periods were assayed. Open bars indicate the durations of no treatment (cpd-B (-)). Shaded bars indicate the durations of oral cpd-B treatment (cpd-B (+)).

Fig. 4 Immunoblot and immunohistochemical analyses of cpd-B-treated animal brains.

(A) Immunoblot analysis of PrP in the brain of non-treated mice (no-treat) or treated mice with 0.1% cpd-B feed (0.1% cpd-B) or 0.2% cpd-B feed (0.2% cpd-B). Each lane represents an aliquot corresponding to 0.13 mg for PrPres (PK+) or 0.83 µg for total PrP...
(PK-) of brain tissue from each mouse sacrificed at a designated day post-cerebral infection (dpi). Molecular size markers on the left show 47, 32, 25, and 16 kDa.

(B) Immunoblot analysis of PrPres of some samples demonstrated in (A), which were diluted and re-assayed to equalize signal intensities of the di-glycosylated PrPres bands as much as possible, for comparison of the signal intensity and the glycoform pattern of PrPres. Molecular size markers on the left show 32, 25, and 16 kDa.

(C) Immunoblot analysis of PrPres to compare the glycoform patterns of the RML prion (RML) and the 263K prion (263K). Analyzed samples were from an RML prion-infected mouse brain and a 263K prion-infected hamster brain. Molecular size markers on the left are the same as those in (B).

(D) Immunohistochemical analysis of abnormal PrP deposition (PrP) and neurodegenerative changes by means of astrocytic glial reaction (GFAP) in the brain of non-infected mice (non-infected), infected but non-treated mice (non-treated), and infected, treated mice with 0.2% cpd-B feed (0.2% cpd-B). Data from each representative mouse sacrificed at a designated day post-cerebral infection (dpi) are shown; every picture is taken from an almost identical area of the thalamus. The sample of 0.2% cpd-B 63 dpi or 0.2% cpd-B 154 dpi is from the same individual mouse for the sample of the right lane of 0.2% cpd-B 63 dpi or the right-most lane of 0.2% cpd-B 154 dpi in (A),
respectively. The scale bar indicates 50 µm.
Tga20-RML model

Days after cerebral inoculation

Survival rate (%)

non-treated (n=6)
0.2% cpd-B (n=6)
0.13% cpd-B (n=6)
0.1% cpd-B (n=7)

ICR-RML model

Days after cerebral inoculation

Survival rate (%)

non-treated (n=14)
0.2% cpd-B (n=13)

Tga20-22L model

Days after cerebral inoculation

Survival rate (%)

non-treated (n=6)
0.2% cpd-B (n=6)

Tga20-Fukuoka-1 model

Days after cerebral inoculation

Survival rate (%)

non-treated (n=8)
0.2% cpd-B (n=5)

Tg7-263K model

Days after cerebral inoculation

Survival rate (%)

non-treated (n=6)
0.2% cpd-B (n=11)

Hamster-263K model

Days after cerebral inoculation

Survival rate (%)

non-treated (n=7)
0.2% cpd-B (n=6)
A

\begin{tabular}{ccc}
\text{no-treat} & 0.1\% cpd-B & 0.2\% cpd-B \\
63 & 63 & 108 \\
63 & 120 & 154 \\
\end{tabular}

PK+

PK-

B

\begin{tabular}{crrrr}
\text{no-treat} & 0.1\% cpd-B & 0.2\% cpd-B \\
\text{dilution} & 63 & x6 & x8 & x15 \\
& 108 & x1 & x2 & x2 \\
& 120 & x1 & x2 & x1 & x4 & x1 \\
\end{tabular}

C

\begin{tabular}{c}
RML 263K \\
\end{tabular}

D

\begin{tabular}{cccc}
\text{non-infected} & \text{non-treated} & 0.2\% cpd-B & 0.2\% cpd-B \\
63 dpi & 63 dpi & 63 dpi & 154 dpi \\
\end{tabular}

\begin{tabular}{c}
PrP \\
GFAP \\
\end{tabular}
### Table 1. Tested compounds in the study and their inhibition activity of abnormal PrP formation in ScN2a cells.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Chemical structure</th>
<th>Molecular weight</th>
<th>Octanol-water distribution coefficient $^a$ (LogD$_{6.5}$)</th>
<th>Inhibition activity of abnormal PrP formation (approximate EC$_{50}$) $^b$ (nM)</th>
<th>Maximum tolerant dose $^c$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cpd·B</td>
<td><img src="image1" alt="Chemical structure" /></td>
<td>264</td>
<td>4.1</td>
<td>0.06</td>
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<td>Cpd·D1</td>
<td><img src="image2" alt="Chemical structure" /></td>
<td>347</td>
<td>2.2</td>
<td>10</td>
<td>&gt; 10</td>
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<td>Cpd·D2</td>
<td><img src="image3" alt="Chemical structure" /></td>
<td>342</td>
<td>3.6</td>
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<tr>
<td>Cpd·D3</td>
<td><img src="image4" alt="Chemical structure" /></td>
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<td>3.2</td>
<td>&gt; 10</td>
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<tr>
<td>Cpd·D4</td>
<td><img src="image5" alt="Chemical structure" /></td>
<td>319</td>
<td>Not determined</td>
<td>1</td>
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<tr>
<td>Cpd·D5</td>
<td><img src="image6" alt="Chemical structure" /></td>
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<td>306</td>
<td>2.4</td>
<td>10</td>
<td>&gt; 10</td>
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</table>

$^a$ The distribution coefficient, a measure of a compound's hydrophilicity or hydrophobicity, was estimated using ChemAxon's calculator plugin software (Budapest, Hungary). The coefficients of medicines used for brain diseases are generally around 3.0.

$^b$ The approximate dose giving 50% inhibition of abnormal PrP formation relative to the control.

$^c$ Maximal tolerant dose that does not affect the rate of cell growth to confluence.
Table 2. Infectivity assays of the brain of non-treated mice or cpd-B-treated mice.

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Non-treated mice (63 dpi)</th>
<th>Cpd-B treated mice (63 dpi)</th>
<th>Cpd-B treated mice (154 dpi)</th>
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<tr>
<td></td>
<td>Mouse ID no.</td>
<td>No. of diseased mice/total</td>
<td>Mean incubation time (days) ± SD</td>
</tr>
<tr>
<td>10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>cnt-1 8/8 77.4 ± 4.5</td>
<td>bc-1&lt;sup&gt;d&lt;/sup&gt; 7/7 284.1 ± 54.6</td>
<td>bl-1 7/7 122.0 ± 14.0</td>
</tr>
<tr>
<td></td>
<td>cnt-2 6/6 84.4 ± 6.9</td>
<td>bc-2&lt;sup&gt;c&lt;/sup&gt; 8/8 136.8 ± 19.2</td>
<td>bl-2&lt;sup&gt;f&lt;/sup&gt; 7/7 92.6 ± 8.9</td>
</tr>
<tr>
<td></td>
<td>cnt-3 7/7 75.7 ± 7.4</td>
<td>bc-2&lt;sup&gt;e&lt;/sup&gt; 1/8 &gt;140&lt;sup&gt;c&lt;/sup&gt;</td>
<td>bl-3 7/7 89.1 ± 4.0</td>
</tr>
<tr>
<td>10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>cnt-1 8/8 77.4 ± 4.5</td>
<td>bc-1&lt;sup&gt;d&lt;/sup&gt; 0/7 &gt;140&lt;sup&gt;c&lt;/sup&gt;</td>
<td>bl-1 7/7 &gt;140&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>cnt-2 6/6 75.3 ± 2.4</td>
<td>bc-2&lt;sup&gt;e&lt;/sup&gt; 1/8 &gt;140&lt;sup&gt;c&lt;/sup&gt;</td>
<td>bl-2&lt;sup&gt;f&lt;/sup&gt; 7/7 97.4 ± 9.6</td>
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<tr>
<td></td>
<td>cnt-3 7/7 75.7 ± 7.4</td>
<td>bc-2&lt;sup&gt;e&lt;/sup&gt; 1/9 &gt;140&lt;sup&gt;c&lt;/sup&gt;</td>
<td>bl-3 9/9 101.6 ± 7.4</td>
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<tr>
<td>10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>cnt-1 7/7 88.4 ± 7.3</td>
<td>bc-1&lt;sup&gt;d&lt;/sup&gt; 1/8 &gt;140&lt;sup&gt;c&lt;/sup&gt;</td>
<td>bl-2&lt;sup&gt;f&lt;/sup&gt; 3/7 &gt;140&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>cnt-1 6/6 155.7 ± 55.3</td>
<td>bc-2&lt;sup&gt;e&lt;/sup&gt; 1/9 &gt;140&lt;sup&gt;c&lt;/sup&gt;</td>
<td>bl-3 3/7 &gt;140&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>cnt-2 7/7 105.4 ± 16.5</td>
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<td></td>
<td>cnt-3 7/7 95.9 ± 7.1</td>
<td></td>
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<tr>
<td>10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>cnt-1 1/7 &gt;420&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>10&lt;sup&gt;7&lt;/sup&gt;</td>
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<td>cnt-1 2/7 &gt;420&lt;sup&gt;b&lt;/sup&gt;</td>
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<td></td>
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</tbody>
</table>

<sup>a</sup> Days post-cerebral inoculation.
<sup>b</sup> Observed up to 420 days post-inoculation.
<sup>c</sup> Observed up to 140 days post-inoculation.
<sup>d</sup> This is the mouse for the sample of the left lane of 0.2% cpd-B 63 dpi in Fig. 4A.
<sup>e</sup> This is the mouse for the sample of the right lane of 0.2% cpd-B 63 dpi in Fig. 4A.
<sup>f</sup> This is the mouse for the sample of the right-most lane of 0.2% cpd-B 154 dpi in Fig. 4A.