Orally Administered Amyloidophilic Compound Is Effective in Prolonging the Incubation Periods of Animals Cerebrally Infected with Prion Diseases in a Prion Strain-Dependent Manner

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The establishment of effective therapeutic interventions for prion diseases is necessary. We report on 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% [wt/wt] in feed) was given orally to cerebrally RML prion-inoculated mice from inoculation until the terminal stage of disease, it extended the incubation periods by 2.3 times compared to the control. The compound exerted therapeutic efficacy in a prion strain-dependent manner such as that 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 an earlier start of administration. The glycoform pattern of the abnormal prion protein in the treated mice was modified and showed predominance of the diglycosylated form, which resembled that of 263K prion, suggesting that diglycosylated 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 the 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.

Caughey and colleagues first found Congo red and sulfated glycans to inhibit abnormal PrP formation in vitro (5, 6), although Congo red was much earlier described as a staining device for prion amyloid rods (23). Since the discovery of the therapeutic activity of Congo red, amyloidophilic compounds such as amyloid dye derivatives and glucosaminoglycan mimetics have been noted as one class of possible therapeutic candidates for prion diseases (4, 32). Recently, the most advanced progress with amyloidophilic compounds, which have an excellent ability to permeate 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 brains 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 antiprion compounds and prolong the incubation periods of animals cerebrally infected with prion disease (14). We also reported that a new class of amyloidophilic chemicals, styrylbenzoazole derivatives, which have better penetration through the blood-brain barrier and which show more discrete labeling of amyloid deposition in brain tissues affected by either Alzheimer’s disease or prion diseases, are effective as antiprion chemicals (15, 19). However, the efficacy of these amyloidophilic compounds, intravenously administered weekly, was not remarkable but was rather limited. In addition, their effectiveness was suggested to be prion strain dependent, 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 study, 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 antiprion compounds was synthesized and tested for either antiprion activity in vitro or therapeutic efficacy in vivo when administered orally as a mixture with feed.
TABLE 1. Tested compounds and their inhibition of abnormal PrP formation in ScN2a cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>Chemical structure</th>
<th>Mol wt</th>
<th>Octanol-water distribution coefficient&lt;sup&gt;a&lt;/sup&gt; (log &lt;i&gt;D&lt;sub&gt;4,0&lt;/sub&gt;&lt;/i&gt;)</th>
<th>Inhibition of abnormal PrP (approximate Et&lt;sub&gt;50&lt;/sub&gt; nM)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Maximum tolerance dose&lt;sup&gt;c&lt;/sup&gt; (µM)</th>
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<td>347</td>
<td>2.2</td>
<td>100</td>
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<tr>
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<td><img src="image" alt="cpd-D2 structure" /></td>
<td>342</td>
<td>3.6</td>
<td>10</td>
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<td>293</td>
<td>3.2</td>
<td>1</td>
<td>&gt;10</td>
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<td>Not determined</td>
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<tr>
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<td>4.7</td>
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<td>2.4</td>
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<sup>a</sup> 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.

<sup>b</sup> The approximate dose giving 50% inhibition of abnormal PrP formation relative to the control.

<sup>c</sup> The maximal dose that does not affect the rate of cell growth to confluence.

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 structures of the compounds are shown in Table 1. The compounds were dissolved in 100% dimethyl sulfoxide 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 antiprion compounds, either mouse neuroblastoma cells (N2a cells) or N2a cells with fivefold PrP overexpression (N2a-58 cells) which were persistently infected with a distinct prion strain were used, as follows: N2a cells infected with RML scrapie prion (ScN2a cells) (25), N2a cells with 22L scrapie prion (N167 cells), N2a-58 cells with RML scrapie prion (N002 or Ch2 cells), or N2a-58 cells with Fukuoka-1 GSS prion (F3 cells) (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 animal disease models after 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 cases of GSS, which were kindly provided by 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-µm-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 a fluorescein isothiocyanate 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 consisting of amino acids 214 to 296 (1:200; Immuno-Biological Laboratories Co. Ltd., Gunma, Japan), followed by incubation with EnVision System horseradish peroxidase 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. Antiprion activity was evaluated by assaying the content of protease-resistant PrP (PrPres) in the cellular models, 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 consisting of amino acids 214 to 296 (1:200; Immuno-Biological Laboratories Co. Ltd., Gunma, Japan), followed by incubation with EnVision System horseradish peroxidase labeling polymer (Dako, Glostrup, Denmark). The reaction product was developed with 3,3'-diaminobenzidine tetrahydrochloride solution and counterstained with hematoxylin.
using a monoclonal antibody, SAF83 (1:5000; SPIO-Bio, Massy, France), followed by an alkaline phosphatase-conjugated goat anti-mouse antibody (1:20,000; Pro-mega Corp., Madison, WI). Immunoactivity 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 by 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 and incubated with SAF83 (1:500) or isotype-matched control immunoglobulin G1 for 20 min on ice. Cells were washed with 0.5% fetal calf serum in PBS and incubated with goat F(ab)'2 fragment anti-mouse immunoglobulin G (heavy plus light chain)-phycocerythrin (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 previously (20) after a 1-week feeding with 0.2% cpd-B ad libitum. All animals were sacrificed at 9:00 a.m. of day 8 by excision of the carotid artery under deep ether anesthesia to remove as much blood as possible, and the brain was collected, rinsed with saline, and weighed. Because preliminary studies found no significant difference in the data for perfused brains and nonperfused 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 9 volumes of 20 mM phosphate buffer, pH 6.5 (PB), and then filtered to obtain the sample for analysis. The sample was then applied to a conditioned C18 solid-phase extraction cartridge. The compound was eluted with methanol and was diluted with an equal volume of PB. The compound then was analyzed using an EPICS XL-ADC flow cytometer (Beckman Coulter Inc., CA).

RESULTS

Antiprion activity in vitro. The antiprion activities of newly synthesized compounds were 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 concentration (EC50) of about 60 pM, cpd-B inhibited PrPres formation (Table 1 and Fig. 1A). Other related compounds were also potent within a nontoxic dose range of up to 10 μM.

To investigate whether the efficacies of the compounds depend on the pathogen strain, 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 effective only in N002 (EC50, 320 nM) and Ch2 (EC50, 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 that 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 of less than 10 μM. However, at a dose of 10 μM, a marginal reduction of the PrPres signals was observed in both cells. At a dose of 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 noninfected N2a cells treated with 1 μM cpd-B, using either immunoblot analysis of the cell lysate without protease digestion or flow cytometry analysis of the cell surface PrP (Fig. 1B and C). In addition, cpd-B did not facilitate the digestion of abnormal PrP by protease K, nor did it interfere with immunodetection, because PrPres signals were not modified after cpd-B was mixed and incubated with a cell lysate of nontreated ScN2a cells before protease K digestion (data not shown).

Pharmacological properties. Abnormal PrP amyloid imaging by cpd-B was performed on brain sections of GSS cases to examine the 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
The nontreated infected mice started exhibiting abnormal signs such as staggering, rotating, irritation, and motionlessness at about 2 months after the infection; the mice then wasted into the terminal stage of disease in a week. Treatment by feeding cpd-B-containing feed ad libitum was initiated immediately after the infection and continued until the terminal stage of disease. The cpd-B-treated mice did not exhibit such abnormal signs as described above and wasted gradually into the terminal stage of disease. As shown in Fig. 2A, oral cpd-B treatment significantly prolonged the incubation periods of infected Tga20 mice in a dose-dependent manner; these were 68.5 ± 5.9 days in the nontreated 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 of 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 [percentage 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 observed only in Tga20 mice infected with RML prion \((14, 15)\). ICR mice were then examined for the therapeutic efficacy of oral cpd-B treatment to investigate whether effectiveness of amyloidophilic compounds is restricted only to Tga20 mice. Nontreated control ICR mice that had been cerebrally infected with RML scrapie prion were in the terminal stage of disease at 154.2 ± 18.4 days postinfection, whereas the mice treated with 0.2% cpd-B feed lived significantly longer \((P < 0.01)\). Even though the oral cpd-B treatment was discontinued at day 187 postinfection when the last of the nontreated animals reached the terminal stage of disease, more than half of the treated mice survived to day 270 postinfection (Fig. 2B).
Next, the therapeutic efficacy of oral cpd-B treatment against other prion strains was investigated. The cpd-B treatment significantly prolonged the incubation periods of Tga20 mice that had been cerebrally infected with 22L scrapie prion \( (P < 0.01) \); these were 96.3 \( \pm \) 5.9 days in the nontreated control mice and 126.3 \( \pm \) 10.3 days in the mice treated with 0.2% cpd-B feed, indicating a 1.3-fold extension of the incubation period (Fig. 2C). Control mice started exhibiting distinguished opisthotonus with head rotating a week before the terminal stage of disease, whereas cpd-B treated mice showed no such clinical sign, even in the terminal stage.

cpd-B was also effective against Fukuoka-1 GSS prion. Cerebrally infected Tga20 mice lived significantly longer with oral cpd-B treatment \( (P < 0.05) \), i.e., 101.6 \( \pm \) 12.1 days for the nontreated control mice and 142.2 \( \pm \) 21.0 days for the mice treated with 0.2% cpd-B feed, indicating a 1.4-fold extension of the incubation period (Fig. 2D). Staggering was observed as an initial clinical sign in the control mice more than 1 week before the terminal stage of disease, although this clinical sign was not seen in the cpd-B treated mice.

In contrast to the case for 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 \( (52.7 \pm 2.8 \text{ days}) \) were significantly but very marginally longer than those of the nontreated mice \( (48.0 \pm 3.0 \text{ days}) \) \( (P < 0.05) \). 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 attributable chiefly 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 nontreated control hamsters that had been cerebrally infected with 263K prion \( (P < 0.05) \); 107.0 \( \pm \) 2.5 days in the cpd-B treated hamsters and 97.4 \( \pm \) 6.9 days in the non-
mice were treated with 0.2% cpd-B feed at different times and for different durations, and the 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 (+)].

These results indicate that oral cpd-B treatment is not as effective for 263K prion.

**Timing and duration of dosing.** The effectiveness of cpd-B at 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 nontreated mice were 63.0 ± 1.8 days, whereas the incubation periods of the mice treated with 0.2% cpd-B feed were inversely correlated with the postinfection durations to the commencement of cpd-B treatment (r = −0.79; P < 0.01); 174.5 ± 7.6 days when started at day 0 postinfection, 117.2 ± 7.0 days when started at day 35 postinfection, and 88.7 ± 17.3 days when started at day 49 postinfection. 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 when treated for 35 days from the infection, and 142.5 ± 7.8 days when treated 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 brains of cpd-B-treated mice was analyzed by immunoblotting and compared with that in the nontreated control mice (Fig. 4A). The PrPres signals in the nontreated mice were very strong at the terminal stage of disease (day 63 postinfection). In contrast, in the mice treated with 0.1% cpd-B feed from the start of infection, PrPres signals were faint at day 63 postinfection and distinct at the terminal stage of disease (day 108 postinfection). However, the PrPres signals at the terminal stage of disease did not reach the high level shown by the nontreated control mice at that stage. Comparison of the signal intensities of the diglycosylated PrPres form showed that 6- to 15-fold-diluted samples from the nontreated terminal mice exhibited signal intensities similar to those of undiluted or 2-fold-diluted samples from the 0.1% cpd-B-treated terminal mice (Fig. 4B). Similarly, in the mice treated with 0.2% cpd-B feed from the start of infection, PrPres signals gradually increased according to the time course after infection: no signals were detected at day 63 postinfection, distinct signals were detected at day 120, and similar or more distinct signals were detected at the terminal stage of infection (day 154 postinfection). The PrPres signals were 63.0 ± 1.8 days when started at day 0 postinfection, and 117.2 ± 7.0 days when started at day 35 postinfection, and 88.7 ± 17.3 days when started at day 49 postinfection. On the other hand, the incubation periods of the mice treated with 0.2% cpd-B feed were inversely correlated with the postinfection durations to the commencement of cpd-B treatment (r = −0.79; P < 0.01); 174.5 ± 7.6 days when started at day 0 postinfection, 117.2 ± 7.0 days when started at day 35 postinfection, and 88.7 ± 17.3 days when started at day 49 postinfection. 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 when treated for 35 days from the infection, and 142.5 ± 7.8 days when treated 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.

The prion signal intensities of the samples from the cpd-B-treated mice at the terminal stage of disease were indistinguishable from those of the cpd-B-treated mice at the terminal stage of disease.

**Infectivity analysis.** Infectivity levels are inversely correlated with incubation periods (24). Therefore, infectivity levels of the brains 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^−5-fold-diluted brain homogenates from the cpd-B-treated mice at day 63 postinfection exhibited incubation periods similar to those of the 10^−9-fold- or greater diluted brain homogenates from the nontreated mice; the 10^−2-fold-diluted brain homogenates from the cpd-B-treated mice at the terminal stage of disease (day 154 postinfection) showed incubation periods similar to those of the 10^−5-fold- or 10^−4-fold-diluted brain homogenates from the nontreated mice. The data indicate that the brains of mice treated with 0.2% cpd-B feed had much lower infectivity levels than those of the nontreated mice at the same time point after infection and even at the terminal stage of disease. A 100-fold to 1,000-fold difference in infectivity levels was apparent between the nontreated 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 in the infectivity levels and the PrPres levels between the cpd-B-treated mice at day 63 postinfection and those at the terminal stage of disease. The gap in infectivity levels between these two groups was around 10-fold; 10-fold dilution of the samples from the cpd-B-treated terminal mice similarly produced no signals on the immunoblot, as observed in the samples from the cpd-B-treated mice at day 63 postinfection (data not shown).
DISCUSSION

In this study, the newly synthesized chemical cpd-B was discovered as an orally available antiprion compound that is effective for prolonging the incubation periods of animals cerebrally infected with prion diseases. This compound has no similarity in chemical structure to previously reported antiprion compounds, although the compound shares the following properties with antiprion amyloidophilic chemicals we previously reported, such as \((\text{trans}, \text{trans})\)-1-bromo-2,5-bis-(3-hydroxycarbonyl-4-hydroxy)styrylbenzene 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 antiprion effects in RML prion-infected cells rather than 22L prion-infected or Fukuoka-1 prion-infected cells, and prolonging the incubation period in the RML prion-infected Tga20 mouse model but never or only marginally in the 263K prion-infected hamster model. Molecular size markers on the left are the same as those in panel B. (D) Immunohistochemical analysis of abnormal PrP deposition (PrP) and neurodegenerative changes by means of astrocytic glial reaction (glial fibrillary acidic protein [GFAP]) in the brains of noninfected mice, infected but nontreated mice, and infected mice treated with 0.2% cpd-B feed. Data from each representative mouse sacrificed at a designated day after cerebral infection (dpi) are shown; every picture was taken from an almost identical area of the thalamus. The samples of 0.2% cpd-B at 63 dpi and 0.2% cpd-B at 154 dpi are from the same individual mouse as the samples in the right lane of 0.2% cpd-B 63 dpi and the rightmost lane of 0.2% cpd-B 154 dpi in panel A, respectively. Bar, 50 μm.

Amyloidophilic chemicals are not the only class of antiprion compound that exhibits the therapeutic efficacy in a prion strain-dependent manner. The 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. The results of this study showed that prions producing predominantly diglycosylated PrPres molecules were less sensitive or resistant to cpd-B treatment, which suggests that either the conformational structure responsible for PrPres or the diglycosylation 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 antiprion compounds must be examined using various prion strains to learn more about prion strain dependency.

Even in the terminal stage of disease, 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 nontreated control mice. It remains unclear why this gap occurs. One possibility is that the treated mice prematurely fell into the terminal stage because of accumulated cpd-B toxicity. This inference, however, does not seem to be correct, because the noninfected mice treated with 0.2% cpd-B feed for more than 1 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 nontreated animals.

A large quantity of cpd-B was needed for efficacy in vivo; disease progression was not halted even though the treatment commenced immediately after the infection and continued to the terminal stage of disease. 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 permeativity 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 loss of efficacy 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; an earlier start of administration is

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### TABLE 2. Infectivity assays of the brains of nontreated mice or cpd-B-treated mice

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<th>Mean incubation time (days) ± SD</th>
<th>Mouse no.</th>
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<td>0/7</td>
<td>$&gt;140^f$</td>
<td>bc-2&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1/8</td>
<td>$&gt;140^f$</td>
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<tr>
<td></td>
<td>cnt-2</td>
<td>6/6</td>
<td>$75.3 ± 2.4$</td>
<td>bc-1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>7/7</td>
<td>$14.0$</td>
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<td></td>
<td>cnt-3</td>
<td>7/7</td>
<td>$75.7 ± 7.4$</td>
<td>bc-2&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1/8</td>
<td>$&gt;140^f$</td>
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<sup>a</sup> Days after cerebral inoculation.
<sup>b</sup> Observed up to 420 days postinoculation.
<sup>c</sup> Observed up to 140 days postinoculation.
<sup>d</sup> Mouse for the sample in the left lane of 0.2% cpd-B at 63 dpi in Fig. 4A.
<sup>e</sup> Mouse for the sample in the right lane of 0.2% cpd-B at 63 dpi in Fig. 4A.
<sup>f</sup> Mouse for the sample of the rightmost lane of 0.2% cpd-B at 154 dpi in Fig. 4A.
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, multidrug combination chemotherapy using several antiprion 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 a cholesterol-lowering drug 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 antiprion compounds are absolutely preferable and practical.

The compounds tested in the study were originally designed as therapeutic lead chemicals for the treatment of Alzheimer’s disease. In fact, cpd-B and related 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. The search for and development of drugs for prion diseases reportedly do not interest pharmaceutical companies because of the limited number of patients, but the possible use of 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 the 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.

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