

than other TSEs. However, at present there is no evidence to indicate that younger age is a positive prognostic factor for survival of TSE patients. Experience shows that administration of PPS early in the clinical course of the disease, rather than young age, may significantly extend the survival of TSE patients.

Proof of efficacy of PPS in TSE has been difficult due to the lack of specific and objective criteria for measuring the response to treatment.

Further clinical, neuroradiological and laboratory investigations will be essential for the evaluation of possible benefits of PPS administration.

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Chelating Compound, Chrysoidine, Is More Effective in Both Antiprion Activity and Brain Endothelial Permeability Than Quinacrine

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SUMMARY

1. As an extension of our previous study of quinacrine and its derivatives, chelating chemicals were screened to obtain more effective, better brain-permeable antiprion compounds using either prion-infected neuroblastoma cells or brain capillary endothelial cells.

2. Eleven chemicals were found to have antiprion activity. Most of them shared a common structure consisting of benzene or naphthalene at either end of an azo bond. Structure–activity data suggest that chelating activity is not necessary but might contribute to the antiprion action.

3. Chrysoidine, a representative compound found here, was about 27 times more effective in the antiprion activity and five times more efficiently permeable through the brain capillary endothelial cells than quinacrine was.

4. These chemicals might be useful as compounds for development of therapeutics for prion diseases.

KEY WORDS: prion; chrysoidine; blood–brain barrier; aromatic azo compounds; therapy; chelating agents; brain endothelial cells; prion-infected neuroblastoma cells.

INTRODUCTION

Transmissible spongiform encephalopathies or prion diseases are fatal neurodegenerative disorders that include Creutzfeldt–Jakob disease and Gerstmann–Sträussler–Scheinker syndrome 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

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(PrP), which is putatively a main component of pathogens or the pathogen itself, and which is rich in beta-sheet structure and resistant to digestion with proteinase K (Prusiner, 1991). Recent outbreaks of variant Creutzfeldt–Jakob disease and iatrogenic Creutzfeldt–Jakob disease through use of cadaveric growth hormone or dural grafts in younger people have necessitated the development of suitable therapies.

We previously found quinacrine and its derivatives to have potent antiprion activity in prion-infected cells (Doh-Ura *et al.*, 2000; Murakami-Kubo *et al.*, 2004). The common structure of these chemicals, a quinoline ring with a side chain containing a nitrogen atom located at a particular distance from another nitrogen atom in the ring indicates that the chemicals have chelating activity, but the involvement of chelating metals in their antiprion activity has never been confirmed. Quinacrine has been used recently for clinical trials of patients with prion diseases in several countries. Orally administered quinacrine is reportedly effective in transiently improving cognitive functions of patients (Nakajima *et al.*, 2004), but it frequently causes such adverse effects as liver dysfunction. For that reason, either improving its penetration into the brain (the target organ of prion diseases) or reducing its uptake into the liver is suggested for producing more beneficial results (Dohgu *et al.*, 2004).

Here, to obtain more effective antiprion compounds with better brain permeability than quinacrine, we screened chelating chemicals in prion-infected neuroblastoma cells. We investigated the brain permeability of a representative chemical using an *in-vitro* model for the blood–brain barrier.

MATERIALS AND METHODS

Chemicals and Cells

Chemicals used in the study were purchased from Sigma-Aldrich Corp. (St. Louis, MO), Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan), and Wako Pure Chemical Industries Ltd. (Osaka, Japan). All chemicals, except for chrysoidine, were dissolved in 100% dimethyl sulfoxide (DMSO), although chrysoidine was dissolved in distilled water.

Acetylated Yellow AB was obtained as follows. Yellow AB was dissolved in dichloromethane and mixed with excess glacial acetic acid. After its complete acetylation was observed by thin layer chromatography, the acetylated product was purified using silica gel column chromatography (dichloromethane/ethyl acetic acid: 9/1 (v/v)). The residual solid was lyophilized and identified as acetylated Yellow AB by both fast atom bombardment mass spectrometry and elemental analysis.

Murine neuroblastoma (NB) cells that had been persistently infected with the scrapie prion strain RML (ScNB cells) (Race *et al.*, 1988) were used for the assay of antiprion activity and grown in Opti-MEM (Invitrogen Corp., CA) containing 10% fetal bovine serum. For the assay of brain endothelial permeability, immortalized endothelial cells from the murine brain capillary (MBEC4 cells) (Tatsuta *et al.*, 1992) were used and grown in DMEM (Invitrogen Corp., CA) containing 10% fetal bovine serum, 100 $\mu\text{g}/\text{mL}$ streptomycin, and 100 units/mL penicillin.

Antiprion Activity Assay

Antiprion activity of a chemical was assayed by measuring its 50% inhibition dose (IC_{50}) for abnormal PrP formation in ScNB cells, as described previously (Doh-Ura *et al.*, 2000; Ishikawa *et al.*, 2004). Each chemical was added at designated concentrations when cells were passed at 10% confluency. The final concentration of DMSO in the medium was maintained at less than 0.5%. The cells were allowed to grow to confluence and were lysed with a lysis buffer (0.5% sodium deoxycholate, 0.5% Nonidet P-40, PBS). The lysates were digested with 10 $\mu\text{g}/\text{mL}$ proteinase K for 30 min and centrifuged at $100,000 \times g$ for 30 min at 4°C . The pellets were resuspended in the sample loading buffer and boiled. The samples were separated using electrophoresis on a 15% Tris-glycine-SDS-polyacrylamide gel and electroblotted. Detection of PrP was done using an antibody PrP-2B, followed by an alkaline phosphatase-conjugated secondary antibody. Immunoreactive signals were visualized with CDP-Star detection reagent (GE Healthcare Bio-Science, NJ) and were analyzed densitometrically. Three independent assays were performed in each experiment.

Cellular PrP Assay

The total level of normal cellular PrP was assayed similarly in noninfected NB cells treated with a chemical. Briefly, the cells were treated with a chemical as described earlier and lysed with the lysis buffer. Four volumes of the lysate were added to one volume of the five times concentrated sample loading buffer and boiled. Then, the samples were analyzed by immunoblotting as described earlier. The cell surface level of normal cellular PrP was assayed by flow cytometry described previously (Kim *et al.*, 2004). Briefly, NB cells dispersed by the treatment with ice-cold PBS containing 0.1% collagenase (Wako Pure Chemicals, Osaka, Japan) were washed with 0.5% fetal bovine serum in PBS (FBS/PBS) and incubated with an antibody SAF83 (1:500) (SPI-BIO, Massy, France) for 30 min on ice. Cells were washed with FBS/PBS and incubated with goat F(ab')₂ fragment antimouse IgG(H + L)-PE (Beckman Coulter, CA) for 30 min. After washing, cells were analyzed using an EPICS XL-ADC flow cytometer (Beckman Coulter, CA).

Surface Plasmon Resonance Assay

Binding assay of a chemical with recombinant PrP was performed using an optical biosensor (Biacore AB, Uppsala, Sweden), as described previously (Kawatake *et al.*, 2006). Briefly, recombinant mouse PrP (amino acids 121–231; PrP121–231) was immobilized on a biosensor chip at a density of *ca.* 3,000 resonance units (RU) using amine coupling. Test chemicals were diluted to 50 μM with the running buffer (3% DMSO in PBS, pH 7.4) and were injected over both the PrP flow cell and the reference at a flow rate of 20 $\mu\text{L}/\text{min}$. The dissociation phase was monitored with injection of the running buffer at a flow rate of 20 $\mu\text{L}/\text{min}$. The flow cell was washed with 10 mM NaOH for 30 s between sample injections. Buffer blanks for double referencing were injected before sample analyses.

Brain Endothelial Permeability Assay

Permeability assay was performed as described previously (Dohgu *et al.*, 2004). Briefly, MBEC4 cells were cultured on the collagen-coated polycarbonate membrane of a Transwell insert (Corning Coster Corp., MA). Before assay, the cells were washed with Krebs–Ringer buffer (118 mM NaCl, 4.7 mM KCl, 1.3 mM CaCl₂, 1.2 mM MgCl₂, 1.0 mM NaH₂PO₄, 25 mM NaHCO₃, 11 mM D-glucose, pH 7.4). Then, the buffer (1.5 mL) was added outside of the insert (abluminal side), and the buffer (0.5 mL) containing 100 μ M of a chemical was loaded on the luminal side of the insert. Samples (0.5 mL) were recovered from the abluminal chamber at 10, 20, 30, and 60 min and replaced immediately with fresh Krebs–Ringer buffer. Sodium fluorescein (Na-F, MW 376; Sigma-Aldrich Corp., MO) was used as a paracellular transport marker, and chrysoidine (Tokyo Kasei Kogyo Co. Ltd., Tokyo, Japan) as a test chemical, in addition to quinacrine as a control. The chemical concentration was measured by either determining the fluorescent intensity of Na-F (Ex(λ) 485 nm; Em(λ) 530 nm) and quinacrine (Ex(λ) 450 nm; Em(λ) 530 nm) or determining the absorbance of chrysoidine at 450 nm. The permeability coefficient was calculated using the slope of clearance curve for each chemical obtained during the 60-min period according to the method described by Dehouck *et al.* (Dehouck *et al.*, 1992). Statistical analysis was performed using one-way analysis of variance followed by Tukey–Kramer method for multiple comparisons.

RESULTS

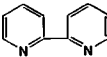
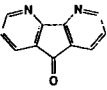
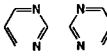
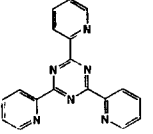
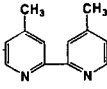
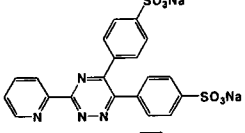
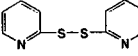
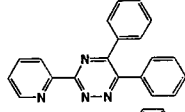
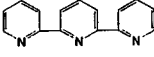
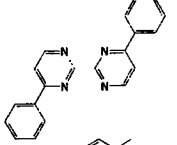
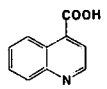
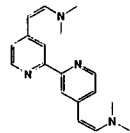
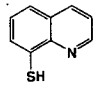
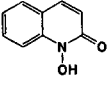
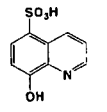
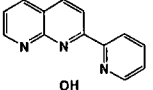
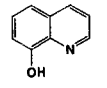
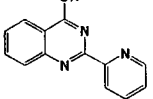
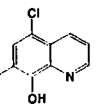
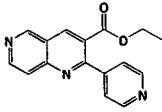
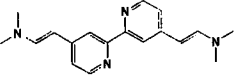
Antiprion Screening *in vitro*

To evaluate functional groups of antiprion chelating chemicals, various chelating chemicals were examined for whether they inhibited abnormal PrP formation in prion-infected ScNB cells. Thirty-five chelating chemicals were analyzed; 11 of them were effective in inhibiting abnormal PrP formation for doses at which cell toxicity was not observed (Tables I and II). Nine of the 11 effective chemicals had a common structure, which consisted of aromatic rings (terminals 1 and 2 in Table II) at both ends of an azo bond. Although both 4-methyl-2-(2-thiazolylazo)phenol and 4-(2-pyridylazo)resorcinol were not effective, they also exhibited this structure, with a thiazole ring and a pyridine ring in the terminal 1 portion, respectively. Their lack of effectiveness might be attributable to cell toxicity, which occurred at lower doses than for chemicals carrying a benzene ring in the terminal 1 portion. On the other hand, all chemicals carrying either a benzene ring or a naphthalene ring in the terminal 2 portion were effective. Therefore, the data suggest that a structure with such an aromatic ring as benzene or naphthalene in either end of an azo bond might be responsible for inhibiting abnormal PrP formation in ScNB cells.

Mechanism of Antiprion Action

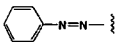
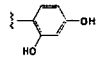
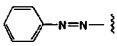
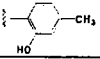
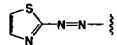
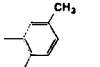
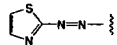
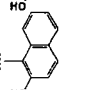
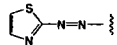
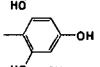
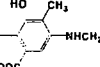
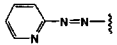
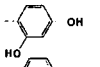
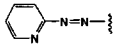
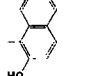
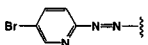
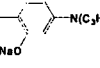
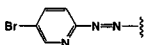
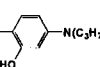
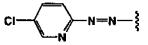
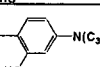
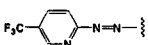
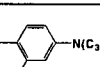
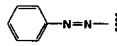
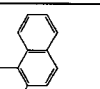
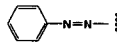
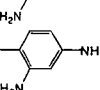

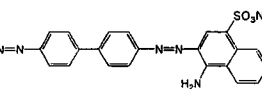
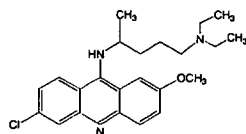
We tested whether the effective chemicals cause any alteration of the cellular PrP level in the treated cells because reduction in the cellular PrP level engenders

Table I. Antiprion Activity in ScNB Cells of Chelating Compounds

Compound	IC ₅₀ (μM)	CM (μM)	Compound	IC ₅₀ (μM)	CM (μM)
	-	75		-	>100
	-	>100		-	25
	-	10		-	>100
	-	10		-	10
	-	0.5		5	25
	-	>100		-	5
	-	5		-	25
	-	>100		25	>200
	-	5		-	10
	-	10		-	>100
				-	5

Note. IC₅₀: approximate dose giving 50% inhibition of abnormal PrP formation relative to the control. CM: approximate maximal dose that does not affect the rate of cell growth to confluence.

Table II. Antiprion Activity in ScNB Cells of Chelating Azo Compounds

Compound	Terminal 1 -N=N-	Terminal 2	IC ₅₀ (μM)	CM(μM)
Phenylazoresorcinol			0.3	50
2-Phenylazo-4-methylphenol			0.3	75
4-Methyl-2-(2-thiazolylazo)phenol			-	0.5
1-(2-Thiazolylazo)-2-naphtol			-	0.5
4-(2-Thiazolylazo)resorcinol			3	5
TAMSMB			-	>100
4-(2-Pyridylazo)resorcinol			-	0.25
1-(2-Pyridylazo)-2-naphtol			-	1
5-Br-PAPS			15	20
5-Br-PADAP			4	10
5-Cl-PADAP			2	5
5-CF3-PADAP			4	10
Yellow AB			0.5	100
Chrysoidine			0.015	>100
Congo red			0.014	not tested
Quinacrine			0.4	2

Note. IC₅₀: approximate dose giving 50% inhibition of abnormal PrP formation relative to the control. CM: approximate maximal dose that does not affect the rate of cell growth to confluence. TAMSMB: 4-methyl-5-sulfomethylamino-2-(2-thiazolylazo)benzoic acid. PAPS: 2-(2-pyridylazo)-5-[N-n-propyl-N-(3-sulfopropyl)amino]phenol, disodium salt. PADAP: 2-(2-pyridylazo)-5-diethylaminophenol.

reduction in abnormal PrP formation. The results revealed no reduction in the cellular PrP level of the cells (Fig. 1(A) and (B)). Furthermore, either to examine whether the chemicals directly destabilize or denature the abnormal PrP structure or to exclude the possibility of interference with preparation and immunodetection of the abnormal PrP, the cell lysate either alone or mixed with the chemicals was incubated at 37°C for 1 h prior to proteinase K digestion; it was then processed ordinarily to obtain the abnormal PrP. The results indicated that the chemicals did not affect the abnormal PrP signals (Fig. 1(C)).

Because it was predicted that the chemicals might exert their antiprion action through a certain mechanism involving chelating metals, the most effective chemical found here, chrysoidine, was preincubated before addition to the ScNB culture medium with an equivalent dose or lower doses of various metal ions, including copper, zinc, cobalt, and aluminum ions. The results revealed no change in the inhibition activity of the chemical (Fig. 2). Furthermore, to examine whether chelating activity is necessary for antiprion action, we modified Yellow AB in such a manner that its amino base was acetylated to remove its chelating activity. The acetylated Yellow AB was tested in ScNB cells, and it was one-eighth as effective in inhibiting abnormal PrP formation as Yellow AB (Fig. 3(A)). Finally, as a chemical bearing the effective structure but lacking chelating activity, the chemical azobenzene, which is most similar in the structure to the chemical chrysoidine, was tested. It was about 30 times less effective than chrysoidine (Fig. 3(B)). These findings suggest that chelating activity is not essential for the antiprion action but might influence it.

Interaction with Recombinant PrP

We previously reported that more potent antiprion agents have higher affinity to recombinant PrP121–231 in surface plasmon resonance (SPR) analysis (Kawatake *et al.*, 2006). Therefore, we examined whether this is also demonstrated in the effective chelating chemicals found here. Six of the chemicals (each at 50 μ M) were tested. The SPR sensorgrams of the chemicals except 4-(2-pyridylazo)resorcinol showed similarly weak signal responses of less than 100 RU as quinacrine did (Fig. 4). However, neither 4-(2-thiazolylazo)resorcinol nor Yellow AB reached the equilibrium state at the association phase; neither 4-(2-thiazolylazo)resorcinol nor 2-phenylazo-4-methylphenol returned to the baseline at the dissociation phase. In contrast, 4-(2-pyridylazo)resorcinol showed the strongest response of more than 200 RU and neither reached the equilibrium state at the association phase nor returned to the baseline at the dissociation phase. The binding response value from the sensorgram (equilibrium or maximum response value divided by molecular weight), which is an index for estimating the interaction of a chemical with the molecules sited on a biosensor chip (Frostell-Karlsson *et al.*, 2000), showed no apparent relationship with the IC₅₀ value of antiprion activity (data not shown), suggesting that the chemicals found here might exert their antiprion action in a manner that differs from those of previously reported antiprion chemicals such as antimalarias and amyloid binding dyes.

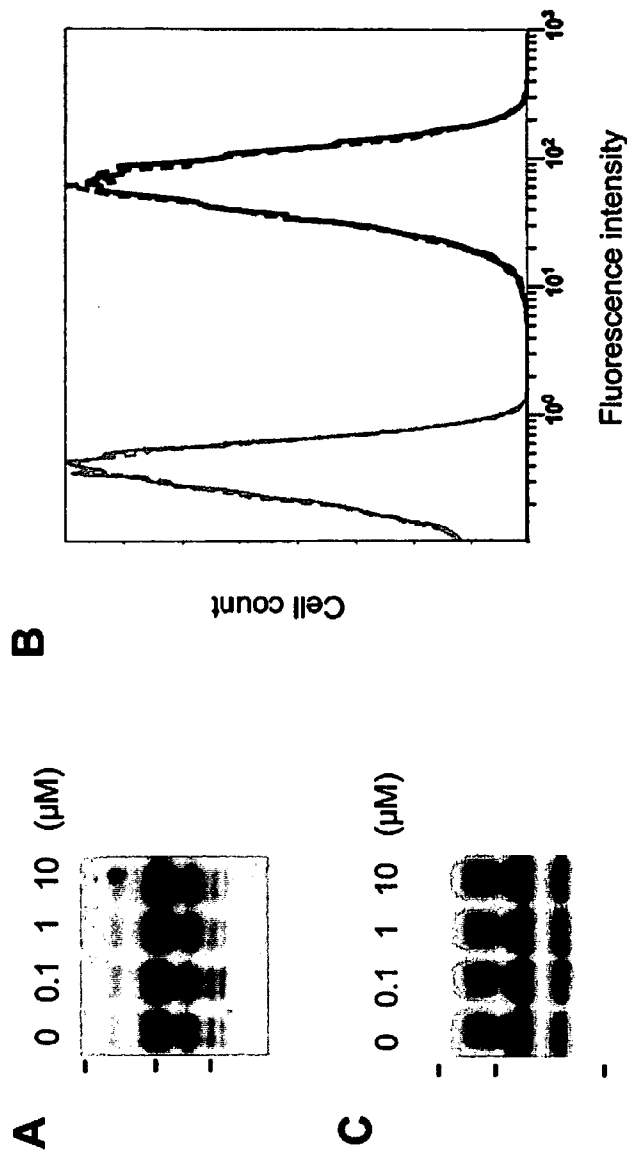


Fig. 1. Effects of a representative chemical, chrysoidine, on the cellular PrP (A, B) and the cell lysate abnormal PrP (C). (A) Immunoblot data of the total cellular PrP in noninfected NB cells treated with a designated dose of chrysoidine. Bars on the left indicate molecular size markers at 81, 42, and 32 kDa. (B) Flow cytometry data of the cell surface PrP in noninfected NB cells treated with 1 μM chrysoidine. Solid line and broken line indicate chrysoidine-treated cells and nontreated cells, respectively. Grey line peaks on the left show their respective isotype controls. (C) Immunoblot data of the abnormal PrP from ScNB cell lysate preincubated with a designated dose of chrysoidine prior to protease digestion. Molecular size markers on the left are 42, 32, and 18 kDa.

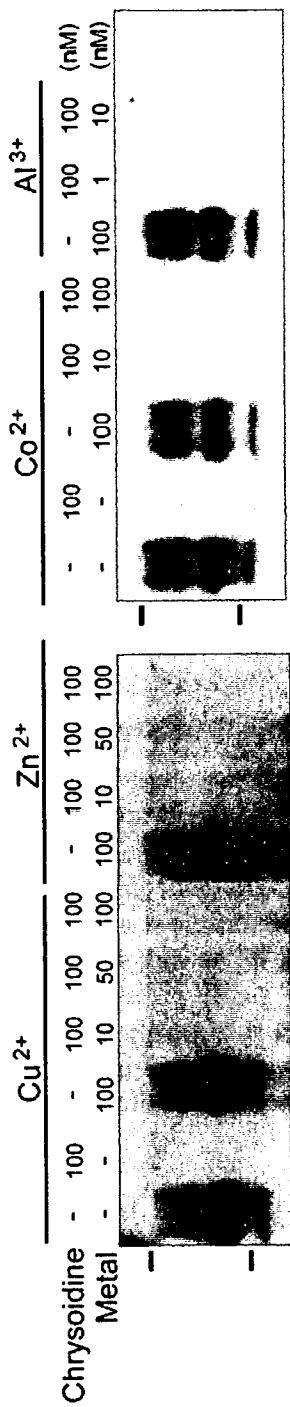


Fig. 2. Antiprion activity in ScNB cells of chrysoidine preincubated with metal ions. Immunoblot data of the abnormal PrP are shown. Bars on the left indicate molecular size markers at 37 and 25 kDa.

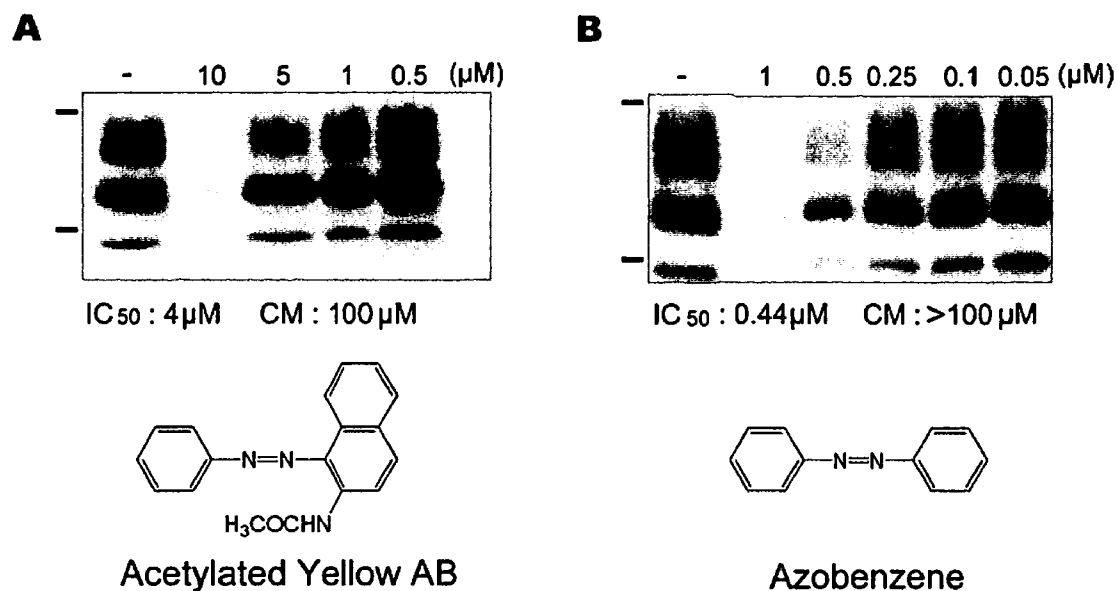


Fig. 3. Antiprion activity in ScNB cells of acetylated Yellow AB (A) and azobenzene (B) Immunoblot data of the abnormal PrP are shown. Bars on the left indicate molecular size markers at 37 and 25 kDa. IC₅₀ is approximate dose giving 50% inhibition of abnormal PrP formation. CM is approximate maximal dose that does not affect the rate of cell growth.

Brain Endothelial Permeability

The brain is the main organ that is affected in prion diseases. Therefore, therapeutic compounds must penetrate into the brain. To examine the permeability of a chemical through the blood–brain barrier, we used a simple analytical model consisting of brain capillary endothelial MBEC4 cells. As a representative of the effective chemicals found in the study, chrysoidine was examined in this model and compared with a paracellular marker, Na-F, as well as a control, quinacrine, which has been used for clinical trials of patients with prion diseases. The results showed that the respective permeability coefficients of Na-F, quinacrine, and chrysoidine were 2.17×10^{-3} , 0.96×10^{-3} , and 4.63×10^{-3} cm/min (Fig. 5). Therefore, chrysoidine penetrated the brain capillary endothelial cells about five times more efficiently than quinacrine.

DISCUSSION

Here, we revealed that chelating chemicals, especially aromatic azo compounds, have antiprion activity. Mechanisms of their antiprion action apparently include neither alteration of cellular PrP level nor direct modification of abnormal PrP. Taken together with previous findings related to the interaction of PrP with metals (review in Brown, 2004), the data obtained through the present study suggest that the chelating activity might influence the antiprion action but is not essential for

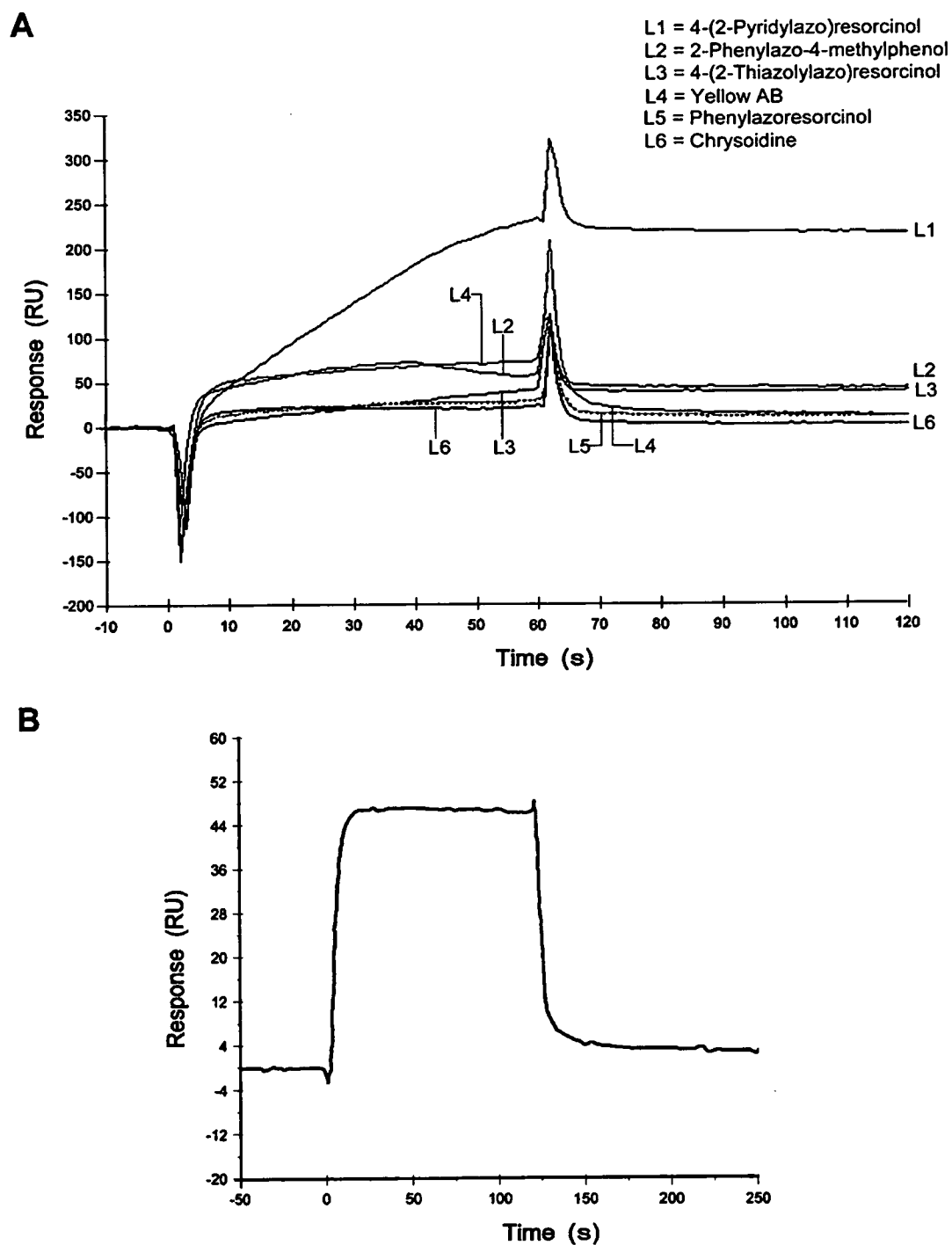


Fig. 4. SPR sensorgrams of chelating compounds (A) and quinacrine (B) interacting with PrP121-231. Each chemical at $50 \mu\text{M}$ was analyzed using a *ca.* 3,000 RU PrP-bound biosensor chip. Each phase of association and dissociation was monitored for 60 s in (A) or 125 s in (B).

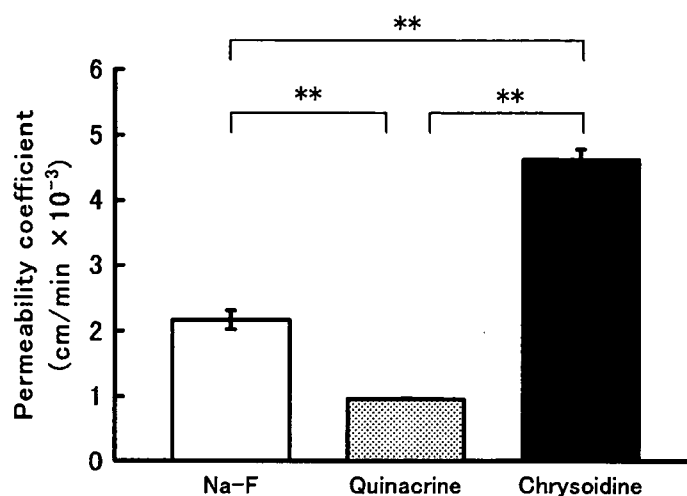


Fig. 5. Permeability coefficients of Na-F, quinacrine, and chrysoidine through MBEC4 monolayer. Each chemical at 100 μM was analyzed. The values are mean \pm SEM ($n = 3\text{--}4$ inserts). ** $p < 0.01$; significant difference between each group.

it. This inference is consistent with our previous results from quinacrine derivatives carrying chelating activities (Murakami-Kubo *et al.*, 2004).

Chrysoidine, a representative chemical found in this study, is far superior to quinacrine in both the antiprion activity and the brain endothelial permeability. The respective antiprion activities of chrysoidine and quinacrine in ScNB cells were 15 nM and 400 nM in IC_{50} , indicating that chrysoidine is about 27 times more effective than quinacrine. Furthermore, chrysoidine penetrated brain capillary endothelial cells about five times more efficiently than quinacrine. In addition, chrysoidine is much less toxic than quinacrine because a maximal dose at which the ScNB cell growth to confluence is still tolerant was more than 100 μM in chrysoidine or 2 μM in quinacrine (Table II). These findings suggest that chrysoidine might be more beneficial *in vivo* than quinacrine, but the *in vivo* efficacy of chrysoidine remains to be evaluated.

Results from the SPR analysis obtained here were not consistent with those of our previous study (Kawatake *et al.*, 2006), where the SPR binding response correlates with the inhibition activity of abnormal PrP formation in ScNB cells. Chrysoidine, the most effective chemical in the study, has a similar structure to either half of a symmetrical compound, Congo red, whose antiprion activity ($\text{IC}_{50} = 14$ nM) is as prominent as that of chrysoidine ($\text{IC}_{50} = 15$ nM) (Table II) but whose permeability into the brain is reportedly very poor because of low lipophilicity and high charge in its acidic groups (Klunk *et al.*, 2002). Interaction with recombinant PrP121–231 differs greatly between chrysoidine and Congo red. Congo red has very high affinity ($K_D = 1.6$ μM) and strong binding response (1.7 RU/Da at 10 μM using a *ca.* 3,000 RU PrP-bound biosensor chip) to the PrP121–231 (Kawatake *et al.*, 2006), whereas chrysoidine shows a sensorgram pattern of low affinity compounds and has very low binding response (0.1 RU/Da at 50 μM using a similar

biosensor chip). These facts suggest that chrysoidine exerts its antiprion action in a manner that differs from that of Congo red, but this inference demands further evaluation.

The brain endothelial permeability assay using MBEC4 cells revealed that the permeability coefficient of quinacrine was much lower than that of Na-F. The results are consistent with those of our previous experiments (Dohgu *et al.*, 2004). Quinacrine transport through the blood–brain barrier is mediated by both the efflux system (P-glycoproteins) and the influx system (organic cation transporter-like machinery). Therefore, quinacrine entry into the brain is controlled by three factors: P-glycoprotein-mediated active efflux at the apical side of the plasma membrane; highly concentrative uptake system; large storage capacity in the cytoplasm of the brain endothelial cells. On the contrary, Na-F is transported through paracellular routes (tight junctions) at the blood–brain barrier, and neither active efflux nor concentrative uptake system is involved in the Na-F permeability. These differences might explain the reason why quinacrine is less efficiently permeabilized than Na-F.

Chrysoidine is used in various fields as a yellowish fluorescent dye. This chemical was suggested to relate with bladder cancer in humans (Cartwright *et al.*, 1983; Sole and Sorahan, 1985), but it is still controversial because the data of a later conducted case-control study denied its relation to the cancer (Sorahan and Sole, 1990). There are no data on the genetic and related effects of the chemical in humans, but it is mutagenic to bacteria and toxic to rat hepatocytes *in vitro* (Sandhu and Chipman, 1990). In the mice orally administered, it produced liver carcinoma, leukemia, and reticulum cell sarcomas (Anonymous, 1975). These findings suggest that clinical use of chrysoidine or related chemicals might be inadequate.

In conclusion, we screened chelating chemicals and found that chrysoidine was much more effective in both antiprion activity and brain endothelial permeability than quinacrine, and it was much less toxic in NB cells. The mechanism of antiprion action of this compound did not apparently include alteration of cellular PrP level, direct modification of abnormal PrP, or chelation of metals. Its interaction with PrP121–231 differed greatly from that of Congo red, despite their structural similarity. These findings will contribute to the development of therapeutic compounds for prion diseases.

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Prophylactic Effect of Dietary Seaweed Fucoidan against Enteral Prion Infection[∇]

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Dietary seaweed fucoidan delays the onset of disease of enterally infected mice with scrapie when given orally for 6 days after infection, but not when given before the infection. This effect was not modified at a tested fucoidan dose range and appeared to reach the maximum level at a concentration of 2.5% or less in feed. Daily uptake of fucoidan might be prophylactic against prion diseases caused by ingestion of prion-contaminated materials, although further evaluation of its pharmacology remains to be done.

Transmissible spongiform encephalopathies, or prion diseases, are fatal neurodegenerative disorders that include Creutzfeldt-Jakob disease (CJD) and Gerstmann-Sträussler-Scheinker syndrome in humans and scrapie, bovine spongiform encephalopathy (BSE), and chronic wasting disease in animals. Recent outbreaks of BSE and variant CJD (vCJD), both of which are considered to occur through ingestion of BSE-contaminated materials (reviewed in reference 21), have necessitated the development of preventive measures against these diseases.

Sulfated polysaccharides, such as heparin, dextran sulfate, and pentosan polysulfate (PPS), are known either to prolong incubation periods in animals with prion diseases or to inhibit formation of pathogen-related abnormal prion protein (PrP) in prion-infected cells (reviewed in reference 3). Their therapeutic effects are attributed to inhibition of the conversion of normal PrP to abnormal PrP by either competitively binding to the normal PrP (4) or reducing normal PrP on the cell surface through stimulation of endocytosis (20). These large-molecule compounds are not taken up well from the gut to blood or from blood to the brain (a target organ of prion diseases). Therefore, these compounds are effective in cases of peripheral infection when given intraperitoneally, intravenously, or subcutaneously (8) and even in cases of intracranial infection when given intracerebroventricularly (5). Recently, PPS intracerebroventricular injection has been utilized for clinical trials of patients; the clinical outcome remains to be determined (17).

Fucoxidans, complex sulfated fucosylated polysaccharides, are known to have various biological activities: anticoagulant, antiviral, antiparasital, anti-inflammatory, contraceptive, and so on, because of their ability to imitate patterns of sulfate substitution on glycosaminoglycans and other sulfated glycans (2). Some fucoxidans are present in large quantities in dietary brown seaweed food products, which are eaten frequently in Asian countries (9). Here, we report that fucoidan from pop-

ularly eaten brown algae has antiprion activity and delays disease onset when it is ingested after the enteral prion infection.

Fucoxidan was prepared from the brown seaweed *Cladophora okamuranus* Tokida (Fig. 1A) and subsequently tested as described previously (15). Briefly, the brown seaweed was suspended in distilled water adjusted to pH 3.0 with 30% HCl and heated at 100°C for 30 or 60 min. The suspension was centrifuged (10,000 × g) at room temperature, and the supernatant was filtered using Microza UF membrane (Asahi Kasei Chemicals, Japan). Then the retentate was washed with distilled water and lyophilized. The levels of fucose, uronic acid, and sulfate in the lyophilized preparation were determined by examining the results of the phenol-H₂SO₄ reaction and carbazole reaction and by ion chromatography, respectively. The purity and molecular mass of the lyophilized preparation were determined by gel filtration high-performance liquid chromatography. Two fucoxidan preparations were used in the experiment: sample 1, with an average mass of 42.6 kDa and 87.8% fucoxidan content; and sample 2, with an average mass of 140.4 kDa and 87.1% fucoxidan content.

Inhibition of abnormal PrP synthesis *in vitro* was investigated as described previously (7, 12) in three different prion-infected neuroblastoma (NB) cells, each of which was persistently infected with a distinct prion strain from scrapie (RML or 22L) or human prion disease (Fukuoka-1). The cells were cultured for 3 days in the presence of fucoxidan, and proteinase K-resistant abnormal PrP in the cell lysate was recovered by ultracentrifugation and analyzed by immunoblotting with three different anti-PrP antibodies, SAF83 against human PrP142-160 (SPI-BIO, France), PrP-2B against mouse/hamster PrP89-103 (5), and PrP-3B against mouse/hamster PrP218-232 (not published). The three antibodies produced the same immunoblot data in the following studies. Therefore, all immunoblot figures presented are of SAF83. The results from prion-infected NB cells showed that sample 2 of the higher-molecular-mass fucoxidan more strongly inhibited abnormal PrP formation at half-maximum effective dosages ranging from 2.5 μg/ml to 5.0 μg/ml in all cells (Fig. 1B), suggesting that fucoxidan exerts its antiprion activity in a prion strain-independent manner. The inhibition was irreversible, and even the most effective

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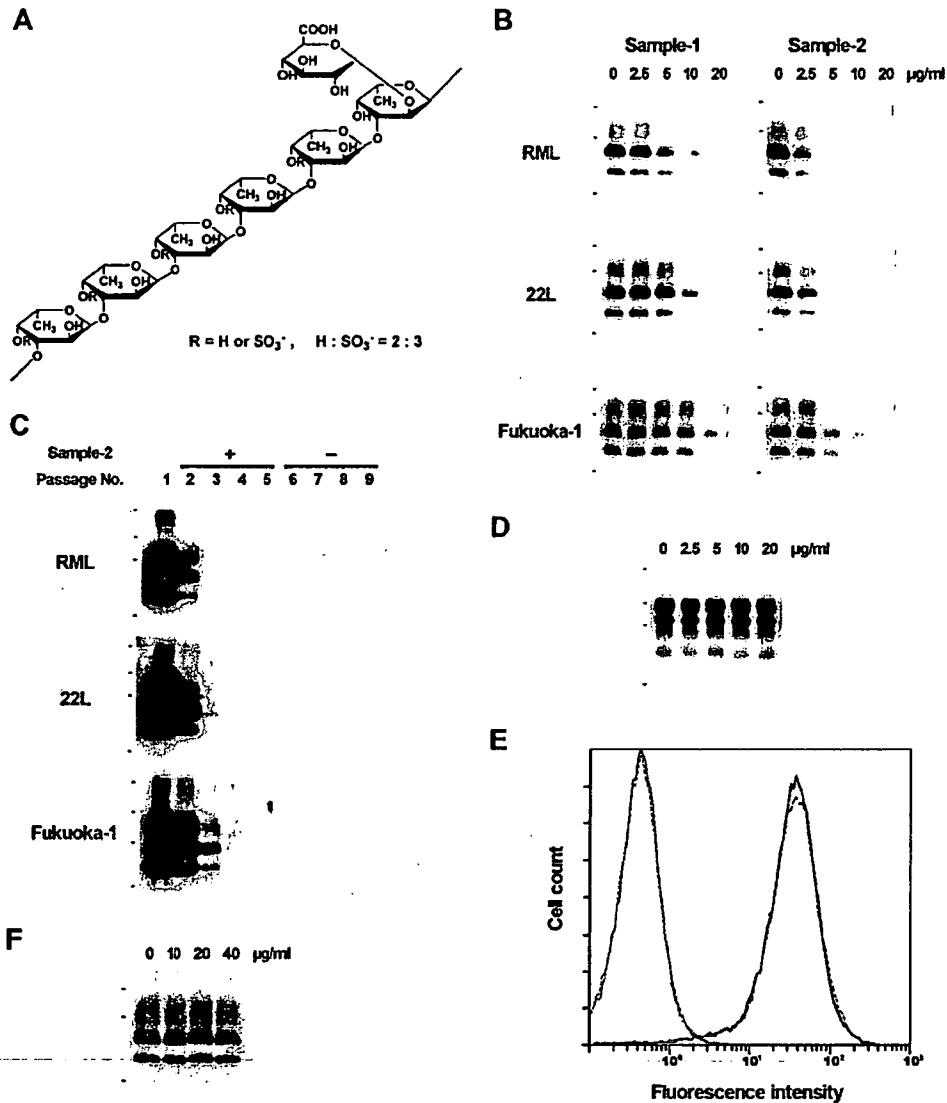


FIG. 1. Fucoidan and its effects on prion-infected or noninfected cells. (A) Chemical structure of fucoidan from *Cladosiphon okamuranus* Tokida. (B) Immunoblot analyses of abnormal PrP in the prion-infected NB cells treated with fucoidan. The small black bars to the left of the blots indicate the positions of molecular size markers at 42, 32, and 17 kDa. (C) Immunoblot analyses of abnormal PrP in the prion-infected NB cells serially passaged in the presence (+) and subsequently in the absence (-) of fucoidan. Overexposed images are shown. The small black bars to the left of the blots indicate the positions of molecular size markers at 81, 42, 32, and 17 kDa. (D) Immunoblot analysis of total normal PrP in noninfected NB cells treated with fucoidan. The molecular size markers to the left of the blot are the same as in panel C. (E) Flow cytometric analysis of normal PrP on the cell surface in noninfected NB cells treated with fucoidan. The solid line and broken line indicate fucoidan-treated cells and nontreated cells, respectively. Gray line peaks on the left show their respective isotype controls. (F) Immunoblot analysis of abnormal PrP from RML-infected cell lysate incubated with fucoidan prior to protease digestion. The molecular size markers to the left of the blot are the same as in panel B. All immunoblot data shown here are of SAF83.

abnormal PrP preparation technique, sodium phosphotungstic acid precipitation (18), never demonstrated that abnormal PrP signals that had once disappeared after treatment with 10 μg/ml of sample 2 fucoidan could reappear after serial passages in the absence of fucoidan (Fig. 1C). The inhibition mechanism included no alteration of either the total or cell surface level of normal PrP. That fact was demonstrated in noninfected NB cells treated with 20 μg/ml sample 2 fucoidan by either immunoblot analysis of the cell lysate without protease digestion or flow cytometric analysis performed as de-

scribed previously (6) (Fig. 1D and E). In addition, apparent modification of the abnormal PrP with fucoidan was not observed in the immunoblot data when the RML-infected cell lysate was incubated with 20 μg/ml sample 2 fucoidan at 37°C for 1 h and processed to obtain the abnormal PrP as described (Fig. 1F). The findings are consistent with those of heparan sulfate mimetics (1, 19), but not those of PPS and dextran sulfate, which stimulate endocytosis of normal PrP on the cell surface and engender reduction of the total and cell surface normal PrP (20). Thus, reduction of normal PrP is not neces-