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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 μg/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 μg/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 μL/min. The dissociation phase was monitored with injection of the running buffer at a flow rate of 20 μL/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 $CaCl_2$, 1.2 mM $MgCl_2$, 1.0 mM NaH_2PO_4, 25 mM $NaHCO_3$, 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 μM 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

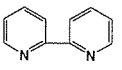
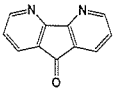
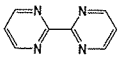
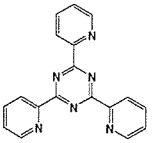
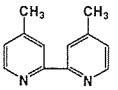
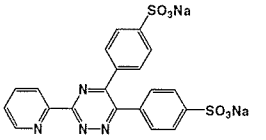
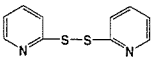
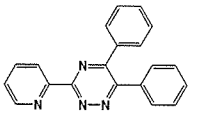
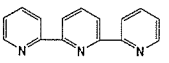
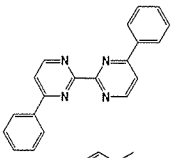
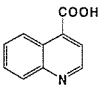
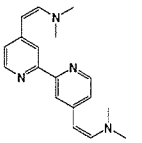
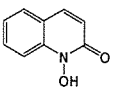
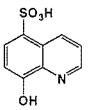
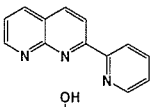
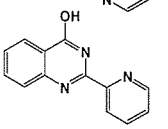
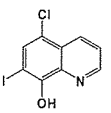
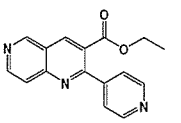
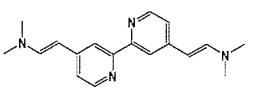
Antiprion Screening *in vitro*

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

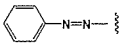
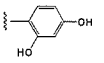
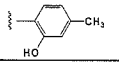
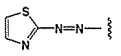
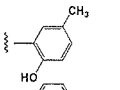
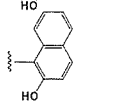
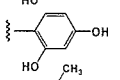
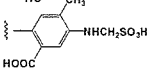
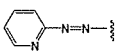
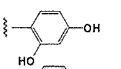
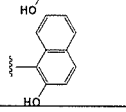
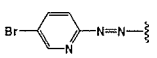
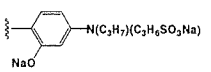
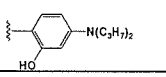
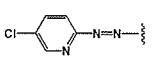
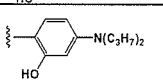
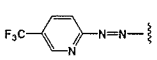
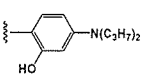
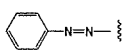
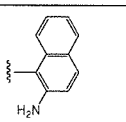
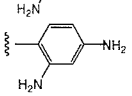
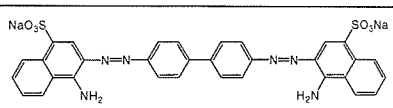
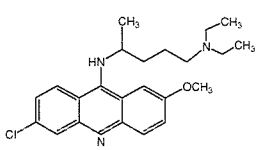
We tested whether the effective chemicals cause any alteration of the cellular PrP level in the treated cells because reduction in the cellular PrP level engenders

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. IC_{50}: 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-CF_3-PADAP			4	10
Yellow AB			0.5	100
Chrysoidine			0.015	>100
Congo red			0.014	not tested
Quinacrine			0.4	2

Note. IC_{50}: 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 μM) 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 IC_{50} 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.

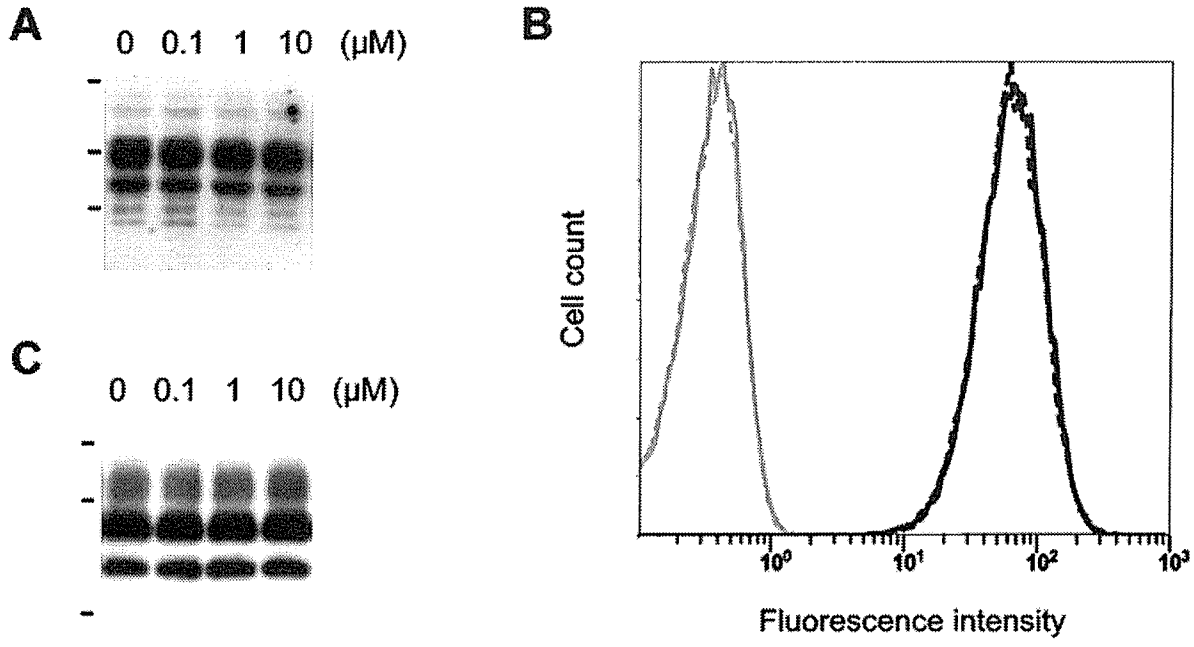


Fig. 1. Effects of a representative chemical, chrysoidine, on the cellular PrP (A, B) and the cell lysate abnormal PrP (C). (A) Immunoblot data of the total cellular PrP in noninfected NB cells treated with a designated dose of chrysoidine. *Bars* on the *left* indicate molecular size markers at 81, 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.

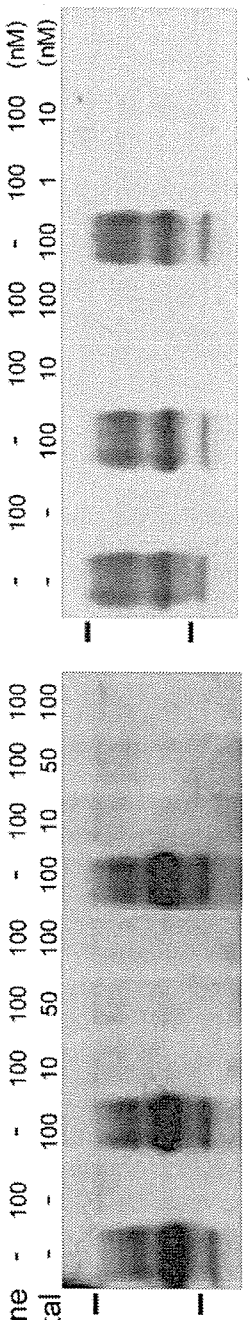


Fig. 2. Antiprion activity in ScNB cells of chrysoidine preincubated with metal ions. Immunoblot data of the abnormal PrP are shown. *Bars* on the *left* indicate molecular size markers at 37 and 25 kDa.

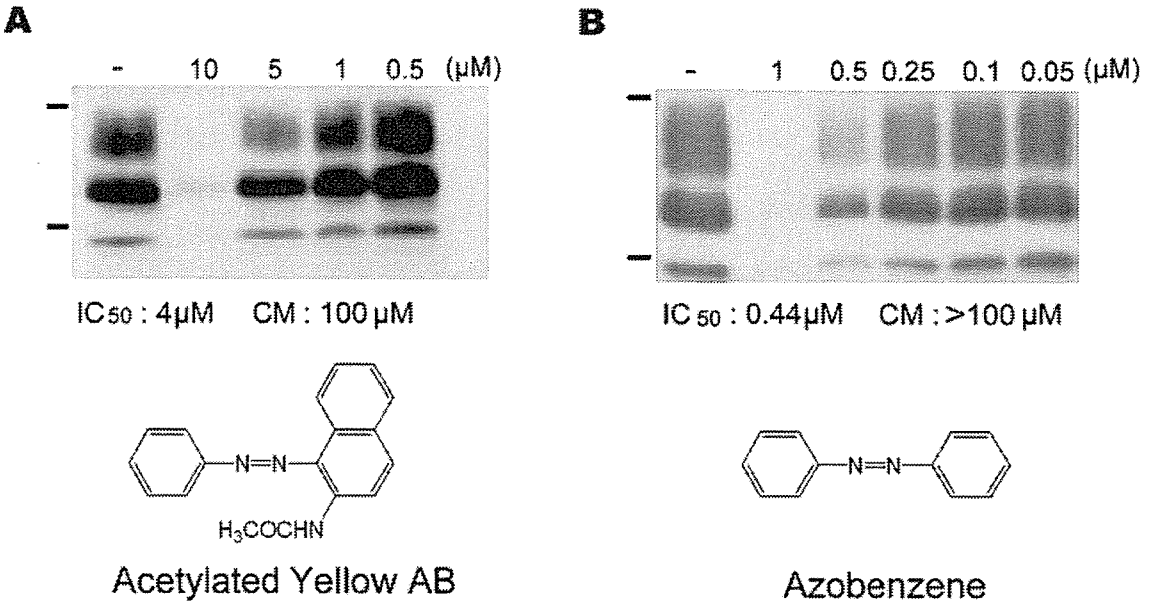


Fig. 3. Antiprion activity in ScNB cells of acetylated Yellow AB (A) and azobenzene (B) Immunoblot data of the abnormal PrP are shown. *Bars* on the *left* indicate molecular size markers at 37 and 25 kDa. IC_{50} is approximate dose giving 50% inhibition of abnormal PrP formation. CM is approximate 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

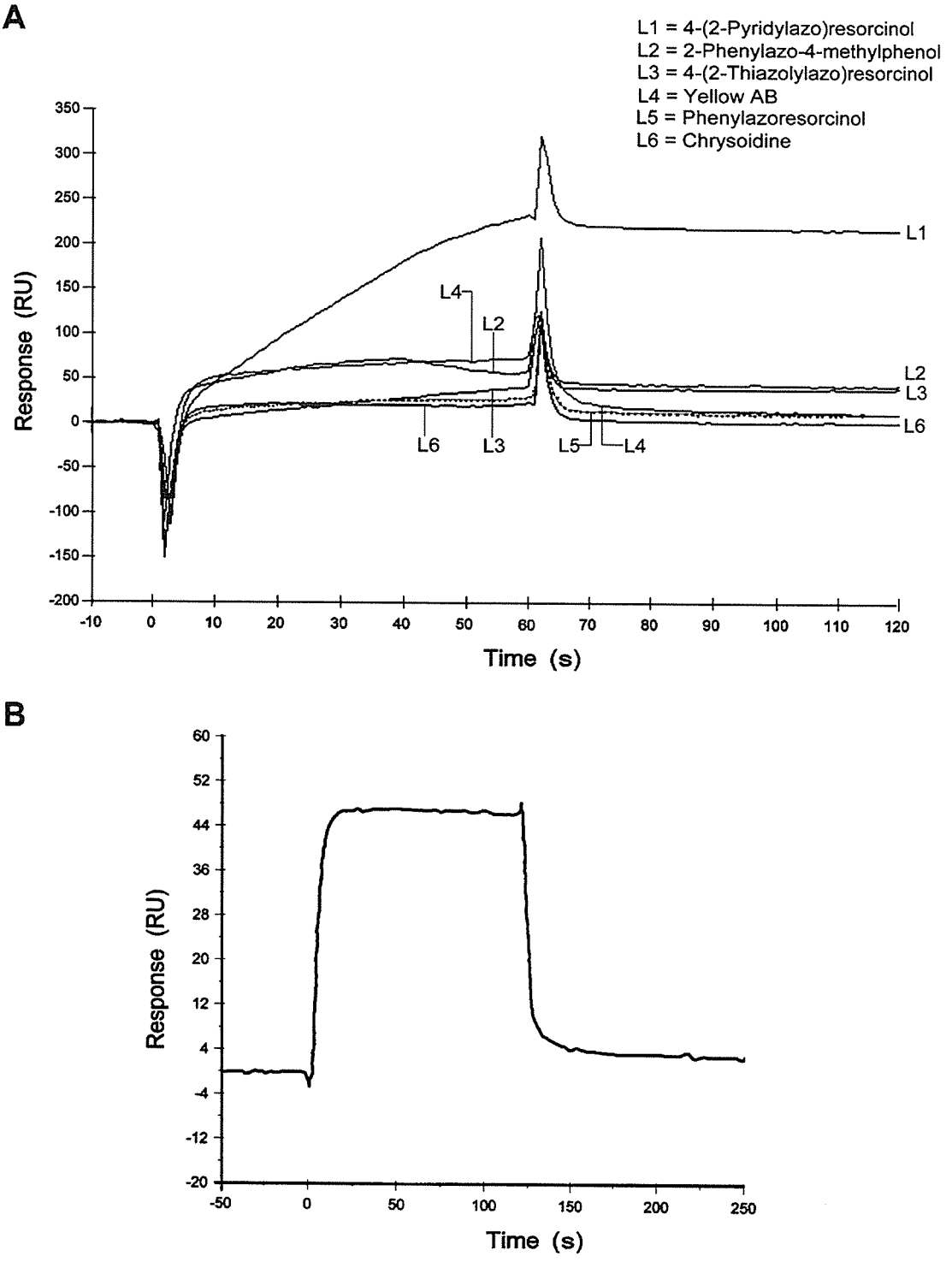


Fig. 4. SPR sensorgrams of chelating compounds (A) and quinacrine (B) interacting with PrP121–231.Each chemical at 50 μM was analyzed using a *ca.* 3.000 RU PrP-bound biosensor chip. Each phase of association and dissociation was monitored for 60 s in (A) or 125 s in (B).

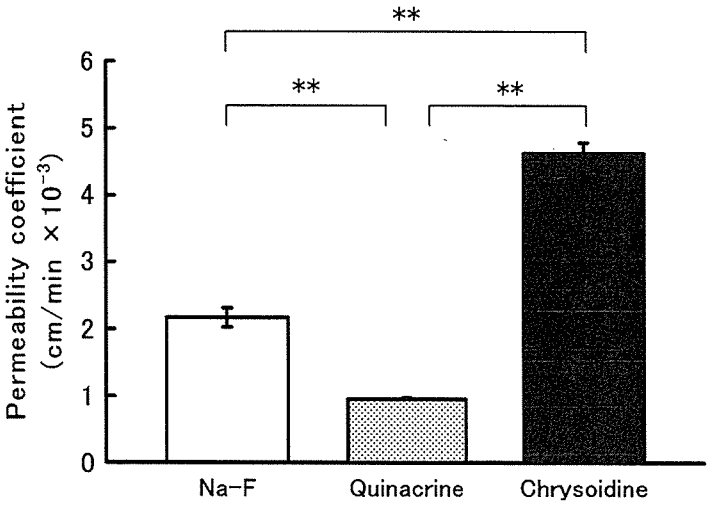


Fig. 5. Permeability coefficients of Na-F, quinacrine, and chrysoidine through MBEC4 monolayer. Each chemical at 100 μM was analyzed. The values are mean ± SEM (n = 3–4 inserts). $^{**}p < 0.01$; significant difference between each group.

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 (IC_{50} = 14 nM) is as prominent as that of chrysoidine (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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プリオン病の治療 10
—その現状と展望—

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プリオン病は，いまだ有効な治療法がない，発症すると確実に死に至る進行性の神経変性疾患である．現在，プリオン本体およびその感染のメカニズムについて急速に理解が進んではいるが，治療薬・治療法についてはいまだ有効なものが見出されていない．英国を中心に欧州で新たに出現した変異型クロイツフェルト・ヤコブ病は，現在小康状態にあるものの，血液製剤を介して二次感染の可能性も考えられている．プリオン病の発症前での診断・検査法，そして予防薬・治療薬の早急な開発が求められている．

Key words
プリオン病
クロイツフェルト・ヤコブ病
治療
予防

はじめに

プリオン病あるいは伝達性海綿状脳症(transmissible spongiform encephalopathy；TSE)は進行性の神経変性疾患であり，有効な治療法が確立されていない現在，治療不可能の致死性の難病となっている．孤発性クロイツフェルト・ヤコブ病(Creutzfeldt-Jakob disease；CJD)を代表とするヒトプリオン病は，年間100万人に1人の割合で発症し，その多くのものは数週間から数ヵ月で急激に症状が進行し，死に至る．発症すると，脳内に海綿状の神経変性とともにプリオンと呼ばれる感染性を持つ蛋白質(PrP^{Sc})の蓄積を生じる．ヒトプリオンが実験動物へと感染し得ることは以前よりよく知られていたが，1980年代に英国を中心に蔓延していた牛海綿状脳症(bovine spongiform encephalopathy；BSE)が，1996年，ヒトへも経口感染し，新たなヒトプリオン病，変異型CJDを引き起こすことが報道されると，欧州全体を巻き込んだ大きなパニックを引き起こした．わが国でもBSE感染牛が2001年に発見され，2005年には英国旅行者の邦人1人が変異型CJDであることが確認された．また，米国や日本ではプリオンに感染した硬膜などの生体材料の移植によって医原性CJDが発生し，特にわが国では対策の遅れもあり，100人を超える症例が報告され，薬害ヤコブ病として大きな社会問題となっている．

現在，プリオン病に対する理解が進み，また感染防止対策がうまく機能していることもあり，BSEや変異型CJDの発生は小康状態あるいは減少へと転じている．しかし，発症していないにもかかわらず感染性を有する，いわゆるキャリアの存在と，血液製剤を介した変異型CJDの二次感染の例が報告され，今後，変異型CJDの発生が拡大していく可能性も出てきた．

近年，プリオン病の基礎研究や治療薬のスクリーニング・検出技術に大きな進展があり，いくつかの薬剤についてはすでに臨床試験にて検討されているが，有効なプリオン病予防薬および治療薬の開発までには至っていない．

プリオン病とは

プリオン病は通常のウイルスや細菌の感染とは異なり，プリオンと呼ばれる蛋白質によって感染が引き起こされる[1]．プリオンはプロテアーゼや高熱に耐性を持つ，不溶性の異常感染型プリオン蛋白(PrP^{Sc})である．生体には同じアミノ酸配列からなる正常プリオン蛋白(PrP^{C})と呼ばれる膜蛋白が存在する．これらの蛋白質の高次構造(コンフォメーション)は大きく異なり，PrP^{Sc}はβシートに富み，アミロイドと呼ばれる不溶性の多量体を形成するが，PrP^{C}はαヘリックスに富む可溶性の単量体として存在する．ヒトにおいては，特定のアミノ酸置換や挿入を持つPrP^{C}変異体が知られており，PrP^{Sc}の感染の有無にかかわらずプリオン病を自然発症する(遺伝性プリオン病)．外科手術などの医療行為よりPrP^{Sc}に汚染された生体材料より感染する場合もある．しかし，ヒトプリオン病の8割は孤発性であり，その原因は不明である．動物実験では，感染体と同じアミノ酸配列を持つPrP^{C}を導入することや，PrP^{C}を過剰発現させることによって，感染しやすくなることが知られている．プリオン病は，人獣共通感染症として知られるが，PrP^{Sc}と同じアミノ酸配列を持つPrP^{C}を発現させることによって，異なる種間の感染をより簡単に成立させることも可能になっている．PrP^{C}，PrP^{Sc}以外の宿主因子の存在も指摘されているが，現在のところ不明である．PrP^{C}の存在はプリオン感染の成立だけでなく，神経変性にも必須であり，感染に付随する神経変性の原因は脳，神経に蓄積するPrP^{Sc}というよりも，PrP^{C}からPrP^{Sc}へと構造変換する過程で生じるという説が有力である．

治療薬スクリーニング法の開発

プリオン病の治療薬の開発には，プリオン持続感染培養細胞を用いてPrP^{Sc}の産生量を測定するアッセイ法と，プリオンを接種したハムスターやマウスの潜伏期の長さを指標にしたアッセイ法が主に用いられている．最近になって，酵母プリオンを用いた方法や，*in vitro* で起こるPrP^{C}からPrP^{Sc}様のプロテアーゼ耐性産物を測定する方法も開発され，治療薬スクリーニングに利用されている．

培養細胞を用いる方法[2]は，多数の検体を安価に迅速に処理することが可能である．最近では，サブクローニングによって高いプリオン感染能を持つ細胞を使用し，さらにメンブレンフィルターに転写することによって，多検体を迅速に定量的に測定できるように改良されている[3]．しかし，培養細胞で認められた治療効果が，動物実験では必ずしも再現できない場合が多く，多検体からの一次スクリーニングとして使用されることが多い．

動物を用いたバイオアッセイは，感染価をエンドポイント希釈することによって発症を指標に測定する方法(IC_{50})や，感染価と潜伏期の長さが反比例関係にあることを利用して感染価を測定する方法を用いて，感染価を推定することができる．これまで開発されたプリオン検出系の中で最も感度が良いことが知られているが，確定するのに時間と費用がかかることが欠点である．ハムスターを用いたバイオアッセイ[4]は，プリオン仮説の提唱者であるPrusinerによって採用され，プリオンの精製の際に使用された．このハムスターを用いたバイオアッセイは，最短60日で感染を検出できるバイオアッセイであったが，PrP^{C}を過剰発現するトランスジェニックマウスの開発によって，ほぼ同等に潜伏期を短縮することに成功している．加えて，PrP^{Sc}と同じアミノ酸配列を有するPrP^{C}を発現するように作製されたトランスジェニックマウスによって，種の異なるプリオン感染も再現できるようになった．また，腹腔内にプリオンを接種後，感染初期にPrP^{Sc}の蓄積が認められる脾臓中のPrP^{Sc}の産生を測定することによって，感染を30日で検出することも可能になっている[5]．

その他に，[PSI+]や[URE3]という酵母プリオンを用いたスクリーニング系[6]や，PrP^{C}とPrP^{Sc}の相互作用を蛍光相関分光法を用いて測定するハイスループットスクリーニング法[7]や，

表面プラズモン共鳴法によるPrP^Cとの相互作用を利用したスクリーニング法[8]が新たに開発されている．

高感度プリオン検出法の開発

PrP^{Sc}の検出法には，プロテアーゼ処理によって混在するPrP^Cを除去し，プロテアーゼ耐性のPrP^{Sc}をメンブレンあるいはプレート上で免疫化学的に測定するウェスタンブロット法やELISA法が従来使用されてきた．その後，PrP^{Sc}に特異性を持つポリマーでコーティングしたプレートを用いるELISA法や，コンフォメーションを認識する抗体を用いたELISA法(CDI法)[9]など，プロテアーゼ処理を省略した検出法も開発されている．また，PrP^{Sc}との相互作用によってコンフォメーション変化を起こし，蛍光を発するよう工夫されたペプチドを用いた高感度検出法や，DNAの増幅に使われているPCR法によく似た原理によって*in vitro*でPrP^{Sc}を増幅するという検出法(PMCA法)[10]など，新しい原理による検出法も開発されている．PMCA法は，PrP^{Sc}からなる凝集体がある条件下でPrP^Cを重合して，より大きなPrP^{Sc}凝集体を形成することを利用している．一定時間ごとに超音波処理と重合反応を繰り返すことによりPrP^{Sc}を増幅するという方法で，これまで最も感度の良かったバイオアッセイをしのぐ感度を有しており，ごく低濃度のPrP^{Sc}の検出などにおいて，現在最も高感度の測定法となっている．現在のところ，増幅できるプリオンは限定されるなど，まだ克服すべき点があるが，反応の自動化も完了しており，血液や尿など低濃度のプリオンを含む試料の高感度検出とプリオン病の早期発見への応用に期待されている．

プリオン病の治療薬候補

プリオン病の治療薬候補として，多くの薬剤，化合物が報告されている．最近になって，さまざまなアッセイ系によってこれまでスクリーニングされたプリオン病予防薬・治療薬について体系的に検討された結果が，Brain誌に報告されている[11]ので参照されたい．

①アミロイド結合性を持つCongo red[11]，

②抗アミロイド活性を持つ抗癌剤ヨードドキソルビシン(4'-deoxy-4'-iododoxorubicin；IDOX)[12]とその構造類似体のテトラサイクリン(tetracycline)[13]，

③抗ウイルス薬からスクリーニングによって見出されたアンフォテリシンB(amphotericin B；AmB)[14]などのポリエン系抗生物質，

④アミロイド結合性を持つペントサンポリサルフェート(pentosan polysulfate；PPS)[15]などのポリアニオン誘導体，

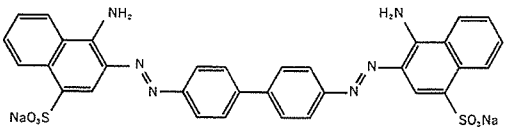
⑤アミロイドなどのβシート構造を破壊するペプチド[16]，

⑥蛋白質と結合し，立体構造に変化を与えるポルフィリン(porphyrin)などのテトラピロール類[17]，

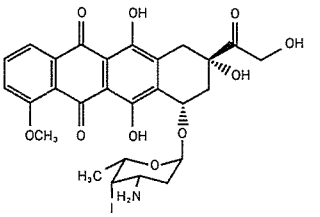
⑦特定の構造を持つトランスフェクシ

表1　プリオン病治療薬候補

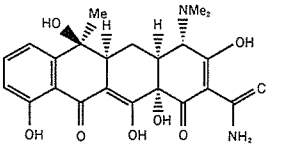
プリオン病治療薬候補化合物	化合物名	発見の経緯，化合物の特徴
アミロイド結合性化合物	Congo red	アミロイド結合性
四環系化合物	IDOX，テトラサイクリン	抗癌剤，抗アミロイド活性
ポリエン系抗生物質	アンフォテリシンB	抗ウイルス薬よりスクリーニング
ポリアニオン系化合物	ペントサンポリサルフェート	同上
βシート破壊ペプチド	iPrP13	アミロイド構造の破壊
テトラピロール系化合物	FeTSP	蛋白質構造変化促進，Congo red様構造
ポリアミン化合物	PAMAM	トランスフェクション試薬，抗プリオン作用
三環系化合物	キナクリン，クロルプロマジン	抗マラリア薬，抗精神病薬，BBB透過
抗体，免疫賦活剤	抗PrP抗体6H4，D18	PrP^Cへの結合
その他	メマンチン，フルピルチン	NMDA受容体アゴニスト，抗神経細胞死



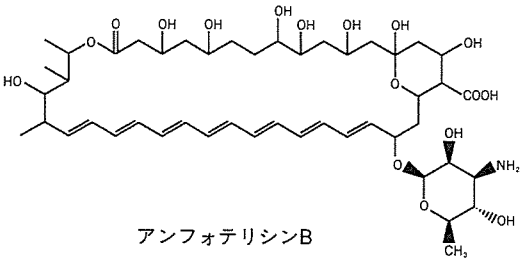
Congo red



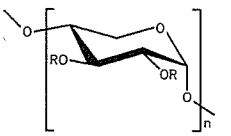
ヨードドキソルビシン



テトラサイクリン



アンフォテリシンB

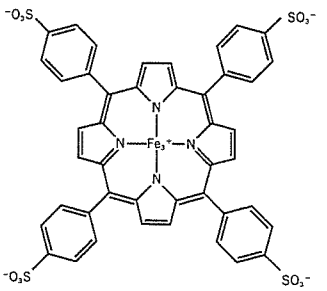


ペントサンポリサルフェート
(R=SO_3Na, n=12)

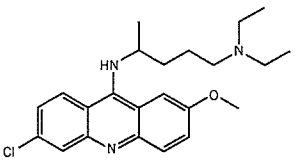
PrP ^{115}A AA AG AV V^{122}

iPrP13 DAPAAPAGPAVPV

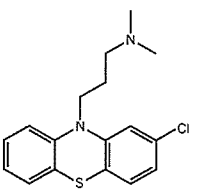
βシート破壊ペプチド



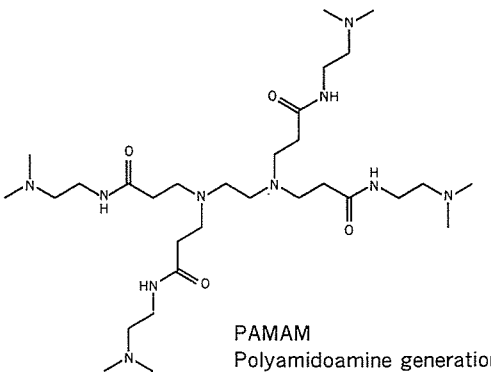
FeTSP
Iron(Ⅲ)meso-tetra(4-sulphonatophenyl)Porphine



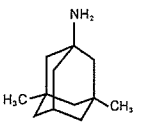
キナクリン



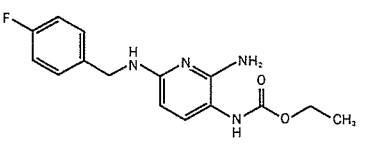
クロルプロマジン



PAMAM
Polyamidoamine generation 0



メマンチン



フルピルチン

図1　さまざまなプリオン病治療薬

ョン試薬が抗プリオン活性を示すことから見出された分岐性ポリアミン，
⑧抗プリオン蛋白抗体[10]，
⑨血液脳関門(BBB)を透過する既存の治療薬からスクリーニングにより見つかった抗マラリア薬キナクリン(quinacrine)[18]や抗精神薬クロルプロマジン(chlorpromazine)[19]など，
⑩神経細胞死を抑制する薬剤からNMDA受容体拮抗薬メマンチン(memantine)[20]や中枢性鎮痛薬フルピルチン(flupirtine)[20]，
などがある(表1，図1)．これらのなかで，実際に患者に応用されている薬剤および化合物には，AmB，PPS，キナクリン，フルピルチンなどがある．本稿では，キナクリン，PPSおよびフルピルチンについて解説する．

キナクリンは，長年にわたって抗マラリア薬として広く使用されてきた薬剤であり，安全性も高いことから，米国，フランス，英国などにて臨床研究が進められている．わが国においても孤発性CJD 22例，医原性CJD 5例，遺伝性プリオン病4例の計31例の症例に対し，300 mg/dayを経口，あるいは経管投与にて12週間の連続投与というプロトコールで臨床研究が行われた．その結果，12例で覚醒度や自発語，注視など，臨床症状に一過的な改善が認められたが(効果が認められた例：孤発性9例，医原性2例，遺伝性1例)，その後，効果は消失した．16例に肝機能障害や溶血性貧血などの重大な副作用が認められ，より毒性の低い塩酸キニーネに変更されたが，持続的な薬効を保つことと副作用を抑えることが困難であった[21]．

PPSについては，英国を中心に13例の治療が行われてきた．わが国では福岡大学にて臨床試験が行われている[22]．PPSはBBBを透過しないため，微量注入器具を用いて脳室内へ連続投与することが検討され，動物実験では脳内接種後10日からの投与で173％，30日からの投与で93％の潜伏期の延長が認められた[15]．本薬剤は，2003年，英国において1例の変異型CJD患者にて臨床研究が開始されたが，脳の萎縮の進行は止まらなかったものの副作用は認められず，臨床症状は落ち着いており，現在まだ経過を観察中である．わが国でも，さまざまなプリオン病患者で臨床研究が開始されており，その効果と副作用について解析が進められている[23]．PPSを用いた脳室内持続投与療法は，英国や日本以外の国でも開始され，全世界で20例近い患者に実施されており，現在最も期待されている治療法のひとつといえる．今後，最適の治療プロトコールの完成と，臨床症状の改善が判断できる症例での検討が求められる．

フルピルチンは，非オピオイド系の中枢性鎮痛薬として臨床で用いられてきたが，細胞培養実験などから神経細胞死を抑制する効果が再発見されて以来，神経変性疾患への治療に応用されるようになってきた．CJD患者については，28名の患者において二重盲検試験として臨床試験が行われた[24]．その結果，フルピルチンはCJDの認知機能の低下を有意に抑制したものの，CJDの進行を抑制することはできなかった．

おわりに

プリオン病は通常の細菌やウイルスなどを介した感染症と異なり，感染してからの潜伏期が長く，発症直前まで感染の有無を知ることができない．加えて，急速に進む神経変性を治療していくことは非常に難解な問題となっている．末梢から中枢神経への感染を予防することは，現在開発されているいくつかの薬剤でも可能と思われるが，それには発症前に治療を開始することが必須であり，現在のところ，医原性プリオン病や遺伝性プリオン病など，将来発症する可能性のある人には応用することができるものの，実際的ではない．MRI拡散強調画像や脳脊髄液中の14-3-3蛋白質などが神経変性初期の診断の助けになることが報告されているが，より早い時期での診断のためのマーカーの検索や検出法の開発が求められている．血液や尿中のPrP^{Sc}の高感度検出は，感染初期あるいは発症初期の検出法となるかもしれない．従来，PrP^{Sc}が神経変性の原因と考えられていたが，いくつかの実験から，PrP^{C}あるいはPrP^{C}からPrP^{Sc}に変化する中間体が神経変性を引き起こすとの仮説が提唱されている．必ずしもPrP^{Sc}の増殖を防げなくとも，神経変性のメカニズムを明らかにし，神経変性の進行を抑制する薬剤の探索や治療法を開発することは，プリオン病の治療法のひとつのゴールでもある．こちらの展開にも期待したい．

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