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Uncaria rhynchophylla, a Chinese Medicinal Herb, Has Potent Antiaggregation Effects on Alzheimer's β-Amyloid Proteins

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Because the deposition of β-amyloid protein (Aβ) is a consistent pathological hallmark of Alzheimer's disease (AD) brains, inhibition of Aβ generation, prevention of Aβ fibril formation, or destabilization of preformed Aβ fibrils would be attractive therapeutic strategies for the treatment of AD. We examined the effects of several medicinal herbs used in traditional Chinese medical formulae on the formation and destabilization of Aβ fibrils by using the thioflavin T binding assay, atomic force microscopic imaging, and electrophoresis. Our study demonstrates that several of these herbs have potent inhibitory effects on fibril formation of both $A\beta_{1-40}$ and $A\beta_{1-42}$ in concentration-dependent manners; in particular, *Uncaria rhynchophylla* inhibited Aβ aggregation most intensively. Significant destabilization of preformed $A\beta_{1-40}$ and $A\beta_{1-42}$ fibrils was also induced by *Uncaria rhynchophylla* as well as some other herb extracts. Three-dimensional HPLC analysis indicated that the water extract of this herb contains several different chemical compounds, including oxindole and indol alkaloids, which have been regarded as neuroprotective. Our results suggest that *Uncaria rhynchophylla* has remarkably inhibitory effects on the regulation of Aβ fibrils, and we conclude that this medicinal herb could have the potency to be a novel therapeutic agent to prevent and/or cure AD. © 2006 Wiley-Liss, Inc.

Key words: Alzheimer's disease; medicinal herb; *Uncaria rhynchophylla*; β-amyloid; thioflavin T

Alzheimer's disease (AD) is the most prevalent cause of dementia, characterized by loss of memory and cognition as well as behavioral and occupational instability in old age (Multhaup, 1997). One of the pathological characteristics of AD is a progressive deposition of insoluble β-amyloid protein (Aβ) in the form of senile plaques (Selkoe, 2002). This protein comprises peptides of approximately 39–43 amino acid residues derived from the transmembrane amyloid precursor protein (APP; Selkoe, 2002). Aβ can form monomers and a variety of different aggregate morphologies, including dimers, small soluble oligomers, protofibrils, diffuse plaques, and fibrillar deposits seen in the senile plaques. All of these variable aggregated forms of Aβ seem to be dominated by a so-called β-sheet structure (Tierney et al., 1988; Barrow and Zagorski, 1991). The increasing evidence that formation of these aggregates causes primary neurodegeneration in AD has led to the amyloid hypothesis, which states that the accumulation of Aβ in the central nervous system is highly neurotoxic and leads to deterioration of synaptic functions (Selkoe, 2002; Wirths et al., 2004). Moreover, several findings

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suggest that Aβ accumulation begins at relatively early stages in AD patients (Anderton et al., 1998; Selkoe, 2002). Therefore, formation, deposition, and aggregation of Aβ in the brain are primary targets for complete amelioration of dementia. Currently, drugs available for dementia such as acetylcholinesterase inhibitors exert only a temporary benefit in cognitive dysfunction (Millard and Broomfield, 1995; Park et al., 2000; Darreh-Shori et al., 2004), and they do not prevent or reverse the formation of Aβ deposits. We believe that the essential requirement for a truly effective antidementia drug would be the prevention of Aβ fibril formation or destabilization of aggregated Aβ or a combination of both.

Herbal remedies used worldwide (particularly in East Asian countries) have a long history of use in alleviating various symptoms of many diseases. Recently, clinical trials in AD patients have also shown that some of these traditional treatments improve scores on the Mini-Mental State Examination (MMSE), P300 latency, and blood flow in the cerebral cortex (Le Bars et al., 1997). Although inconclusive, these provocative studies suggest that even old remedies may be beneficial in AD and related disorders. We have reported that several traditional Chinese herbal medicines such as *Formula licnalis angelicae compositae* (Kami-untan-to in Japanese; Suzuki et al., 2001; Nakagawasai et al., 2004), *Pilulae octo-medicamentorum rehmanniae* (Hachimi-jio-gan in Japanese; Iwasaki et al., 2004), and *Pulvis depressionis hepatis* (Yoku-kan-san in Japanese; Iwasaki et al., 2005) improve symptoms of dementia. Moreover, Terasawa et al. (1997) showed that *Pulvis uncariae* (Choto-san in Japanese) improved cognitive functions in patients with vascular dementia. The hooks and stems of *Uncaria rhynchophylla* (*Uncaria uncus cum ramulus*; Choto-ko in Japanese), a major medicinal plant, *Pulvis uncariae*, are reported to have hydroxyl radical-scavenging activity and to prevent delayed neuronal death in vivo (Yokoyama et al., 2004). However, underlying the mechanism of Chinese medical herbs, including *Uncaria rhynchophylla*, for the formation and metabolism of Aβ fibrils has not yet been investigated. In the present study, we examined the effects of *Uncaria rhynchophylla* on formation of Aβ aggregates and destabilization of preformed Aβ fibrils in vitro by using fluorescence spectroscopy with thioflavin T, atomic force microscopy (AFM), and electrophoresis. Our results provide strong evidence that some Chinese herb extracts, including *Uncaria rhynchophylla*, have inhibitory and destabilizing effects on Aβ fibrils.

MATERIALS AND METHODS

Reagents

Aβ proteins (1–40 and 1–42) and thioflavin T were obtained from the Peptide Institute (Osaka, Japan) and from Sigma (St. Louis, MO), respectively. All the reagents and drugs used were of analytical grade.

Preparation of Medicinal Herb Extracts

Water, methanol, and ethanol extracts of medicinal herbs were prepared by refluxing 10 g of sliced dry herbs in 100 ml of each solution. The decoction after cooling to room temperature was evaporated completely under reduced pressure to yield dried or oily extracts. The extracts were weighed and dissolved in dimethylsulfoxide (DMSO) at a concentration of 100 mg/ml and then stored at –20°C. When being assayed, these extracts were dissolved in the 50 mM potassium phosphate buffer (pH 7.4) and the solutions were adjusted if necessary to pH 7.4.

Analysis of 3D HPLC Fingerprints of Water Extract of *Uncaria rhynchophylla*

Uncaria rhynchophylla (0.5 g) was extracted with 30 ml of distilled water under ultrasonication for 30 min. The solution was filtered and then submitted to HPLC analysis. HPLC equipment was controlled with an HPLC pump (LC-10AD; Shimadzu, Kyoto, Japan) using a TSK-GEL 80_{TS} column (4.6 × 250 mm), and elution was performed with solvents (A) 50 mM $AcOH\text{-}AcONH_4$ and (B) CH_3CN. A linear gradient of 90% A and 10% B changing over 60 min to 0% A and 100% B was used. The flow rate was controlled with the LC-10AD at 1.0 ml/min. After the eluate was obtained from the column, the 3D data were processed with a diode array detector (SPD-M10A; Shimadzu, Kyoto, Japan).

Thioflavin T Measurement

Thioflavin T measurement was performed by the method described by Suemoto et al. (2004), with slight modifications. For Aβ aggregate-formation assay, Aβ (20 μM) dissolved in 50 mM potassium phosphate buffer (pH 7.4) with a test herbal extract was incubated at 37°C for 96 hr ($A\beta_{1-40}$) or 24 hr ($A\beta_{1-42}$). For destabilization assay of preformed Aβ aggregates, after incubation of $A\beta_{1-40}$ (96 hr) or $A\beta_{1-42}$ (24 hr) without a test herbal extract, the mixture of aggregated Aβ and each herbal extract was incubated for 30 min at 37°C.

At the end of the incubation, 3 μM thioflavin T dissolved in the 100 mM glycine buffer (pH 8.5) was added to the mixture. Fluorescence of thioflavin T bound to Aβ aggregates was measured with a microplate reader (Spectramax Gemini XS; Molecular Devices, Sunnyvale, CA) with a filter set (excitation at 442 nm and emission at 485 nm) after incubation for 30 min at room temperature. Percentage inhibition was calculated by comparing these fluorescence values with those found in control solutions with no test herbal extracts.

AFM Imaging

Highly ordered pyrolytic graphite (HOPG) was used as an AFM substrate. Thirty microliters of $A\beta_{1-42}$ sample containing a test extract was placed on HOPG for 15 min. Images were acquired in liquid using a MultiMode SPM NanoScope III system (Veeco/Digital Instruments, Santa Barbara, CA) operating in tapping mode.

Destabilization Assay of SDS-Insoluble Aβ Fibrils

$A\beta_{1-42}$ dissolved in the 50 mM potassium phosphate buffer (pH 7.4) was incubated at 37°C for 24 hr. After incu-

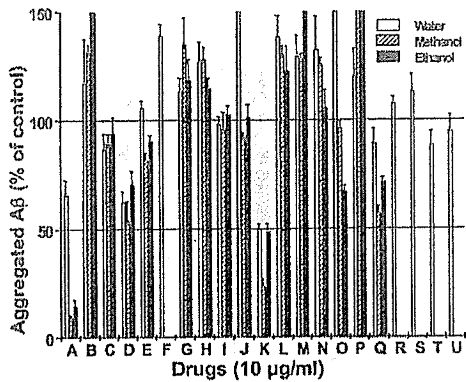


Fig. 1. Effects of different medicinal herbs on destabilization of $A\beta_{1-42}$ fibrils. The $A\beta_{1-42}$ aggregation was assessed by the thioflavin T method and expressed as a percentage of control aggregation, which was observed in the absence of these extracts. A, *Curcuma longa*; B, *Astragalus membranaceus*; C, *Polygala tenuifolia*; D, *Cinnamomum cassia*; E, *Cornus officinalis*; F, *Zizyphus jujuba*; G, *Dioscorea japonica*; H, *Rehmannia glutinosa*; I, *Acoru calamus*; J, *Alisma orientale*; K, *Uncaria rhynchophylla*; L, *Citrus unshiu*; M, *Angelica acutiloba*; N, *Panax ginseng*; O, *Ophiopogon japonicus*; P, *Poria cocos*; Q, *Paeonia suffruticosa*; R, *Saussurea lappa*; S, *Euphorid longana*; T, *Aconitum carmihaeri*, U, the processing of *Aconitum carmihaeri*. Values represent the means ± SD from four independent experiments. $^*P < 0.01$, compared with extract-untreated control.

bation, Aβ was added into each test herbal extract and incubated at 37°C for 1 hr. After centrifuging, Aβ pellets were added to 400 μl of the 0.2% SDS-containing phosphate-buffered saline and incubated at 37°C for 1 hr. After centrifuging again, Aβ pellets were added to 400 μl of the potassium phosphate buffer and sonicated. The fluorescence of intrinsic tyrosine and phenylalanine of each Aβ protein was measured with a Hitachi F-2000 fluorescence spectrophotometer (excitation and emission at 275 nm and emission at 300 nm, respectively).

Electrophoresis

The Aβ samples mentioned above were resuspended in the sample buffer, boiled for 5 min, electrophoresed on 10–20% Tris-tricine gel, and subjected to Coomassie brilliant blue staining.

Data Analysis

The data were expressed as mean ± SD. Statistical comparisons were made via Student's *t*-test. $P < 0.05$ was considered to be significant.

RESULTS

Screening of Medicinal Herbs That Influence Aggregation of Aβ Fibrils

We referred to old Chinese literature and selected 20 different medicinal herbs shown in Figure 1, based on the reported benefits for memory and intelligence. The effects of extracts of these medicinal herbs on the kinetics of destabilization of $A\beta_{1-42}$ fibrils are summarized in Figure 1. At a concentration of 10 μg/ml, several extracts showed destabilizing activity for Aβ fibrils, such as *Uncaria rhynchophylla*, *Cinnamomum cassia*, and *Paeonia suffruticosa*. *Uncaria rhynchophylla*, which was extracted by water, methanol, or ethanol, was concentrated under a reduced pressure to give oily residues (1.54 g, 3.21 g, and 2.40 g for water, methanol, and ethanol, respectively).

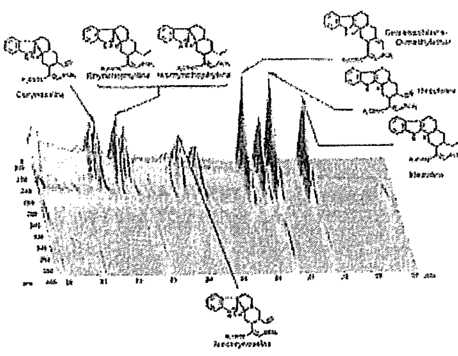


Fig. 2. Three-dimensional HPLC profile of the water extract of *Uncaria rhynchophylla*. Each peak indicates a molecule described in the figure including oxindole and indol alkaloids, which were reported to be neuroprotective.

HPLC Analyses of *Uncaria rhynchophylla*

Three-dimensional fingerprints of water extract of *Uncaria rhynchophylla* performed by 3D HPLC are shown in Figure 2. The results show that the water extract contains several different chemical compounds, including oxindole alkaloids (corynoxeine, rhynchophylline, isorhynchophylline, and isocorynoxeine) and indol alkaloids (geissoschizine methyl ether, hirsuteine, and hirsutine).

Concentration-Dependent Effects of *Uncaria rhynchophylla* on Kinetics of Aβ Fibril Formation and Breakdown

With regard to the inhibitory effect of *Uncaria rhynchophylla* on Aβ fibril formation, concentration dependencies were examined by using the thioflavin T method (Fig. 3). We observed that each of the three different extracts induced a concentration-dependent decline in fluorescence intensity in both $A\beta_{1-40}$ (Fig. 3A) and $A\beta_{1-42}$ (Fig. 3B). $A\beta_{1-40}$ fibril formation was inhibited by 10 μg/ml water (38.9% ± 12.2%), methanol (41.3% ± 4.8%), and ethanol (50.3% ± 46.7%) extracts of *Uncaria rhynchophylla* (Fig. 3A). $A\beta_{1-42}$ fibril formation was also inhibited by each of the three different extracts (10 μg/ml), although the effect was less potent than that on $A\beta_{1-40}$ (Fig. 3B).

To determine whether these extracts have a destabilizing activity on preformed Aβ fibrils, we performed further thioflavin T experiments (Fig. 3C,D). Fluorescence derived from thioflavin T was decreased dose

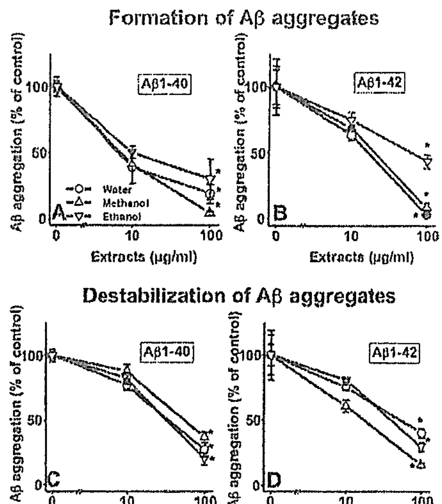


Fig. 3. Effects of three different *Uncaria rhynchophylla* extracts on the kinetics of formation and destabilization of Aβs. **A,B:** Aβ aggregate-formation assay. Reaction mixtures containing 20 μM of $A\beta_{1-40}$ (A) or $A\beta_{1-42}$ (B), 50 mM phosphate buffer (pH 7.4), and various extracts [water (circles), methanol (upward-pointing triangles), and ethanol (downward-pointing triangles)] were incubated at 37°C for 96 hr (A) or 24 hr (B). Aβ aggregation was expressed as a percentage of control, which was observed in the absence of herbal extract. **C,D:** Aβ aggregate-destabilization assay. Reaction mixtures containing 20 μM $A\beta_{1-40}$ (C) or $A\beta_{1-42}$ (D) were incubated at 37°C for 96 hr (C) or 24 hr (D). Then these extracts were added and incubated for 30 min. Aβ aggregation was assessed by the thioflavin T method and expressed as a percentage of control aggregation, which was observed in the absence of herbal extract. Values represent the means ± SD from four independent experiments. $^*P < 0.01$, compared with extract-untreated control.

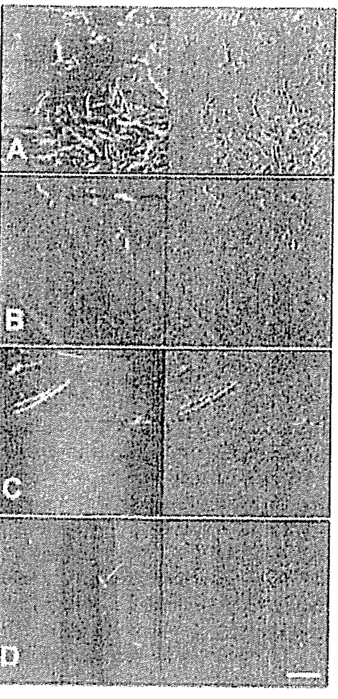


Fig. 4. Atomic force microscopic imaging of $A\beta_{1-42}$ fibrils. After incubation of $A\beta_{1-42}$ for 24 hr for preformed fibrils, the mixture of aggregated Aβ and the *Uncaria rhynchophylla* extract was incubated at 37°C for 30 min. **A:** vehicle (DMSO). **B:** 100 μg/ml water extract. **C:** 100 μg/ml methanol extract. **D:** 100 μg/ml ethanol extract. Left and right panels indicate height and amplitude modes, respectively. Scale bar = 500 nm.

dependently after the addition of each extract of *Uncaria rhynchophylla* to preformed Aβ fibrils to an extent similar to the inhibitory effects on Aβ aggregations. Preformed $A\beta_{1-40}$ (Fig. 3C) fibrils were destabilized by 10 μg/ml of water (77.2 ± 3.7%), methanol (87.7 ± 5.4%), and ethanol (82.3 ± 4.5%) extracts. Moreover, preformed $A\beta_{1-40}$ (Fig. 3C) and $A\beta_{1-42}$ (Fig. 3D) fibrils were destabilized by each of the three different extracts (100 μg/ml) by more than 50%.

AFM Elucidates Inhibitory Effects of *Uncaria rhynchophylla* on Aβ Fibrils

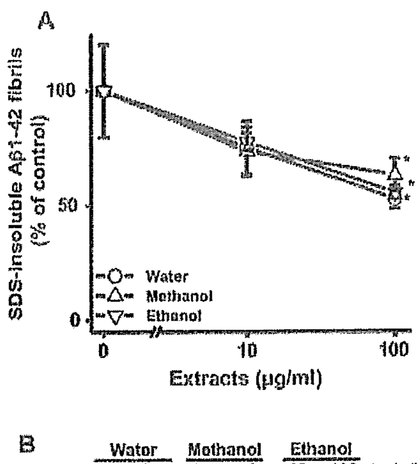
To visualize Aβ fibril breakdown by herbal extracts, the fibrils were observed by AFM. After incubation of $A\beta_{1-42}$ (20 μM) with the water extract of *Uncaria rhynchophylla* (100 μg/ml), preformed Aβ fibrils were largely diminished, although the shapes of the fibrils were hardly changed in the presence or absence of the herbal extract (Fig. 4A,B). A similar tendency was observed after incubation with 100 μg/ml methanol or ethanol extract of *Uncaria rhynchophylla* (Fig. 4C,D).

Centrifugation and Electrophoresis of SDS-Insoluble Aβs

SDS solubility of $A\beta_{1-42}$ fibrils was analyzed in order to investigate the biochemical characteristics of Aβ fibril disassembly by *Uncaria rhynchophylla*. After centrifugation, levels of pellets were assessed by fluorescence of aggregated $A\beta_{1-42}$ (Fig. 5A). SDS-insoluble pellets in the absence of extract served as the control. SDS-insoluble pellets were reduced to approximately 60% by treatment with 100 μg/ml of these extracts. Furthermore, the electrophoresis analysis also revealed that *Uncaria rhynchophylla* decreased SDS-insoluble $A\beta_{1-42}$ fibrils (Fig. 5B).

DISCUSSION

Pulvis uncariae and *Pulvis depressionis hepatis* are representative traditional Chinese herbal medicines that have been used for neuropsychiatric disorders. The former has been administered to relatively aged patients with physical weakness and subjective symptoms such as headache, dizziness, vertigo and tinnitus, and the latter has been used as a remedy for restlessness and agitation in children. *Uncaria uncus cum ramulus* originating from the hooks and stems of *Uncaria rhynchophylla* (Oliv.)



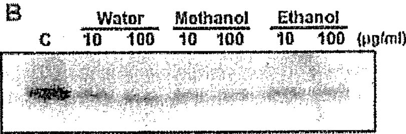


Fig. 5. Effects of three different extracts of *Uncaria rhynchophylla* on SDS-insoluble $A\beta_{1-42}$. **A:** After reaction mixtures containing 20 μM $A\beta_{1-42}$ were incubated at 37°C for 24 hr, the *Uncaria rhynchophylla* extracts [water (circles), methanol (upward-pointing triangles), and ethanol (downward-pointing triangles)] were added and incubated for 1 hr. The aggregates dissolved in SDS-contained PBS were assessed by fluorescence measurement. Values represent the means ± SD from four independent experiments. $^*P < 0.05$, compared with extract-untreated control. **B:** Levels of SDS-insoluble $A\beta_{1-42}$ fibril were assessed by electrophoresis. The mixtures containing 20 μM $A\beta_{1-42}$ and each extract were run on a 10–20% Tris-tricine gel, followed by staining with Coomassie brilliant blue.

Havil. is regarded as a main medicinal plant comprising *Pulvis uncariae* and *Pulvis depressionis hepatis*. It was reported that *Uncaria rhynchophylla*, which we mainly investigated in this study, has neuroprotective effects in vitro and in vivo by reducing oxidative damage to neurons (Dohi et al., 2003; Mahakunakorn et al., 2004, 2005). In addition, *Uncaria rhynchophylla* contains approximately 0.09% (=2 mM) alkaloids such as corynoxeine, rhynchophylline, isorhynchophylline, isocorynoxeine, geissoschizine methyl ether, hirsuteine, and hirsutine (shown in Fig. 2), which are reported to protect neurons against glutamate toxicity in cultured cerebellar granule cells by the inhibition of Ca^{2+} influx (Shimada et al., 1999). Thus, these alkaloids are considered to play important roles in neurotrophic, neuroprotective, and antiapoptotic mechanisms in neurons and other kinds of cells (Shi and Kenneth, 2002; Kang et al., 2004), although the relationship between these compounds with amyloid aggregation is still unknown. Studies of antiamyloid effects of these alkaloids and how much of the active compounds of *Uncaria rhynchophylla* could pass through the blood–brain barrier are now underway.

In the present study, we examined the effects of water, methanol, and ethanol extracts of several medicinal herbs contained in traditional Chinese herbal medicines on fibrillation and destabilization of Aβs. *Curcuma longa* (extract A in Fig. 1), *Cinnamonum cassia* (D), *Uncaria rhynchophylla* (K), and *Paeonia suffruticosa* (Q) destabilized $A\beta_{1-40}$ and $A\beta_{1-42}$ fibrils with IC_{50} values of approximately 10 μg/ml. The major molecule contained in *Curcuma longa* is curcumin, which was previously reported to inhibit Aβ fibril formation and destabilize preformed Aβ fibrils in vitro and in vivo (Ono et al., 2004; Yang et al., 2005). Therefore, *Curcuma longa* could also be a good candidate for treatment of AD.

The extracts of *Uncaria rhynchophylla* inhibited aggregation of both $A\beta_{1-40}$ and $A\beta_{1-42}$ to a similar extent. These results suggest that the inhibitory effect of *Uncaria rhynchophylla* on amyloidogenesis of Aβ is not dependent on the amino acid sequence of its C-terminal. However, *Citrus unshiu* (L) caused inhibition of $A\beta_{1-40}$ fibril formation and destabilization of preformed $A\beta_{1-40}$ fibrils, whereas it did not affect $A\beta_{1-42}$. Furthermore, some of the other Chinese herbal extracts show distinct effects on $A\beta_{1-40}$ from those on $A\beta_{1-42}$ so that mechanisms of herbal medicines on aggregation and metabolism of Aβs could not be the same.

All the results obtained from thioflavin T fluorescence, AFM, and SDS-insoluble fibril analyses have proved the inhibitory effects of *Uncaria rhynchophylla* extracts (water, methanol, and ethanol) on the assembly of Aβ fibrils. In the thioflavin T experiments, the three extracts induced a decrease in fluorescence intensity of thioflavin T in the μg/ml range. We confirmed that these extracts did not quench thioflavin T fluorescence at the diluted concentration in our preliminary experiment. These results would suggest two possibilities; one is that *Uncaria rhynchophylla* indeed destabilizes Aβ fibrils, and the other is that this extract antagonizes binding of thioflavin T to Aβ. It is reported that absorbance of Congo red is increased by binding to Aβ protein as well as thioflavin T, although the binding site in Aβ to Congo red is different from that to thioflavin T. In our preliminary experiments, each of three different *Uncaria rhynchophylla* extracts decreased absorbance of Congo red, suggesting that the decrease in thioflavin T fluorescence by *Uncaria rhynchophylla* extracts is caused by destabilization of Aβ fibrils. Furthermore, our AFM results also strongly support this notion, because destabilization of Aβ fibrils by *Uncaria rhynchophylla* extracts was indeed visualized morphologically.

Legleiter and colleagues (2004) have previously reported from AFM study that the m266.2 antibody directed against the central domain of Aβ prevents formation of fibrils, and our AFM study also revealed that the number of $A\beta_{1-42}$ fibrils was significantly reduced by treatment with each of the extracts of *Uncaria rhynchophylla*. In addition, *Uncaria rhynchophylla* affected both $A\beta_{1-40}$ and $A\beta_{1-42}$, which are different in the amino acid sequences of the C-terminal, to a similar extent. These results suggest that the extracts of *Uncaria rhynchophylla* could inhibit Aβ fibril formation through blocking Aβ proteins to bind to each other at the central domain of the protein.

The levels of SDS-insoluble $A\beta_{1-42}$ fibril measured by Li and colleagues (1999) indicated that human AD brains express predominantly SDS-insoluble Aβ compared with the SDS-soluble Aβ fraction. These phenomena would suggest that the SDS-insoluble form of Aβ, as predicted to be present in the AD brain where massive amyloid plaques are formed, plays a key role in the pathogenesis of AD. Because our data revealed that SDS-insoluble pellets of Aβ were decreased by the extracts of *Uncaria rhynchophylla* in a concentration-dependent manner, these extracts are considered to be candidates for a therapeutic strategy for AD by eliminating senile plaques in the brain.

It is proposed that Aβ toxicity is more strongly linked to the formation of oligomeric aggregates (Kirkitadze et al., 2002). In our preliminary experiments, the treatment of SK-N-SH cells with *Uncaria rhynchophylla* extracts significantly protected the cells against $A\beta_{1-40}$ or $A\beta_{1-42}$ toxicity, in close agreement with concentrations that inhibited Aβ aggregation. It is not clear whether this protective effect of *Uncaria rhynchophylla* extracts is mediated by inhibition of polymerization of insoluble Aβs or by a decrease in oligomeric Aβ formation from soluble monomeric Aβs. We are now investigating how each status of Aβs, that is, monomers, oligomers, and polymers, is modulated by *Uncaria rhynchophylla* extracts.

In conclusion, our study suggests that *Uncaria rhynchophylla* not only inhibits Aβ fibril formation but also disassembles preformed Aβ fibrils. Therefore, the extracts of *Uncaria rhynchophylla* could have the ability to become a therapeutic drug for AD patients as well as a primary or secondary preventive agent for healthy individuals and patients with mild cognitive impairment. This medical plant is reported to have protective effects against neuronal cell death, and it is considered that *Uncaria rhynchophylla* has very little toxicity, because no obvious adverse effects of *Pulvis uncariae* and *Pulvis depressionis hepatis* have been reported although these have been used as therapeutics for other diseases for more than 1,000 years. Therefore, *Uncaria rhynchophylla* may be a new class of therapeutic and preventive drug for AD through regulation of the formation and the clearance of senile plaques.

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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	—N=N—	OH, HO	0.3	50
2-Phenylazo-4-methylphenol		CH_3, HO	0.3	75
4-Methyl-2-(2-thiazolylazo)phenol		CH_3, HO	-	0.5
1-(2-Thiazolylazo)-2-naphtol	S, N, —N=N—	HO	-	0.5
4-(2-Thiazolylazo)resorcinol		OH, HO	3	5
TAMSMB		CH_3, $NHCH_2SO_3H$, HOOC	-	>100
4-(2-Pyridylazo)resorcinol	N, —N=N—	OH, HO	-	0.25
1-(2-Pyridylazo)-2-naphtol		HO	-	1
5-Br-PAPS	Br, N, —N=N—	$N(C_3H_7)(C_3H_6SO_3Na)$, NaO	15	20
5-Br-PADAP		$N(C_3H_7)_2$, HO	4	10
5-Cl-PADAP	Cl, N, —N=N—	$N(C_3H_7)_2$, HO	2	5
$5\text{-}CF_3$-PADAP	F_3C, N, —N=N—	$N(C_3H_7)_2$, HO	4	10
Yellow AB	—N=N—	H_2N	0.5	100
Chrysoldine		NH_2, H_2N	0.015	>100
Congo red	NaO_3S, NH_2, —N=N—, —N=N—, SO_3Na, H_2N		0.014	not tested
Quinacrine	CH_3, HN, CH_3, N, CH_3, OCH_3, Cl, N		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.

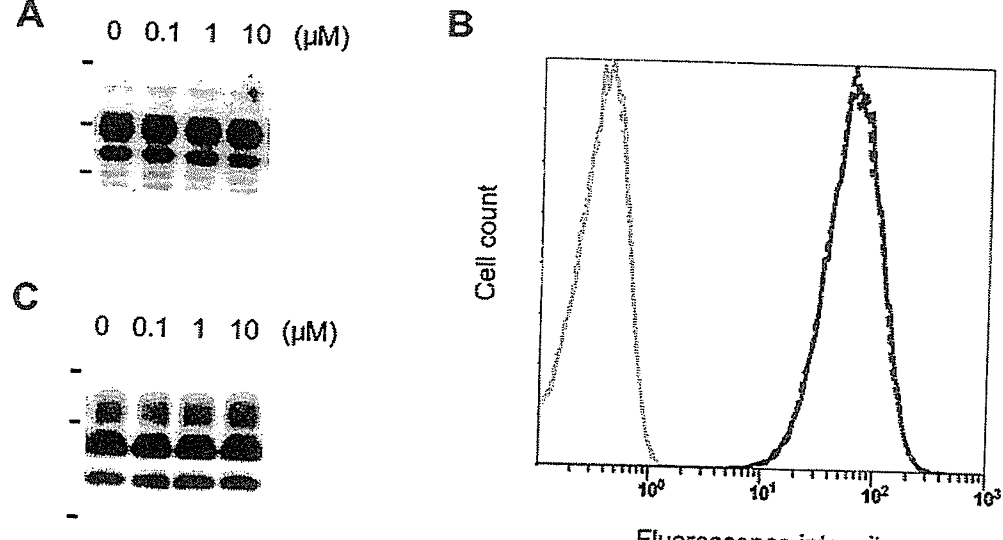

Fig. 1. Effects of a representative chemical, chrysoidine, on the cellular PrP (A, B) and the cell lysate abnormal PrP (C). (A) Immunoblot data of the total cellular PrP in noninfected NB cells treated with a designated dose of chrysoidine. *Bars* on the *left* indicate molecular size markers at 81,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

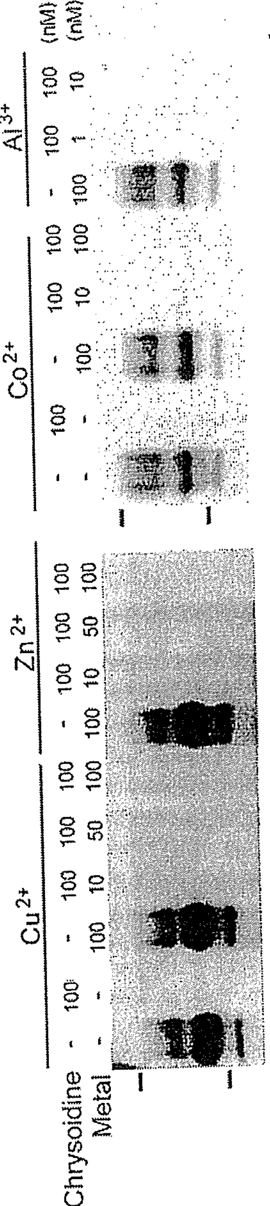

Fig. 2. Antiprion activity in ScNB cells of chrysoidine preincubated with metal ions. Immunoblot data of the abnormal PrP are shown. *Bars* on the *left* indicate molecular size markers at 37 and 25 kDa.

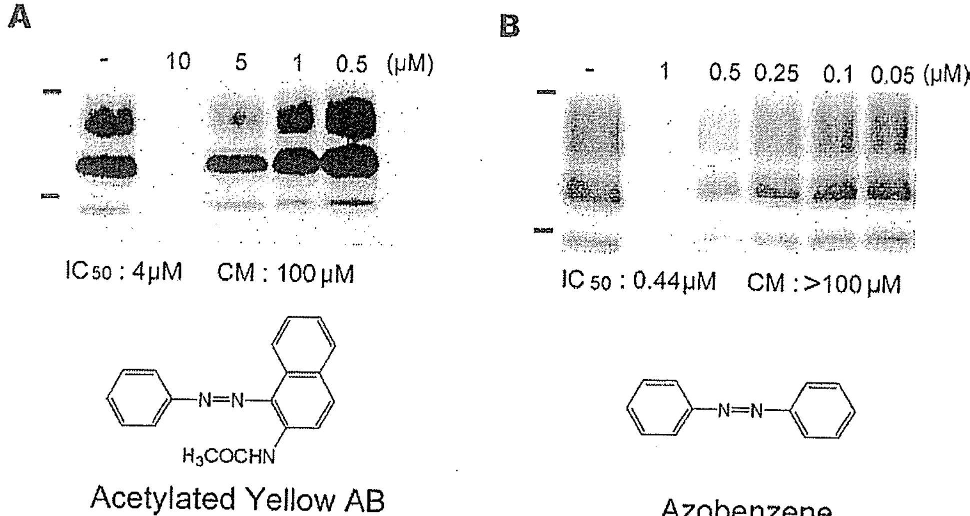

Fig. 3. Antiprion activity in ScNB cells of acetylated Yellow AB (A) and azobenzene (B) Immunoblot data of the abnormal PrP are shown. *Bars* on the *left* indicate molecular size markers at 37 and 25 kDa. IC_{50} is approximate dose giving 50% inhibition of abnormal PrP formation. CM is approximate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

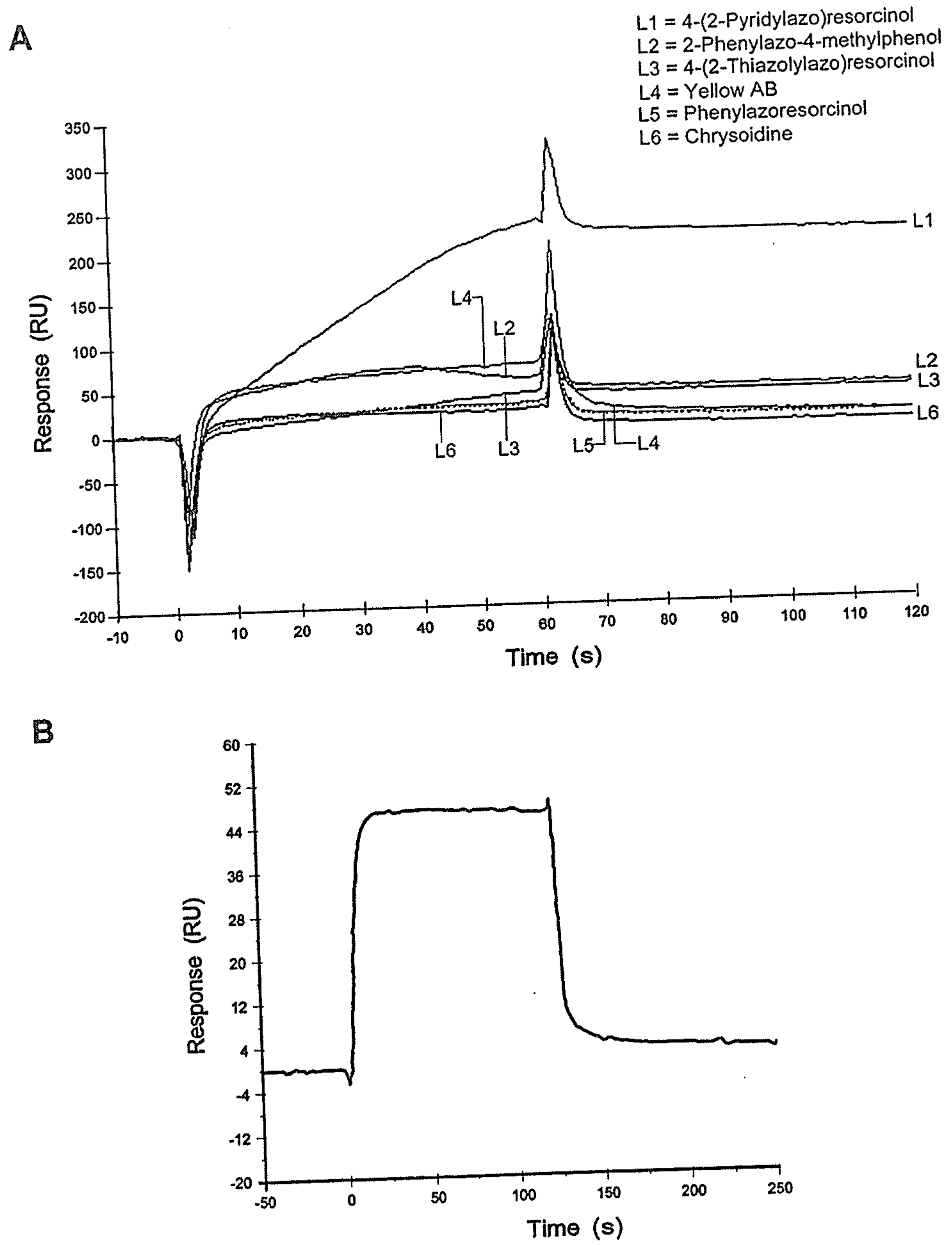

Fig. 4. SPR sensorgrams of chelating compounds (A) and quinacrine (B) interacting with PrP121–231.Each chemical at 50 μM was analyzed using a *ca.* 3,000 RU PrP-bound biosensor chip. Each phase of association and dissociation was monitored for 60 s in (A) or 125 s in (B).

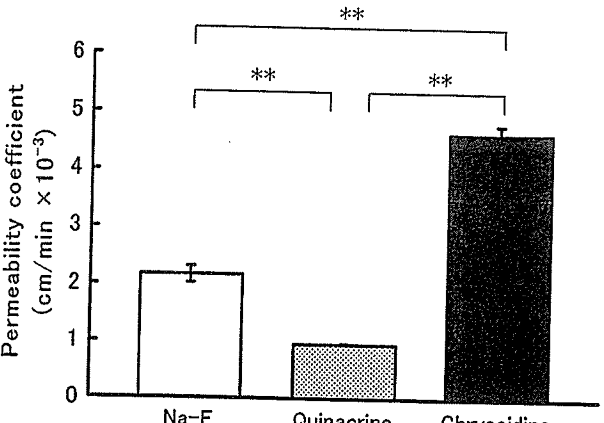

Fig. 5. Permeability coefficients of Na-F, quinacrine, and chrysoidine through MBEC4 monolayer. Each chemical at 100 μM was analyzed. The values are mean ± SEM (n = 3–4 inserts). $^{**}p < 0.01$; significant difference between each group.

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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