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Metal complexes with superoxide dismutase-like activity as candidates for anti-prion drug

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Abstract—Various compounds were evaluated for ability to inhibit the formation of the abnormal protease-resistant form of prion protein (PrP-res) in two cell lines infected with different prion strains. Examination of the structure–activity relationships indicated that compounds with copper-selective chelating ability and whose copper complexes have high SOD-like activity are candidates for anti-prion drug.

Transmissible spongiform encephalophathies (TSEs) or prion diseases are a group of fatal neurodegenerative disorders, and their development is associated with accumulation of aggregated proteins, oxidative damage to the brain, and neuronal cell loss. Prion diseases are characterized by the generation of a protein molecule termed PrP^{Sc} (scrapie isoform of the prion protein), which is a conformational variant of the normal host protein, PrP^{C} (cellular isoform of the prion protein).[1,2] It is believed that the conversion of PrP^{C} into PrP^{Sc} is the key event in the pathogenesis of TSEs.

The octapeptide repeat region of the PrP^{C} binds several copper ions with concentration of the micromolar range[3,4] and their dissociation constant for the ion is reported to be femtomolar range.[5] The biological significance of this interaction is not clear, but it is reported that PrP^{C} has a copper-dependent superoxide dismutase (SOD) activity[6] and PrP^{C} may be involved in copper uptake into cells.[7,8] Recently, there has been increasing interest in the role of metal ions, in particular copper, in prion diseases.[9,10]

In the early 1970s, it was reported that the copper chelator cuprizone induced prion diseases-like histopathological changes in mice.[11,12] On the other hand, Sigurdsson et al. recently found that a copper chelator, D-penicillamine, delayed the onset of prion disease in infected mice, and suggested that chelator-based therapy might attenuate the disease.[13] Copper has been implicated in the pathogenesis of prion disease, but numerous studies have only succeeded in demonstrating the complexity of the effects of copper on the development of prion diseases, and it remains unclear whether this ion promotes or inhibits disease progression.

In the present study, we evaluated the ability of a wide range of compounds[14] to inhibit the formation of the abnormal protease-resistant form of prion protein (PrP-res), using two cell lines, ScN2a cells and F3, infected with different prion strains.[15,16] We then analyzed the structure–activity relationships to investigate what kinds of structure or biochemical characteristics contribute to anti-prion activity.

Spectrophotometric complexation studies.[17–19] The complexes were prepared as previously reported.[20,21] Solutions of 10 mM $Cu(ClO_4)_2$ and 8-hydroxyquinoline were prepared in H_2O. Cu(II)-chelate formation of 8-hydroxyquinoline was demonstrated by Job's method.[18,19] The spectrophotometric complexation studies showed that 8-hydroxyquinoline binds in 2:1 ratio with Cu(II) (Fig. 1A). 2,2′-Biquinoline, neocuproine, bathocuproine, 4,4′-dicarboxy-2,2′-biquinoline, porphyrins, cimetidine and D-penicillamine bind in 1:1 ratio with Cu(II) (2,2′-biquinoline, Fig. 1B; others, data not

Keywords: Prion; 2,2′-Biquinoline; Cimetidine; TPEN; Copper; Chelate; Metal complex; SOD activity.
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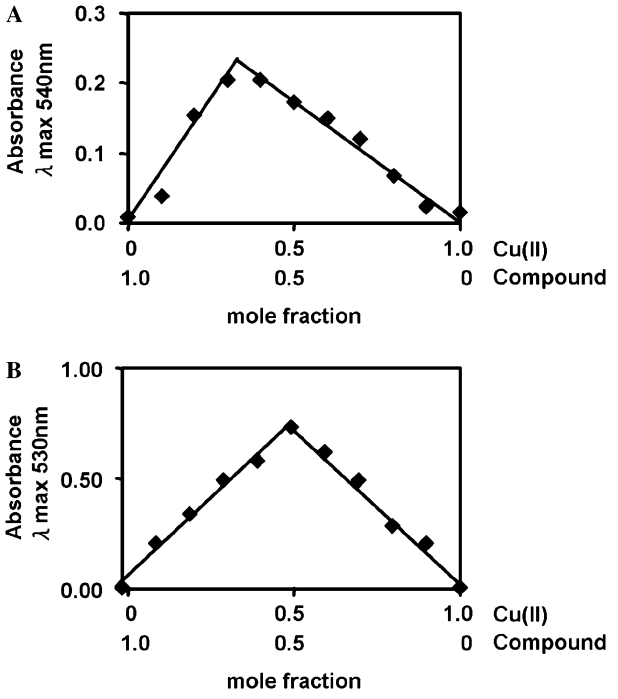


Figure 1. Continuous variation plots for 8-hydroxyquinoline and Cu(II) (A) and 2,2′-biquinoline and Cu(II) (B). (A) 2:1 binding ratio between 8-hydroxyquinoline and Cu(II), (B) 1:1 binding ratio between 2,2′-biquinoline and Cu(II). The plots were obtained by Job's method in aqueous solution.

shown). However, it has been reported that the oxidation state of copper may be altered in the D-penicillamine complex, and the complex prepared in this way contains both Cu(I) and Cu(II).[22]

Inhibition of PrP-res formation in ScN2a cells and F3 cells by metal chelators.[23–26] 1,10-Phenanthroline, 2,2′,2″-terpyridine and 8-hydroxyquinoline did not inhibit PrP-res formation within a nontoxic dose range (Table 1), but were cytotoxic at 100 nM. Chelators of this class can chelate a wide variety of metals.

Neocuproine, bathocuproine, 2,2′-biquinoline and 4,4′-dicarboxy-2,2′-biquinoline are highly specific copper chelators. The chelators of this class, except 4,4′-dicarboxy-2,2′-biquinoline, effectively inhibited PrP-res formation in ScN2a cells and F3 cells in a dose-dependent manner (Fig. 2). The concentrations giving 50% inhibition (IC_{50}) of PrP-res formation in ScN2a cells relative to the DMSO-treated or untreated control ranged from 5 to 80 nM (Table 1). These compounds showed no apparent cytotoxicity at concentrations up to 1 μM. However, neocuproine was ineffective in F3 cells within a nontoxic dose range. Findings from these experiments suggest that compounds having copper-selective chelating ability are more effective inhibitors than non-selective metal-chelating compounds, but not an exclusive factor.

Inhibition of PrP-res formation in ScN2a cells and F3 cells by porphyrins.[23–26] Porphyrins can form 1:1 stable chelates with various metal ions. The order of stability for divalent metal ions is Cu > Fe > Zn > Mn, regardless of the type of substituents on the porphyrin ring. Porphyrins were effective inhibitors of PrP-res formation, with IC_{50} values ranging from 5 to 320 nM in ScN2a cells and F3 cells (Table 2). And Mn(III)–porphyrins complexes showed higher anti-prion activity than the metal-free compounds (Table 2).

SOD-like activity and correlation with anti-prion activity. It is known that Mn(III)–porphyrin complexes show high SOD-like activity in vitro and in vivo.[27,28] We thought that SOD-like activity might contribute to the anti-prion activity of such compounds, since the SOD activity of PrP^{C} is decreased by conversion to PrP^{Sc}. Therefore, we focused on chelators having SOD-like activity. Many low-molecular metal complexes, mainly copper, manganese and iron complexes, have been synthesized and their SOD-like activity examined in vitro and in vivo,[29–33] and some of them showed activity in vivo.[34–36] As shown in Table 3, SOD-like activity of these compounds was measured in vitro by our methods.[37] The SOD-like activity in cell lysates was significantly increased when these metal-free compounds were added to the cell cultures (data not shown). Therefore, the chelators that showed anti-prion activity formed metal complexes and had SOD-like activity.

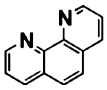
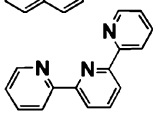
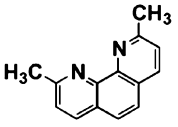
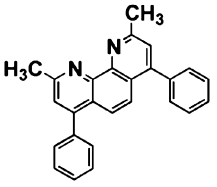
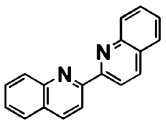
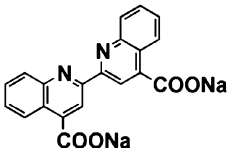
Among these compounds, we chose cimetidine[34,38] and TPEN[39] for further examination, as well as Mn-TCPP (Mn-TBAP), which we had already examined. Cimetidine effectively inhibited PrP-res formation, with IC_{50} values of 5 nM in ScN2a cells and 200 nM in F3 cells. TPEN inhibited PrP-res formation, with IC_{50} values of 5 nM in ScN2a cells and 200 nM in F3 cells.

We found that the compounds, shown in Tables 1 and 2, with higher anti-prion activity in ScN2a cells had higher SOD-like activity (Table 3). Statistical analysis exhibited a significant linear correlation between these two activities ($r = 0.93$) (Fig. 3).

Despite numerous studies, it remains unclear whether copper ions promote[13] or inhibit[40] prion disease. In Alzheimer's disease, another neurodegenerative disease, the copper- and zinc-selective chelator clioquinol was effective in decreasing β-amyloid deposits.[41] However, Doh-ura et al. found that clioquinol and related compounds, quinoline hydrochloride, 8-hydroxyquinoline, and 8-acetoxyquinoline, were ineffective in scrapie-infected mouse neuroblastoma (ScNB) cells.[25] Thus, chelating drugs that are effective in inhibiting β-amyloid formation may not inhibit the conversion of PrP^{C} to PrP^{Sc}.

In this study, we evaluated the anti-prion activity of various compounds having metal-chelating ability in order to identify the requirements for anti-prion activity. We found that many, but not all, compounds having selective copper-chelating ability are effective inhibitors of PrP-res formation in ScN2a cells and F3 cells. Thus, copper-selective chelating ability per se may not be essential for anti-prion activity. This idea is supported by the observation that porphyrins chelating manganese

Table 1. Inhibition of PrP-res formation in ScN2a cells and F3 cells by metal chelators

Compound	Structure	Metal(M)	Inhibition PrP-res IC_{50} (nM)	
			ScN2a cells	F3 cells
1,10-Phenanthroline			N.E.	N.E.
2,2′,2″-Terpyridine			N.E.	N.E.
8-Hydroxyquinoline			N.E.	N.E.
Bis(8-quinolinolato) Copper(II)		Cu^{2+}	N.E.	N.E.
Bis(8-quinolinolato) Zinc(II)		Zn^{2+}	N.E.	N.E.
Neocuproine			80	N.E.
Bathocuproine			80	200
2,2′-Biquinoline			5	250
4,4′-Dicarboxy-2,2′-biquinoline			N.E.	N.E.

N.E., no effect.
IC_{50}, concentration of a compound causing 50% inhibition of PrP-res formation relative to the control.

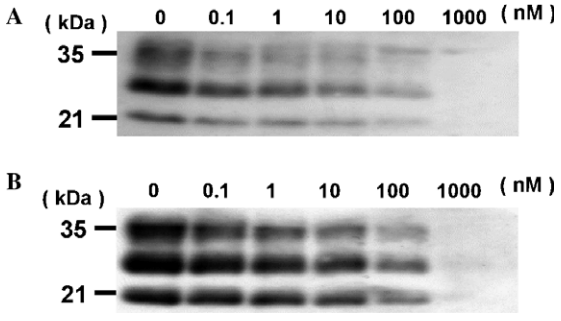
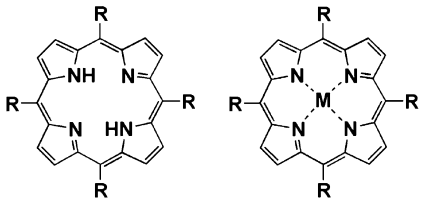


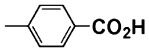
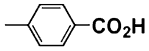
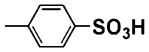
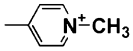
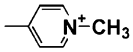
Figure 2. Anti-prion activity of 2,2′-biquinoline in prion-infected cells. Various concentrations of the compound were added to freshly passaged ScN2a cells (A) or F3 cells (B), and the PrP-res levels were analyzed by Western blotting. Lanes: 0, cells treated with DMSO alone; others, treated with the indicated concentration of 2,2′-biquinoline. Bars on the left indicate molecular mass markers at 35 and 21 kDa.

showed greater anti-prion activity than the metal-free compounds. Therefore, we examined whether SOD-like activity was associated with anti-prion activity, and discovered that this was the case.

PrP^C plays an important role in cell protection from oxidative stress, and modulates the activity of antioxidant enzymes by regulating the intracellular copper concentration, but it can also play a direct role owing to its intrinsic SOD activity.[6,42,43] Cells with accumulated abnormal PrP^{Sc} displayed the phenotypes of decreased copper-binding capacity and higher sensitivity to oxidative stress.[16,44] Interestingly, we found a significant correlation ($r = 0.93$) between SOD-like activity and anti-prion activity. Furthermore, we confirmed that the copper complex of D-penicillamine, which has been reported

Table 2. Inhibition of PrP-res formation in ScN2a cells and F3 cells by porphyrins



Compound	R	Metal(M)	Inhibition PrP-res IC_{50} (nM)	
			ScN2a cells	F3 cells
TPP			10	320
TCPP			250	160
Mn-TCPP (MnTBAP)		Mn^{3+}	40	60
TPPS			200	160
TMPyP			130	160
Mn-TMPyP		Mn^{3+}	5	40

IC_{50}, concentration of a compound giving 50% inhibition of PrP-res formation relative to the control.

Table 3. SOD-like activity of metal complexes

Chelating metal	Compound	SOD-like activity IC_{50} (μM)
Cu	8-Hydroxyquinoline	263
	Clioquinol	140
	Neocuproine	50
	Bathocuproine	32
	2,2′-Biquinoline	3
	4,4′-Dicarboxy-2,2′-biquinoline	263
	Cimetidine	0.4
	D-Penicillamine	28
Mn	TCPP	8
	TMPyP	0.3
Fe	TPEN	0.4

IC_{50}, concentration of a compound giving 50% inhibition of WST-1 reduction.

to show anti-prion activity, exhibits SOD-like activity.[13]

It is not easy to find molecules with both good metal-binding ability and high SOD-like activity, because, taking copper ions as an example, the former property means the Cu(II) complex is rather stable, while the latter property implies that the complex is prone to be reduced to the Cu(I)-chelator state.[45] This might explain why compounds such as clioquinol that are good copper chelators are nevertheless ineffective in terms of anti-prion activity.[25]

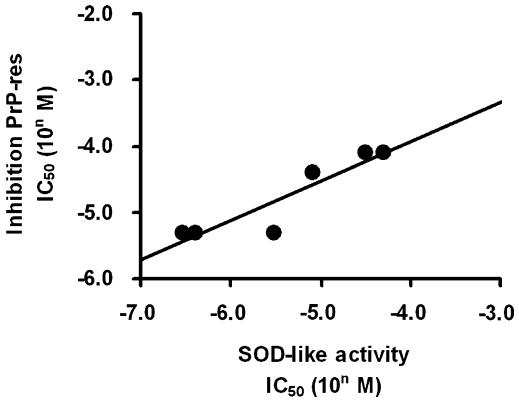


Figure 3. Correlation between SOD-like activity and inhibition of PrP-res formation in ScN2a cells. The plot shows data from seven compounds for which both SOD activity and inhibition of PrP-res formation were determined. ($r = 0.93$) SOD-like activity IC_{50}: concentration of a compound giving 50% inhibition of WST-1 reduction. Inhibition PrP-res IC_{50}: concentration of a compound giving 50% inhibition of PrP-res formation relative to the control.

On the other hand, cimetidine can form complexes with both Cu(I) and Cu(II), and has satisfactory SOD-like activity in both states, so it may be a good candidate for anti-prion activity. Furthermore, cimetidine can cross the blood–brain barrier to act in the central nerve system.[46] This type of compounds may provide a possible therapeutic approach for prion diseases.

In conclusion, we suggest that compounds which have copper-selective chelating ability, and whose copper complexes have high SOD-like activity are candidates for anti-prion drug.

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14. Copper(II) perchlorate hexahydrate 98% and D-penicillamine were purchased from Sigma. Iron(II) sulfate heptahydrate, 2,2′-biquinoline, and cimetidine were purchased from Wako Pure Chemical (Osaka, Japan). 1,10-Phenanthroline monohydrate, 2,9-dimethyl-4,7-dimethyl-1,10-phenanthroline (bathocuproine), 2,9-1,10-phenanthroline (neocuproine), tetraphenylporphine (TPP), tetraphenylporphine tetrasulfonic acid (TPPS), α,β,γ,δ-tetrakis(1-methylpyridinium-4-yl)porphine *p*-toluenesulfonate (TMPyP), tetrakis(4-carboxyphenyl)porphine (TCPP), 2,2′-bicinchoninic acid dipotassium salt, 5-chloro-7-iodo-8-hydroxyquinoline (clioquinol), and 8-hydroxyquinoline were purchased from Tokyo Kasei (Tokyo, Japan). Manganese(III) tetrakis(1-methylpyridinium-4-yl)porphyrin pentachloride (Mn-TMPyP) and Mn(III)tetrakis(4-benzoic acid)porphyrin chloride(Mn-TCPP or Mn-TBAP) were purchased from Calbiochem (California, USA). They were dissolved in 100% dimethylsulfoxide (DMSO) or 95% ethanol just before use.
15. Two types of prion-infected mouse neuroblastoma (N2a) cell lines were used in this study: N2a cells infected with the RML strain (ScN2a) [16] and N2a#58 cells infected with the Fukuoka-1 strain (F3). N2a#58 cells are known to express five times more normal PrP than N2a cells. Both ScN2a cells and F3 cells were grown in six-well culture plates in Opti-MEM (Invitrogen) supplemented with 10% fetal bovine serum. The cells were allowed to reach confluence, and chemicals at various concentrations were added to the medium when 5% of the confluent cells were passaged.The final concentration of either DMSO or ethanol in the medium was less than 0.2%. The cultures were allowed to grow to confluence (3 or 4 days).
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23. The anti-prion activity of each compound was assayed by measuring the 50%-inhibitory concentration (IC_{50}) for PrP-res formation in ScN2a cells and F3 cells, as described in previous reports.[24–26] Briefly, compounds were added at designated concentrations to the medium when cells were passaged at 10% confluency. The cells were allowed to grow to confluence and lysed with lysis buffer (0.5% sodium deoxycholate, 0.5% Nonidet P-40, and PBS). The lysates were digested with 10 μg/ml proteinase K for 30 min at 37 °C and centrifuged at 15,000 rpm for 5 min at 24 °C with GLASSFOG(Q-bio gene, USA). The pellets were resuspended in sample loading buffer and boiled. Samples were separated by electrophoresis on 15% Tris–glycine–SDS–polyacrylamide gel and electroblotted. PrP-res was detected using an antibody, SAF83 (1:5000; SPI-Bio, France), followed by an alkaline phosphatase-conjugated secondary antibody. Immunoreactive signals were visualized using CDP-Star detection reagent (Amersham Biosciences Corp., U.S.A.) and were analyzed densitometrically. At least three independent experiments were performed to estimate IC_{50} of each compound.
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