New Series of Antiprion Compounds: Pyrazolone Derivatives Have the Potent Activity of Inhibiting Protease-Resistant Prion Protein Accumulation

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Abstract: To find effective antiprion compounds, we synthesized and evaluated various pyrazolone derivatives. Seven of 19 compounds showed inhibition of PrP-res accumulation and the remarkably active compound **13** showed an IC_{50} value of 3 nM in both ScN2a and F3 cell lines. Findings from studies on physicochemical and biochemical properties suggest that the action mechanism of these compounds does not correlate with any antioxidant activities, any of hydroxyl radical scavenging activities, or any SOD-like activities.

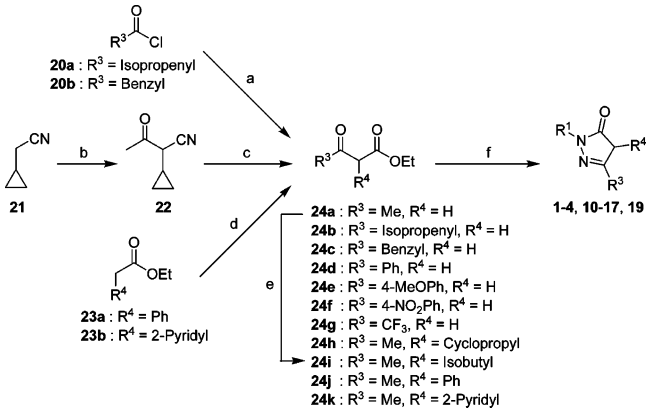
Prion diseases or transmissible spongiform encephalopathyies (TSEs[a]) are invariably fatal neurodegenerative diseases that include Creutzfeldt−Jakob disease (CJD), Gerstmann−Sträussler−Scheinker syndrome (GSS), familial fatal insomnia (FFI), and kuru in humans, scrapie in sheep and goats, chronic wasting disease (CWD) in deer and elk, and bovine spongiform encephalopathy (BSE) in cattle. These diseases are characterized by deposition of the protease-resistant isoform of prion protein (PrP^{Sc}), which is thought to be the main component responsible for the pathogenesis. PrP^{Sc} is known to be an abnormally folded β-rich conformation of cellular prion protein (PrP^{C}) and is resistant to digestion with proteinase K.[1]

Natural and constitutive prion protein, PrP^{C}, is a GPI-anchored membrane glycoprotein. The biological significance of this protein is unclear, but it is reported that the N-terminal octapeptide repeat region of PrP^{C} binds several copper ions with a femtomolar dissociation range (K_d).[2,3] PrP^{C} has copper-dependent superoxide dismutase (SOD) activity[4] and may also be involved in copper uptake into cells.[5,6] Recently, there has been increasing interest in the role of copper in prion diseases.[7,8] In 2003, it was reported 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.[9] 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.

Although there are no suitable therapies for this disorder, outbreaks of variant CJD and iatrogenic CJD through the use of cadaveric growth hormone or dural grafts in younger people have necessitated their development. Furthermore, screening for antiprion compounds in a cell culture model of prion disease has led to the identification of many antiprion compounds,[10] such as quinoline derivatives,[11,12] Congo red and analogues,[13,14] and 2-aminopyridine-3,5-dicarbonitrile compounds;[15] however, their activity is thought to be insufficient to develop therapeutic agents.

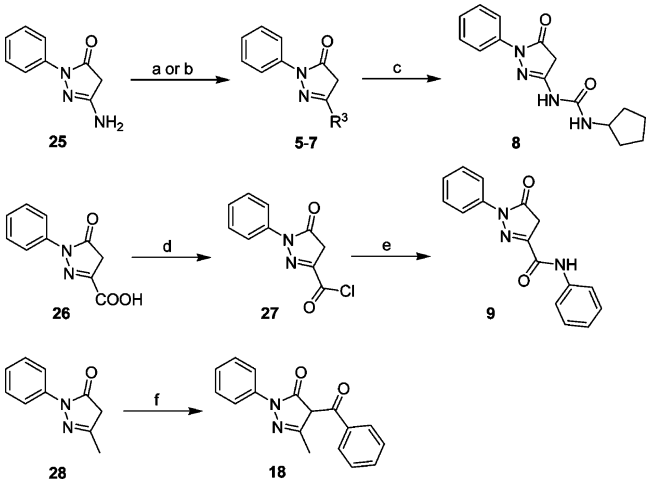
Recently, a new pyrazolone compound, edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one, also known as MCI-186), has been developed as a medical drug for brain ischemia[16,17] and has also been reported to be effective for myocardial ischemia.[18] In this

Scheme 1. Synthesis of Pyrazolone Derivatives **1**−**4**, **10**−**17**, and **19**[a]



[a] Reagants and conditions: (a) (i) malonic acid monoethyl ester potassium salt, $MgCl_2$, Et_3N, MeCN; (ii) 2 M HCl aq, 0 °C, 39−43%; (b) (i) LDA, THF; (ii) Ac_2O, THF, −78 °C, 78%; (c) (i) AcCl, EtOH; (ii) c-HCl, EtOH, 40 °C, 89%; (d) (i) NaH, THF, 60 °C; (ii) Ac_2O, THF, rt, 7−51%; (e) (i) NaOEt, EtOH; (ii) isobutyl iodide, THF, 80 °C, 43%; (f) R^1NHNH_2, EtOH or AcOH, reflux, 11−85%.

Scheme 2. Synthesis of Pyrazolone Derivatives **5**−**9**, **18**[a]



[a] Reagants and conditions: (a) for **5** and **6**, R^1OCOCl, pyridine, 50 °C, 16−23%; (b) for **7**, benzoyl chloride, dioxane, rt, 15%; (c) **6**, cyclopentylamine, xylene reflux, 42%; (d) oxalyl chloride, DMF, CH_2Cl_2; (e) aniline, CH_2Cl_2, rt, 74% (two steps); (f) benzoyl chloride, $Ca(OH)_2$, dioxane, reflux, 79%.

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[a] Abbreviations: TSEs, transmissible spongiform encephalopathies; CJD, Creutzfeldt−Jakob disease; GSS, Gerstmann−Sträussler−Scheinker syndrome; FFI, familial fatal insomnia; CWD, chronic wasting disease; BSE, bovine spongiform encephalopathy; PrP^{Sc}, infectious conformational form of prion protein; PrP^{C}, normal cellular prion protein; GPI, glycosylphosphatidylinositol; SOD, superoxide dismutase; PrP-res, protease-resistant form of prion protein; RML, Rocky Mountain Laboratory; PBS, phosphate-buffered saline; Tris, tris(hydroxymethyl) aminomethane; SDS, sodium dodecyl sulfate.

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Table 1. Inhibition of PrP-res Accumulation in ScN2a Cells and F3 Cells, Oxidation Potentials (E_{pa}), and Hydroxyl Radical Scavenging Activity

cmpd	R^1	R^3	R^4	inhibition PrP-res IC_{50}[a,c,d] (nM) ScN2a cells	inhibition PrP-res IC_{50}[a,c,d] (nM) F3 cells	E_{pa}[e,f] (mV)	pH[g]	scavenging activity IC_{50} (mM)[h]
edaravone	Ph	CH_3	H	>1000	N.E.[b]	483	7.0	0.25
1	cyclohexyl	CH_3	H	13	25	549	7.4	
2	4-CH_3OPh−	CH_3	H	N.E.[b]	N.E.[b]	678	7.8	
3	4-ClPh−	CH_3	H	0.5	N.E.[b]	473	7.4	
4	Ph	isopropenyl	H	158	794	387	7.4	
5	Ph	CH_3OCONH−	H	6	501	454	7.8	
6	Ph	PhOCONH−	H	N.E.[b]	N.E.[b]	397	7.0	0.38
7	Ph	PhCONH−	H	2000	1260	458	7.8	0.22
8	Ph	cyclopentylNHCONH−	H	126	158	372	7.8	
9	Ph	PhNHCO−	H	398	1580	478	7.8	
10	Ph	$PhCH_2$−	H	N.E.[b]	N.E.[b]	269	>8.0	
11	Ph	Ph	H	N.E.[b]	N.E.[b]	397	7.6	
12	Ph	4-CH_3OPh−	H	N.E.[b]	N.E.[b]	397	7.8	
13	Ph	4-NO_2Ph−	H	3	3	419	7.4	0.09
14	Ph	CF_3	H	398	631	673	7.6	0.81
15	Ph	CH_3	cyclopropyl	N.E.[b]	N.E.[b]	275	7.8	0.72
16	Ph	CH_3	isobutyl	N.E.[b]	16	262	>8.0	
17	Ph	CH_3	Ph	40	1	227	7.6	0.79
18	Ph	CH_3	PhCO−	6	1000	640	7.0	0.61
19	Ph	CH_3	2-pyridyl	79	631	403	>8.0	

[a] IC_{50}, concentration of a compound causing 50% inhibition of PrP-res accumulation relative to the control. [b] N.E., no effect. [c] At the concentration range for antiprion activity assay (10^{-10}−10^{-7} M for **2**, **3**, **11**, **12**, and **17** and 10^{-10}−10^{-6} M for the others), no cytotoxicity was observed against both of the two cell lines (Supporting Information). [d] In our system, IC_{50} values of quinine and quinidine in ScN2a cells are 10 μM and 5 μM, respectively. Those values are consistent with a previously report.[11] [e] Conditions for measurement: 10 mM sample in 50 mM NaCl; working electrode, Pt; reference electrode, Ag^+/AgCl; counter electrode, Pt; scan speed, 50mV/s; scan range, −0.2 to 1.0 V. [f] Oxidation potentials were expressed versus Ag^+/AgCl. [g] Oxidation properties (E_{pa}) were measured at indicated pH because of their poor solubility in acidic and neutral aqueous solutions. [h] Conditions for measurement: a mixture of 25 mM H_2O_2, 25 mM DMPO, and a compound was irradiated with UV. ESR spectrometer parameters were as follows: microwave power, 10 mW; modulation width, 0.063 mT; time constant, 0.03 s; sweep width, 7.5 mT; sweep time, 1 min; gain, 320.

study, we focused on and explored the pyrazolone compounds derived from edaravone, as antiprion agents, and found new and highly active antiprion compounds.

Our initial goal was to prepare a small focused library of edaravone derivatives. The preparation of pyrazolone compounds was achieved by refluxing the corresponding β-ketoester and hydrazine compound in ethanol or acetic acid. β-Ketoesters **24b**,**c**,**h**−**k**, which are not commercially available, were synthesized from acyl chlorides **20a**,**b**, nitrile **21**, ethyl acetoacetate **24a**, or ethyl esters **23a**,**b** (Scheme 1). Treatment of amine **25** with chloroformic acid ester or benzoyl chloride gave carbamates **5** or **6** or amide **7** (Scheme 2). Carbamate **6** was then converted to urea **8** by treatment with cyclopentylamine. Amide **9** was synthesized from carboxylic acid **26** via acyl chloride **27**. Compound **18** was prepared from edaravone **28** with benzoyl chloride in the presence of $Ca(OH)_2$.

The antiprion activity of each compound was evaluated as the ability to inhibit the accumulation of the abnormal protease-resistant form of prion protein (PrP-res), as described in previous reports.[11,19,20] In this study, two types of prion-infected mouse neuroblastoma (N2a) cell lines, ScN2a and F3, were used. N2a cells that were infected with the RML strain are called ScN2a,[21] and N2a#58 cells that were infected with the Fukuoka-1 strain are called 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. Compounds were added at the designated concentration to the medium when cells were passaged at 10% confluency. The cells were allowed to grow to confluence (3 or 4 days) 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, CA). The pellets were resuspended in sample loading buffer and boiled. Samples were separated by electrophoresis on 15% Tris-glycine-SDS-polyacryl-amide gel and electroblotted. PrP-res was detected using an antibody, SAF83 (1:5000; SPI-Bio, Montigny-le-Bretonneux, France), followed by an alkaline phosphatases-conjugated secondary antibody. Immunoreactive signals were visualized using CDP-Star detection reagent (Amersham Biosciences Corp., NJ) and were analyzed densitometrically. At least three independent experiments were performed to determine the IC_{50} value of each compound.

The original lead compound, edaravone, showed weak antiprion activity in ScN2a cells. The pyrazolone compounds **3** and **16** were effective in one of two cell lines (Table 1). Compounds **1**, **4**, **5**, **7**, **8**, **9**, **13**, **14**, **17**, **18**, and **19** inhibited PrP-res accumulation in both ScN2a cells and F3 cells, but the others did not (within a nontoxic dose range). Among the synthesized pyrazolone derivatives, 3-(4-nitrophenyl) compound **13** showed the highest activity for inhibiting PrP-res accumulation (IC_{50} = 3 nM), which is 130 times more active than quinacrine (IC_{50} = 400 nM)[19] and was one of the most potent compounds reported so far.[12,14] Although there are no reports that pyrazolone derivatives inhibit PrP-res accumulation in

prion-infected cells, compounds having a pyrazolone ring might be a new series of antiprion activity substances.

Because various types of compounds, such as 1-cyclohexyl compound **1**, 3-isopropenyl compound **4**, 3-(4-nitrophenyl) compound **13**, and 4-benzoyl compound **18**, showed relatively high antiprion activity, the position and class of substituents were not directly correlated with the activity of inhibiting PrP-res accumulation; therefore, we searched for the properties of synthesized compounds.

We had previously determined the oxidation potential and hydroxyl radical scavenging activity of edaravone-related derivatives.[22] Briefly, one-electron oxidation potentials (E_{pa}) of all synthesized derivatives were measured in a 50 mM NaCl solution by cyclic voltammetry (CV). Oxidation currents were observed with all the tested compounds but were irreversible, probably because the one-electron oxidation products were unstable and converted to degraded compounds as reported.[23] Because of the poor solubility of several derivatives in the neutral aqueous solution, the solutions were slightly basified using aqueous NaOH to solubilize these compounds.

Although the derivatives showed a wide variety of oxidation potentials (Table 1), no correlations were observed between oxidation potentials and antiprion activity.

Radical scavenging activity, which is known as the main action of edaravone as a brain-protecting drug,[23] was evaluated using the electron spin resonance (ESR) spin-trapping method with 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) as a spin trap.[22] Hydroxyl radicals were generated by UV irradiation (200 mJ/cm^2) of hydrogen peroxide solution containing DMPO and edaravone derivatives. The inhibitory effect of the derivatives on the formation of hydroxyl radical adducts of DMPO was used as a measure of radical scavenging activity.

IC_{50} values were determined for 7 of 19 derivatives with diverse antiprion activity (**6**, **7**, **13**, **14**, **15**, **17**, and **18**). Compounds **15** and **17** exhibited efficient inhibition of the hydroxyl radical adduct formation to a similar extent, but **15** did not inhibit PrP-res accumulation in contrast with **17**, which showed antiprion activity in the nanomolar range. It was found that there is poor correlation between hydroxyl radical scavenging activity and antiprion activity.

Recently, Fukuuchi et al. found that compounds that have copper-selective chelating ability and whose copper complexes have high SOD-like activity are candidates for antiprion drugs.[24] For example, D-penicillamine has been reported to show moderate antiprion activity[9] and its copper complex exhibits SOD-like activity with an IC_{50} value of 28 μM.[24] The copper complex of 2,2′-biquinoline, whose IC_{50} value of antiprion activity has been reported to be 5 nM,[24] also exhibits SOD-like activity, with an IC_{50} value of 3 μM.[24] We therefore considered if our compounds might show SOD-like activity itself or in the form of a copper complex.

To investigate this idea, we first examined whether the synthesized derivatives could chelate with Cu(II). The chelation study was carried out using Job's method.[25] Solutions of each compound and $Cu(ClO_4)_2$ at a ratio (compound/Cu(II)) of 1:0 to 0:1 were prepared in 95% ethanol, and absorption spectra were measured. Spectrophotometric complexation studies showed that **1**, **15**, and **18** bound with Cu(II) at a 2:1 ratio and **6**, **7**, and **16** bound with Cu(II) at a 1:1 ratio. Compounds **9**, **14**, and edaravone showed no spectral changes in the presence of Cu(II) (**18**, Figure 1A; others, data not shown). It was unclear whether other compounds, such as **3**, **13**, and **17**, can bind with Cu(II) because they showed little spectral shift in the presence of Cu(II) (**13**, Figure 1B; others, data not shown).

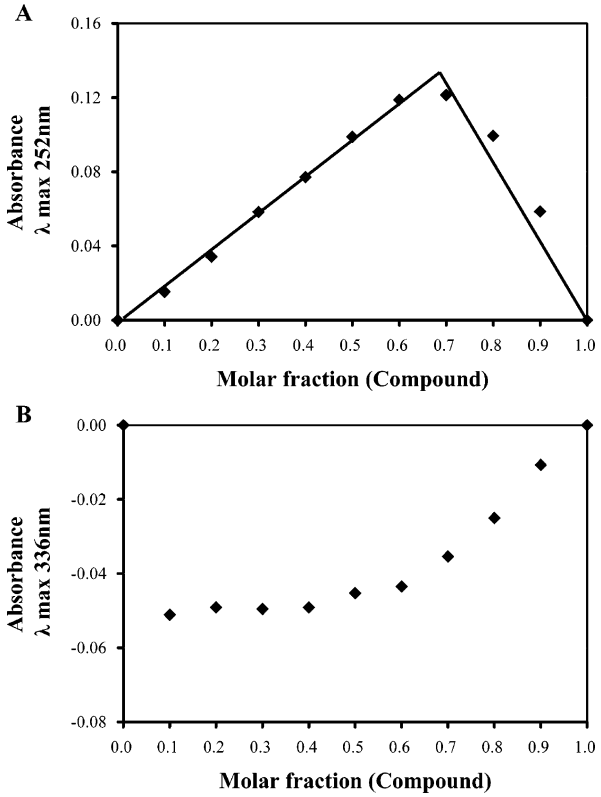
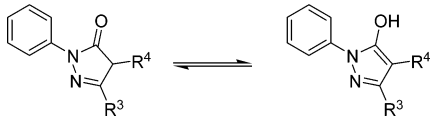


Figure 1. Continuous variation plots for compound **18** and Cu(II) (A) and compound **13** and Cu(II) (B). A: A 2:1 binding ratio between compound **18** and Cu(II); B: binding of compound **13** to Cu(II) was uncertain. Plots were obtained by Job's method in ethanol solution.

Table 2. SOD-Like Activities of Edaravone Derivatives



			SOD-like activity[a] (%)	
cmpd	R^3	R^4	cmpd	with Cu(II)[b]
edaravone	CH_3	H	0.6	5.7
6	PhOCONH—	H	5.6	11.6
7	PhCONH—	H	9.2	3.2
13	4-NO_2Ph—	H	1.8	11.9
14	CF_3	H	13.4	7.4
15	CH_3	cyclopropyl	15.9	35.4

[a] Activity, percentage of inhibition of WST-1 tetrazorium formation by a compound at 1 mM. [b] All compounds were measured at 1 mM and 2 mM of Cu(II).

Findings from these experiments suggest that copper-chelating ability was not essential for antiprion activity, as previously reported.[24,26]

SOD-like activity of synthesized compounds (edaravone, **6**, **7**, **13**, **14**, and **15**) was measured in vitro using SOD-like assay kit-WST (Dojindo Laboratories, Kumamoto, Japan). This method is a xanthine-based photometric assay using tetrazolium salt WST-1. SOD-like activities of derivatives were evaluated at 1 mM (Table 2). Although it was uncertain whether some compounds, such as **13**, bind with Cu(II), SOD-like activities of derivatives in the presence of Cu(II) were also investigated using a solution of 1 mM $Cu(ClO_4)_2$ and 0.5 mM compound in a 0.9% NaCl solution. Because it is known that Cu(II) has superoxide scavenging activity, we also evaluated the SOD-

like activity of Cu(II) itself. A total of 1 mM of Cu(II) inhibited WST-1 formazan formation by 6.7%.

For all measured compounds, SOD-like activity was found to be very weak. Furthermore, because nonantiprion compounds, such as **6** and **15**, showed comparable activity with **13** and were more effective than compounds **7** and **14**, SOD-like activity may not be correlated with antiprion activity of these compounds.

In conclusion, we found that some pyrazolone compounds derivatized from edaravone have the ability to inhibit the accumulation of PrP-res, and 3-(4-nitrophenyl) compound **13** had remarkable activity (IC_{50} = 3 nM). To obtain information about their action mechanism, we investigated their oxidation potentials, copper-complexing, and SOD-like activity. Findings from these experiments suggest that these properties have little correlation with activity.

Further active antiprion derivatives and the mechanistic studies are under investigation.

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Supporting Information Available: Experimental details of the synthesis and characterization data for all compounds and analytical methodologies. This information is available free of charge via the Internet at http://pubs.acs.org.

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