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A multiple free-radical scavenging (MULTIS) study on the antioxidant capacity of a neuroprotective drug, edaravone as compared with uric acid, glutathione, and trolox

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ABSTRACT

Edaravone (3-methyl-1-phenyl-2-pyrazoline-5-one) is a neuroprotective drug that has been used for brain ischemia injury treatment. Because its activity is speculated to be due to free radical scavenging activity, we carried out a quantitative determination of edaravone's free radical scavenging activity against multiple free radical species. Electron spin resonance (ESR) spin trapping-based multiple free-radical scavenging (MULTIS) method was employed, where target free radicals were hydroxyl radical, superoxide anion, alkoxyl radical, alkylperoxyl radical, methyl radical, and singlet oxygen. Edaravone showed relatively high scavenging abilities against hydroxyl radical (scavenging rate constant $k = 2.98 \times 10^{11}$ M^{-1} s^{-1}), singlet oxygen ($k = 2.75 \times 10^{7}$ M^{-1} s^{-1}), and methyl radical ($k = 3.00 \times 10^{7}$ M^{-1} s^{-1}). Overall, edaravone's scavenging activity against multiple free radical species is as robust as other known potent antioxidant such as uric acid, glutathione, and trolox. A radar chart illustration of the MULTIS activity relative to uric acid, glutathione, and trolox indicates that edaravone has a high and balanced antioxidant activity with low specificity.

The generation of a high level of reactive oxygen species (ROS) or free radicals has been shown in ischemia–reperfusion (I–R) injury in vivo, especially immediately after the reperfusion is initiated.[1–3] Therefore, it was speculated that the ROS over-production is the cause of the I–R injury, but the kind and amount of responsible ROS is unknown. Based on its ROS scavenging ability, edaravone (3-methyl-1-phenyl-2-pyrazoline-5-one (Fig. 1)) was developed as a therapeutic drug for human brain I–R injury.[4] The I–R damage occurs immediately after re-oxygenation, suggesting that oxygen-derived free radical species may be involved in these damaging processes.[4,5]

Many investigators have demonstrated in vivo and in vitro that edaravone possesses antioxidant activity or free radical scavenging activity;[6–11] however, the content of the scavenging activity is still unclear partly because there is a limited choice of target ROS. Furthermore, quantitative evaluation of its scavenging activity has been hardly performed. In this Letter, we quantitatively evaluated edaravone's free radical scavenging rate constants against several distinct free radical species.

Based on the newly proposed method (ORAC–ESR: oxygen radical absorbance capacity (ORAC) method used an ESR trapping technique) of the scavenging rate evaluation,[12] a method has been developed to determine the scavenging ability against multiple ROS in biological specimens such as human serum. This method was named as multiple radical scavenging (MULTIS).[13] Selected ROS and methyl radical which are adopted in the MULTIS method include hydroxyl radical ($HO^{\cdot}$), superoxide anion (O_2^{-}), alkoxyl

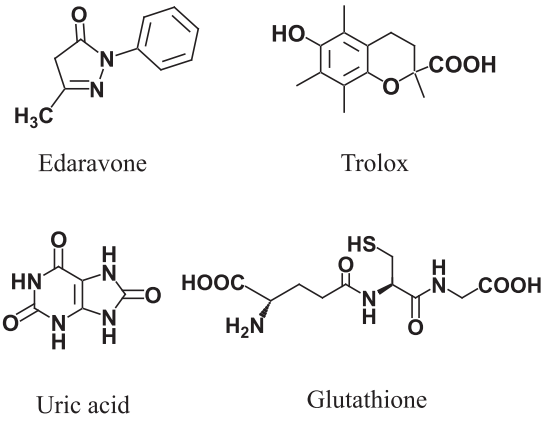

Figure 1. Structures of edaravone, uric acid, glutathione, and trolox.

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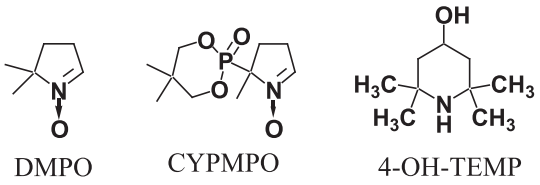

Figure 2. Structures of spin traps.

radical (RO·), alkylperoxyl radical (*t*-BuOO·), methyl radical (H_3C·) and singlet oxygen ($^1O_2$). The essence of the MULTIS method is to use the same photolysis method for the generation of five ROS and H_3C·. Those active species are considered as main causes of oxidative damage in cells and tissues.[1–3]

Briefly, the scavenging rate evaluation with MULTIS is to compare free radical concentration in the presence and absence of the antioxidant. For the determination of free radical concentration, ESR spin trapping method was employed. Strictly speaking, the method of singlet oxygen concentration determination should not be classified as spin trapping; however, for the sake of simplicity this method is also described as spin trapping in this report. Uric acid, glutathione, and trolox shown in Figure 1 are the important antioxidant that can protect cells and tissues from ROS-mediated damage.[14,15] We compared the MULTIS values of edaravone with those of uric acid, glutathione, and trolox.

Spin trapping compounds (spin traps) used in this study are shown in Figure 2. Edaravone, uric acid, reduced glutathione, and trolox were purchased from Aldrich Chemical Company Inc. (Milwaukee WI, USA), Nakalai Tesque (Kyoto, Japan), and Wako Pure Chemistry (Osaka, Japan), respectively. These reagents were used as received. CYPMPO (5-(2,2-dimethyl-1,3-propoxy cyclophosphoranyl)-5-methyl-1-pyrroline *N*-oxide), DMPO (5,5-dimethyl-pyrroline *N*-oxide), and 4-OH-TEMP (4-hydroxy-2,2,6,6-tetramethylpiperidine) were obtained from Radical Research (Hino, Japan), Funakoshi (Tokyo, Japan), and Tokyo Chemical Ind. (Tokyo, Japan), respectively: DMPO and 4-OH-TEMP were used for the detection of H_3C· and $^1O_2$, respectively, and CYPMPO for HO·, $O_2^{-\cdot}$, RO·, and *t*-BuOO·.

Free radicals including singlet oxygen were produced with the UV/Vis light irradiation (RUVF-203S, Radical Research Inc.). Precursors and sensitizers that were used to photo-generate free radicals are listed in Table 1.[13] Those free radical precursors or sensitizers were dissolved in phosphate buffer and subjected to in situ photolysis. A JEOL FA200 X-band spectrometer (Akishima, Japan) was used to record ESR spectra of trapped free radicals (spin adducts).[13] For the rapid analysis of scavenging ability, trapped free radicals were identified and ESR signal intensity was assumed to be proportional to the free radical concentration. Antioxidant's scavenging ability for spin-adducts is negligible because there was no time-decay of spin-adduct concentrations.

In the presence of antioxidants (AOx) like edaravone and the spin trap (ST) like DMPO, the free radical (R·) scavenging reaction should occur as follows:

$$\mathrm{AOx} + \mathrm{R}^{\cdot} \rightarrow \mathrm{Products}, \quad k_{\mathrm{AOX}}$$

$$\mathrm{ST} + \mathrm{R}^{\cdot} \rightarrow \mathrm{Spin\ adduct}, \quad k_{\mathrm{ST}}.$$

Relative scavenging rate constants ($k_{\mathrm{AOx}}/k_{\mathrm{ST}}$) were calculated according to following equation:[12]

$$\frac{I_0 - I}{I} = \frac{k_{\mathrm{AOx}}}{k_{\mathrm{ST}}} \frac{[\mathrm{AOx}]_0}{[\mathrm{ST}]_0} \tag{1}$$

where I and I_0 are ESR signal heights in the presence and absence of antioxidant (AOx), respectively. The $[\ ]_0$ symbol expresses the initial concentration. For example, methyl radical (DMSO+H_2O_2, UV irradiated) reacted with DMPO, showing ESR signal of methyl spin adduct of DMPO (Fig. 3a). When the same experiment was carried out in the presence of edaravone the signal level diminished (Fig. 3b). This difference is equal to $I_0 - I$, from which relative scavenging rate constants $k_{\mathrm{AOx}}/k_{\mathrm{ST}}$ can be calculated according to Eq. 1. By selecting various $[\mathrm{AOx}]_0/[\mathrm{ST}]_0$, a plot of $(I_0 - I)/I$ versus $[\mathrm{AOx}]_0/[\mathrm{ST}]_0$ can be obtained (Fig. 2c). The linear plot shown in Fig. 3 for the methyl radical/edaravone/DMPO system with the slope of $k_{\mathrm{AOx}}/k_{\mathrm{ST}}$ (2.14 ± 0.12) passing through the origin is a clear evidence that the competitive mechanism between edaravone and DMPO against methyl radical is holding. The same mechanism may be safely assumed in other free radicals. The degree of decrease in ESR intensity is dependent on the magnitude of AOx's scavenging ability against the specific free radical. Relative scavenging rate constants ($k_{\mathrm{AOx}}/k_{\mathrm{ST}}$) for other ROS can be evaluated in a similar fashion to methyl radical.

The top panel of Table 2 lists the relative scavenging rate constant $k_{\mathrm{AOx}}/k_{\mathrm{ST}}$ of edaravone, uric acid, glutathione, and trolox against five ROS and H_3C·. Based on these MULTIS numbers, the quantitative comparison of the three antioxidants is possible for each ROS. The $\ln(100k_{\mathrm{AOx}}/k_{\mathrm{edaravone}})$ values for five ROS and H_3C· are illustrated in a radar chart format (Fig. 4), where edaravone's result is expressed as 100%, that is, edaravone's radar chart is a regular hexagon. Obviously the radar chart illustration is very effective in expressing the difference in MULTIS activity.

Before we proceed to the discussion of these numbers or hexagonal shapes, we converted $k_{\mathrm{AOx}}/k_{\mathrm{ST}}$ in Table 2 into absolute values of k_{AOx} by using previously published k_{ST} values. The k_{ST} values for HO·, $O_2^{-\cdot}$, RO· and CH_3· were $k_{\mathrm{DMPO}}(\mathrm{HO}^{\cdot}) = 2.8 \times 10^9\ \mathrm{M}^{-1}\ \mathrm{s}^{-1}$, $k_{\mathrm{CYPMPO}}(O_2^{-\cdot}) = 48\ \mathrm{M}^{-1}\ \mathrm{s}^{-1}$, $k_{\mathrm{CYPMPO}}(\mathrm{RO}^{\cdot}) = 2.27 \times 10^4\ \mathrm{M}^{-1}\ \mathrm{s}^{-1}$, and $k_{\mathrm{DMPO}}(\mathrm{CH_3}^{\cdot}) = 1.4 \times 10^7\ \mathrm{M}^{-1}\ \mathrm{s}^{-1}$, respectively.[16–18] Further, we used the rate constants ($k_{\mathrm{trolox}}(^1O_2) = 6.22 \times 10^7\ \mathrm{M}^{-1}\ \mathrm{s}^{-1}$)[19] between trolox and $^1O_2$ to calculate the rate constants for antioxidants: $k_{\mathrm{glutathione}}/k_{\mathrm{trolox}} = 15.1$ for $^1O_2$. [18] Because k_{ST} of *t*-BuOO· for the CYPMPO trap was not available, the rate constants for *t*-BuOO· could not be evaluated. Thus, calculated rate constant data are listed in the bottom panel of Table 2.

Table 1
Precursors and spin traps for multiple free radical species

Free radical	Precursor/sensitizer	Spin trap
$HO^{\cdot}$	Hydrogen peroxide (H_2O_2 (5 mM))	CYPMPO
$O_2^{-\cdot}$	Riboflavin (60 μM), EDTA (10 mM)	CYPMPO
$RO^{\cdot}$ [a]	2,2′-Azobis(2-amidino-propane) dihydrochloride (AAPH (5 mM))	CYPMPO
$t-BuOO^{\cdot}$	*t*-Butylhyroperoxide (15 mM)	CYPMPO
$CH_3^{\cdot}$	Dimethyl sulfoxide (DMSO (100 mM)), H_2O_2 (20 mM)	DMPO
$^1O_2$	Rosebengal (50 μM)	4-OH-TEMP

[a] Alkoxyl radical derived from AAPH.