Antioxidant Effects of Hydroxybenzalacetones on Peroxynitrite-Induced Lipid Peroxidation in Red Blood Cell Membrane Ghost and SOS Response in *Salmonella typhimurium* TA4107/pSK1002

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Antioxidant activity of a series of hydroxybenzalacetones was determined against peroxynitrite-induced lipid peroxidation in red blood cell membrane and SOS response through DNA damage in bacterial cells. Hydroxybenzalacetone derivatives with hydroxy, methoxy, ethoxy or methyl substitution were analyzed and found to be more effective than the water-soluble vitamin E analogue Trolox. The inhibitory effect against lipid peroxidation correlated well to that against the SOS response, which is dependent on decomposition of peroxynitrite by hydroxybenzalacetones outside of the cell membrane. The antioxidant activity was shown to correlate well with the electric parameter σ^+. Electron-donating substituents with more negative σ^+ values increased the potencies. The result suggests that hydroxybenzalacetones with more electron-donating substituents will protect tissue more effectively against oxidative stress.

Key words antioxidant effect; hydroxybenzalacetone; peroxynitrite; lipid peroxidation; SOS response; *Salmonella typhimurium*

Peroxynitrite is a strong biological oxidant that can be formed *in vivo* by the nearly diffusion-controlled reaction of nitric oxide and superoxide.[1—3] Peroxynitrite initiates lipid peroxidation,[4] damages DNA,[5] oxidizes thiol groups[6,7] and modifies amino acetyl groups on protein.[8] The oxidation and damage induce various pathological conditions.

We have reported that peroxynitrite induces the SOS response through DNA damage in the cells of *Salmonella typhimurium* TA4107/pSK1002 and that antioxidants added scavenge extracellular peroxynitrite.[9] In addition, the SOS system was used for measuring the antioxidant potencies of manganese porphyrins against peroxynitrite, and tissue protection from oxidative stress was suggested.[10]

Hydroxybenzalacetone derivatives (OH-BZs) are compounds related to dehydrozingerone and are regarded as 'half curcumin' (Fig. 1). Curcumin has been reported to be an antioxidant, and to have anti-inflammatory and antitumor-promoting activities.[11—13] We have investigated the potencies of OH-BZs and we have reported the antimutagenic activities[14,15] and antitumor-promoting activities.[16,17] We recently measured the inhibitory effects against lipid peroxidation by *tert*-butyl hydroperoxide (*t*-BuOOH) and γ-irradiation and analyzed the quantitative structure–activity relationship.[18] In the present study, we measured the protective effects of OH-BZs with a substituent(s) (Fig. 1) against lipid peroxidation and DNA damage in bacterial cells induced by peroxynitrite. Here we report that the antioxidant potencies against peroxynitrite increased with the electron-donating substituents,

and a linear relationship was observed between the electron-donating ability and activity.

Experimental

Chemicals Dehydrozingerone (4-OH-3-OMe-BZ) was purchased from Aldrich Chemical (WI, U.S.A.). Trolox was obtained from Tokyo Kasei (Tokyo, Japan). OH-BZs were synthesized by a modified method as described for 4-OH-BZ in ref. 19; reactions were performed at room temperature by using more dilute NaOH (10—20%) solutions. Peroxynitrite solution (ONOO$^-$, >40 mM in 0.3 M NaOH) was from Dojindo (Kumamoto, Japan). The concentration of ONOO$^-$ was determined just before use by characteristic absorption at 302 nm in 0.1 M NaOH ($\varepsilon=1670\,\text{M}^{-1}\text{cm}^{-1}$).[20] 2-Thiobarbituric acid (TBA), *o*-nitrophenyl-β-D-galactopyranoside (ONPG) and ampicillin were obtained from Wako Pure Chemicals (Osaka, Japan). All other chemicals were of the highest grade available. All solutions were prepared with deionized water from Barnstead ultra pure water system.

Preparation of Red Blood Cell (RBC) Membrane Ghost RBC membrane ghosts were prepared using commercially available rabbit blood as described previously.[10] RBCs washed with PBS were lysed in 10 mM phosphate buffer with 0.1 mM EDTA, pH 7.4. After the lysed solution was centrifuged, the precipitate was washed with 10 mM phosphate buffer and suspended in 10 mM phosphate buffer. The RBC membrane ghost suspension was diluted to give 2.0 mg protein/ml and frozen as aliquots at −80 °C until use.

Measurement of ONOO$^-$-Induced Lipid Peroxidation Lipid peroxidation by ONOO$^-$ was monitored colorimetrically measuring TBA-reactive substances produced in the RBC membrane ghost.[10] The reaction mixture (500 μl) contained RBC membrane ghost suspension (100 μl), OH-BZ solution (100 μl) and 0.067 mM phosphate buffer, pH 6.8 (300 μl). The peroxidation was initiated by adding ONOO$^-$ (30 mM, 5 μl) and terminated by HCl (0.05 M, 1 ml) after 30 min (37 °C). The reaction mixture with 0.67% (w/v) TBA solution added (0.5 ml) was heated at 95 °C for 15 min, cooled rapidly and mixed with *n*-butanol containing 15% (v/v) methanol solution (1.5 ml). After centrifugation (1400×*g*, 15 min), the supernatant was measured at 535 nm. The amount of the TBA-reactive substances produced was calculated using a calibration curve for tetramethoxypropane as a malondialdehyde standard.

Measurement of ONOO$^-$-Induced SOS Response The strain *S. typhimurium* TA4107/pSK1002 (*oxyΔ1[oxyΔ(oxyR argH)1]/umuC'-'lacZ*) was used for induction of SOS response by ONOO$^-$.[9] The fused gene (*umuC'-'lacZ*) is induced through the SOS response by DNA damage and results in a hybrid protein with β-galactosidase activity.[21] The overnight cell culture was diluted 20-fold with TGA (1% Bacto tryptone, 0.5% NaCl and 0.2% glucose) medium with 20 μg/ml ampicillin and incubated to OD$_{600}$=0.25—0.3 at 37 °C. The cells were resuspended into PBS
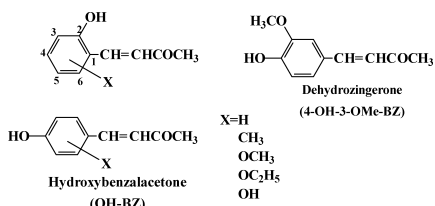
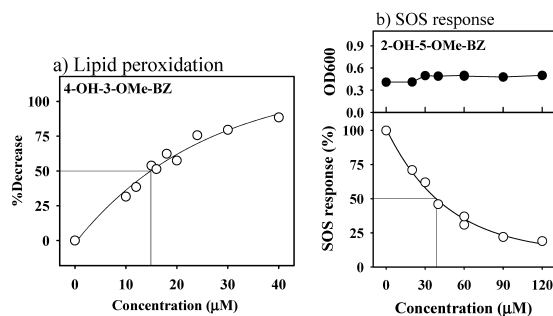Fig. 1. Structures of the Hydroxybenzalacetone Derivatives Used

Fig. 2. Determination of IC$_{50}$ of 4-OH-3-OMe-BZ and 2-OH-5-OMe-BZ against Oxidation by ONOO$^-$
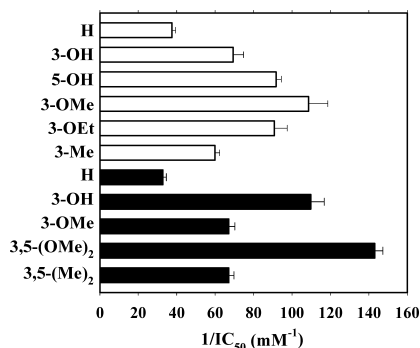

Fig. 3. Inhibitory Effects of OH-BZs on ONOO$^-$-Induced Lipid Peroxidation

Open and closed bars represent 2-OH-BZs and 4-OH-BZs, respectively.

(OD$_{600}$=1.0—1.2). The various concentrations of OH-BZs and PBS (50 μl) were added to the sample tubes and the controls (0.45 ml each), respectively. Following the preincubation, the induction of the SOS response was initiated by addition of ONOO$^-$ (30 mm, 5 μl) and incubated at 37 °C for 30 min. After centrifugation, the cells in TGA (2.5 ml) were incubated for another 90 min. The β-galactosidase activity was calculated using the following equation: units=1000(OD$_{420}$−1.75OD$_{550}$)/(t (30 min)×v (0.2 ml)×OD$_{600}$).[9]

Calculation of 50% Inhibition (IC$_{50}$) against Lipid Peroxidation and SOS Response The antioxidant activities of OH-BZs were examined by their IC$_{50}$ values, the concentrations that inhibit 50% of the lipid peroxidation induced by ONOO$^-$. The value was calculated using the equation from a nonlinear regression for the relationship between the concentrations used and the percentage of the TBA-reactive substances induced (Fig. 2a). The IC$_{50}$ value against the DNA damage was obtained from the relationship between the concentrations used and the percentage of the SOS response produced (Fig. 2b). All data are expressed as mean±S.D. of three or more experiments.

Results and Discussion

Figure 3 shows the inhibitory effects of OH-BZs with substituents at 3- and/or 5-position in the lipid peroxidation. Three (OH)$_2$-BZs, 2,3-, 2,5- and 3,4-substituted derivatives, exhibited 2—3 times the inhibitory effects of 2-OH-BZ and 4-OH-BZ, indicating that the introduction of an OH group into the *ortho*- or *para*-position(s) of the phenolic OH group increased the antioxidant potencies. In addition, the presence of 3-alkoxyl group strengthened the activity by about 2—3 times. In 4-OH-BZs, the activity of 4-OH-3,5-(OMe)$_2$-BZ with two *ortho*-methoxy groups was about twice that of 4-OH-3-OMe-BZ, suggesting that the substituent effect is additive. Taking into account this additivity rule, the effects of the *ortho* substituent on the activity would be in the order OH>OMe>Me.

Table 1 shows IC$_{50}$ values of OH-BZs against lipid peroxi-

Table 1. IC$_{50}$ Values of OH-BZs against ONOO$^-$-Induced Lipid Peroxidation and SOS Response

Compound	IC$_{50}$ (μM)		$\sigma^{+ a)}$
	Lipid peroxidaton	SOS response	
2-OH-BZ			
H	26.7±1.4	87.9±4.2	0
3-OH	14.4±1.1	17.7±1.0	−0.98
5-OH	10.9±0.32	9.89±0.68$^{c)}$	−0.98
3-OMe	9.21±0.85	35.5±0.7	−0.78
4-OMe	25.4±0.9	51.8±1.4	0.05
5-OMe	12.5±1.1	38.5±1.3	−0.78
3-OEt	11.0±0.8	24.9±0.9	−0.78
3-Me	16.7±0.7	42.4±2.5	−0.31
5-Me	21.6±1.2	51.7±2.7	−0.31
4-OH-BZ			
H	30.5±1.7	108±6	0
3-OH	9.11±0.58	17.7±1.5	−0.98
3-OMe	14.9±0.7	53.3±4.8	−0.78
3,5-(OMe)$_2$	6.99±0.21	22.8±1.5	−1.56$^{b)}$
3,5-(Me)$_2$	14.9±0.6	47.4±1.5	−0.62$^{b)}$
Trolox	48.6±2.3	57.0±5.1	—

The reaction mixture contained 0.2% DMSO. Lipid peroxidation and SOS response were induced by 0.3 mm ONOO$^-$. *a*) The data was obtained from refs. 22, 23. *b*) 2σ_o^+. *c*) The compound was toxic for cells.
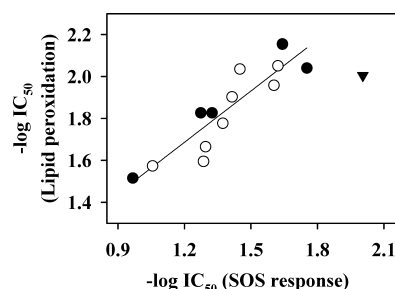
Fig. 4. Correlation between −log IC$_{50}$ Values of Lipid Peroxidation and SOS Response

Open and closed circles represent 2-OH-BZs except 2,5-(OH)$_2$-BZ (triangle, toxic) and 4-OH-BZs, respectively. Correlation coefficient=0.903 (n=13).

dation and SOS response induction by ONOO$^-$. Benzalacetone (BZ), a non-phenolic compound, reduced neither peroxidation nor SOS response (data not shown). In the lipid peroxidation, all of OH-BZs tested inhibited the peroxidation more strongly than Trolox, a water-soluble analog of vitamin E, and in the SOS response, OH-BZs except 2-OH-BZ and 4-OH-BZ were stronger than Trolox. 2,5-(OH)$_2$-BZ showed the strongest inhibition against the SOS response, but was toxic for bacterial cells. The inhibitory effects (−log IC$_{50}$) on the lipid peroxidation and the SOS response showed a good correlation (r=0.903) except 2,5-(OH)$_2$-BZ (Fig. 4).

We previously investigated the quantitative structure–activity relationship for the inhibitory effect against the lipid peroxidation by t-BuOOH and γ-irradiation,[18] showing that the electronic parameter, σ^+,[22] which involves through-resonance effects, plays an important role in describing the potency (−log IC$_{50}$).[23] The values of σ^+ represent the electronic effects of the substituents on the phenolic OH group, and the σ_p^+ values were used as the σ_o^+ values of the corresponding *ortho*-substituent ($\sigma_p^+ = \sigma_o^+$) because the effect of the *ortho*-substituent is supposed to be equivalent with that of the *para*-substituent.[24] By using this assumption, the rela-

Fig. 5. Correlation between Electronic Parameter σ^+ and $-\log IC_{50}$ of OH-BZs against $ONOO^-$-Induced Lipid Peroxidation

Open and closed circles represent 2-OH-BZs and 4-OH-BZs, respectively. Correlation coefficient=0.930 (n=14).

tionship between $-\log IC_{50}$ ($ONOO^-$) and σ^+ was examined, led to a good correlation ($r=0.930$) as shown in Fig. 5. The activity increased as the σ^+ value becomes more negative, showing that more electron-donating substituents increase the antioxidant activity. In Fig. 5, all compounds showing positive deviations from the regression line are those with *ortho* substituents. This would mean that the *ortho* steric effect would even strengthen the activity. On the other hand, the $-\log IC_{50}$ value of the lipid peroxidation was hardly correlated to the hydrophobicity parameter $\log P$ (1.3—2.7).[26] In the SOS response, a poor correlation was also observed. The results suggest that the OH-BZs tested should not create a significant hydrophobic interaction with RBC ghosts and the bacterial cell membrane. Similar results were observed for the antioxidant activities of OH-BZs against lipid peroxidation induced by t-BuOOH and γ-iradiation, although the OH-BZs tested contained derivatives of wider range of hydrophobicity (1.3<$\log P$<4.0).[18] Moreover, Nakao *et al.* has demonstrated that no hydrophobic contribution is involved in the inhibitory activities of hydroxyphenylurea derivatives against lipid peroxidation of rat brain homogenate.[27]

In conclusion, the OH-BZs tested here inhibited the oxidation of membrane lipids and the DNA damage of bacterial cells induced by peroxynitrite. Both oxidation systems were inhibited by OH-BZs more effectively than with Trolox, mainly depending on the electron-donating effect. OH-BZs with substituents of more negative σ^+ values increased the activities in the same way as observed in the inhibitory effects on lipid peroxidation by t-BuOOH and γ-irradiation.[18] The inhibitory activities of OH-BZs against the DNA damage were dependent on the peroxynitrite-scavenging potency just outside cells because antioxidants react with extracellular peroxynitrite and the DNA damage is induced by peroxy-

nitrite through the cell membrane without decomposition.[9] The results suggest that the OH-BZs tested here will protect tissue under oxidative conditions.

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