ESR Study on the Antioxidant Activity of TAK-218 in Biological Model Membranes

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TAK-218 has a 2,3-dihydrobenzofuran-5-amine (coumaran) structure which resembles α-tocopherol, and is a promising candidate as an agent for central nervous system (CNS) trauma and ischemia. The radical scavenging activity of TAK-218 was studied using electron spin resonance (ESR) spectroscopy.

TAK-218 exhibited a more potent scavenging activity towards the hydroxyl radical than did the well-known hydroxyl radical scavengers, mannitol and dimethylsulfoxide. Towards the superoxide radical, TAK-218 showed equal potency to glutathione. TAK-218 reacted rapidly with stable radicals, such as galvinoxyl and 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH), and gave the quinone as a two-electron oxidized product in analogy with α-tocopherol.

To exhibit an excellent antioxidative activity in living systems, the compounds should not only have the intrinsic radical scavenging activity but also good distribution in the biological lipid-bilayer membrane. To examine the antioxidant activity of TAK-218, the inhibition of lipid peroxidation by α-tocopherol and TAK-218 in liposomal membranes was studied using an ESR spin-label technique.

Both α-tocopherol and TAK-218 completely inhibited lipid peroxidation by radicals generated in an aqueous layer using a water-soluble radical initiator, 2,2′-azobis-(2-amidinopropane) hydrochloride (AAPH). At a high incubation temperature (45 °C), α-tocopherol scavenged radicals more effectively than TAK-218 on the surface of the membrane, while TAK-218 scavenged radicals more effectively in the interior of the membrane. The difference between TAK-218 and α-tocopherol for radical scavenging in the membrane system derives from the different distribution pattern of these compounds. TAK-218 can penetrate the membrane freely and can scavenge the radical in the membrane interior.

Furthermore, TAK-218 was shown to inhibit lipid peroxidation initiated by a lipid soluble radical initiator, 2,2′-azobis-(2,4-dimethylvaleronitrile) (AMVN), in a membrane more effectively than α-tocopherol.

Key words TAK-218; ESR; biological model membrane; spin-label technique; antioxidant activity; α-tocopherol

TAK-218 ((*S*)-2,3-dihydro-2,4,6,7-tetramethyl-2-[(4-phenyl-1-piperidinyl)methyl]-5-benzofuranamine dihydrochloride) shown in Fig. 1, is a promising candidate as an agent for central nervous system (CNS) trauma and ischemia.[1] TAK-218 exhibits two functions in living systems: one is the inhibition of dopamine release and the other is the scavenging of free radicals. Cell injury and cell death are caused by the lack of oxygen during brain ischemia and the mechanical damage in traumatic injuries (primary injury), followed by the generation of free radicals after reperfusion.[2—4] The resulting free radicals attack the normal cells surrounding the primary injured cell and cause secondary injury.[2] By inhibiting lipid peroxidation, TAK-218 prevents secondary injury and minimizes the damage. TAK-218 has not only intrinsic radical scavenging activity but also good distribution in the biological lipid-bilayer membrane, which leads to its excellent antioxidative activity in living systems.[5]

In this paper, the scavenging activity for the hydroxyl and the superoxide radical was initially estimated in homogeneous solution using a spin-trapping method. Next, the inhibition of lipid peroxidation by TAK-218 and α-tocopherol in liposomal membranes was studied using an ESR spin-label technique. Two systems were adopted to estimate the inhibition of lipid peroxidation. One was an 2,2′-azobis-(2-amidinopropane) hydrochloride (AAPH) system to simulate the attack of free radicals from the aqueous region and the other was 2,2′-azobis-(2,4-dimethylvaleronitrile) (AMVN) system to simulate the attack of free radicals in membranes. The former is the model of the initial stage of injury, in which the free radicals emanating from the damaged cell attack the membrane of the normal cell. The latter is the model of the progressing stage of injury, in which the chain reaction of the lipid peroxidation starts in the membrane.

The effect of TAK-218 on the membrane fluidity of the liposomes was also examined to estimate the interaction between TAK-218 and the membranes. Finally, ESR spectra of the aminyl radicals derived from TAK-218 were measured and the stability and the electronic structure of this radical are discussed.

Experimental

Radical Scavenging Activities Stable Radical Scavenging Activity: The stable free radicals, galvinoxyl and 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) were used to assay the radical scavenging activity.[6,7] The reaction mixture consisted of galvinoxyl (1.0 mM, 1.0 ml) and TAK-218 (1.0 mM, 1.0 ml) dissolved in methanol. The ESR signal intensity of galvinoxyl in this mixture was measured using an ESR spectrometer. DPPH was used as a stable radical in the same manner as galvinoxyl (1.0 mM, 1.0 ml).

Hydroxyl Radical Scavenging Activity: We used the ESR spin-trapping method to evaluate the hydroxyl radical scavenging activity.[8,9] Hydroxyl radicals (·OH) were produced using Fenton's reaction involving Fe^{2+} and hydrogen peroxide. The resulting hydroxyl radicals were trapped by 5,5-di-

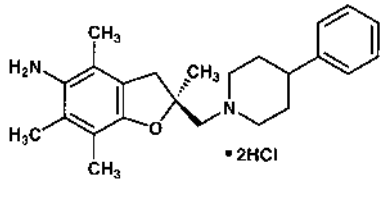

Fig. 1. Chemical Structure of TAK-218

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methyl-1-pyrroline-*N*-oxide (DMPO).

In the standard experiment, each of the following stock solutions was added in the following order, up to a total volume of 1.0 ml:

a) ADP (adenosine 5′-diphosphate sodium salt, from equine muscle grade X, Sigma) as a chelating agent, 20.0 mM, 0.1 ml; b) $FeCl_2$ dissolved in 0.001 N HCl, 1.0 mM, 0.1 ml; c) the test substance dissolved in distilled water, 1.0—10.0 mM, 0.1 ml; d) buffer solution (100.0 mM NaCl+25 mM $NaHCO_3$, pH=3.0); e) DMPO as the spin trap (Aldrich), 500 mM, 0.1 ml; and f) H_2O_2, 0.3%, 0.1 ml.

After addition of H_2O_2, the sample was transferred into a quartz flat cell and put into the standard cavity of the ESR spectrometer. Recording of the spectrum was started 2 min after the addition of H_2O_2. The method to calculate the 50% inhibitory concentration (IC_{50}) is the same as shown below for superoxide radical scavenging activity.

Superoxide Radical Scavenging Activity: The basic methodology and the preparation of the reagents used for these experiments were essentially the same as described in our previous article.[10] Superoxide radicals ($O_2^{-\cdot}$) were produced from hypoxanthine (HPX) and xanthine oxidase (XOD). The generation of $O_2^{-\cdot}$ radicals by the HPX–XOD system was followed by trapping these radicals on DMPO, and detecting the DMPO–O_2^- spin adduct. All the reagents were dissolved in phosphate buffer solution (60.0 mM Na_2HPO_4+6.67 mM KH_2PO_4, pH=7.8). In the standard experiment, each of the following stock solutions was added in the following order, to make up the total volume to 0.2 ml:

a) HPX (Sigma), 2.0 mM, 0.05 ml; b) DETAPAC (diethylene triamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid, Sigma) as a chelating agent, 5.5 mM, 0.03 ml; c) the test substance dissolved in DMF (*N*,*N*-dimethylformamide), 2.0—10.0 mM, 0.05 ml; d) DMPO as the spin trap (Aldrich), 500 mM, 0.02 ml; and e) XOD (from buttermilk, Grade I, Sigma), 0.25 units/ml, 0.05 ml.

After the last solution, XOD, was added, the sample was transferred into a quartz flat cell and put into the standard cavity of the ESR instrument. Recording of the spectrum was started 2 min after the addition of XOD, when the maximum ESR signal intensity of DMPO–O_2^- was expected.

The reactivity of the test substances with $O_2^{-\cdot}$ radicals was estimated from the signal intensities of DMPO–O_2^- relative to the control intensity. The $O_2^{-\cdot}$ scavenging activity was represented as the IC_{50} for the formation of DMPO–O_2^- and used to obtain the rate constants of the reactions between the $O_2^{-\cdot}$ radicals and the scavengers, in the kinetic competition model.[10,11] Assuming the validity of the kinetic competition model, we can consider two main reactions competing in our system as described by Eqs. 1 and 2:

$$O_2^{-\cdot} + S \xrightarrow{k} S - O_2^- \quad (1)$$

$$O_2^{-\cdot} + DMPO \xrightarrow{k'} DMPO - O_2^- \quad (2)$$

where S means the scavenger being tested. Since $k'=17.9\ M^{-1}\ s^{-1}$ [10,11] and we know the final concentration of the scavenger under test as well as the $[IC_{50}]_{final}$ and the final concentration of DMPO, $[DMPO]_{final,}$ we can approximate the value of k ($M^{-1}\ s^{-1}$).

$$k=k'\times[DMPO]_{final}/[IC_{50}]_{final}=3.58\times10^3/IC_{50} \quad (3)$$

where IC_{50} means the same as in Fig. 4 and Table 2.

IC_{50} was calculated as described[10] using the following equation:

$$V=(I_{control}/I_{test})-1 \quad (4)$$

where $I_{control}$ and I_{test} are the spin adduct signal intensities observed in the absence and in the presence of the tested antioxidant, respectively. After we plotted V against the concentration of the added TAK-218, we linearized the V values using the least-squares method. So the reliability of our data was confirmed using the square of regression coefficient (R^2) derived from a curve fitting calculation. The ESR signal intensities used for the calculation of IC_{50} values were those with the best R^2 values obtained from duplicate measurements. IC_{50} is the concentration of the tested substance at which $V=1$.

Inhibition of Lipid Peroxidation The inhibition of lipid peroxidation of TAK-218 in a phosphatidylcholine liposome was studied using a spin label technique. A liposome of phosphatidylcholine was used for the model membrane and azo compounds were adopted as initiators for lipid peroxidation. The amount of radicals in the liposomal membrane was estimated from the time–course of the ESR signal intensity of the DOXYL-stearic acid spin label.

AAPH and AMVN were used as hydrophilic and lipophilic radical initiators, respectively. These azo compounds enabled us to generate radicals that might initiate lipid peroxidation of the liposomal membrane at a constant and known rate, either in the aqueous phase or in the lipid region.[5,6,12,13] AAPH, AMVN and α-tocopherol were purchased from Wako. Four kinds of DOXYL-stearic acid, 5-DOXYL-stearic acid (5DSA), 7DSA, 12DSA and 16DSA were used as spin labels, where the nitroxide group is attached at various positions along the fatty acid chain to locate it at different depths in the lipid bilayer. All the spin label agents were purchased from Aldrich.

The liposome used in this investigation as a model membrane was made from L-α-phosphatidylcholine. It is generally accepted that phospholipids spontaneously form bilayers when dispersed in water, and that these can serve as excellent models for real biological membranes. Commercial L-α-phosphatidylcholine, from egg yolk purchased from Sigma, was used without further purification.

Spin labeled liposome containing antioxidants was prepared by the thin-film method of Niki and his colleagues[12—14] and the ESR spectra were measured as follows.

1) The phosphatidylcholine (100 mg/ml, 0.4 ml) and lipophilic additives such as antioxidants (TAK-218 or α-tocopherol) and spin label agents, or AMVN when necessary, were dissolved in chloroform (5 ml).

2) This chloroform solution was transferred to a round-bottom flask and the solvent was slowly evaporated under reduced pressure to obtain a thin film of phosphatidylcholine on the glass wall.

3) HEPES buffer solution (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid+NaOH, pH=7.76, 5.0 ml) was added to the flask. The mixture was vigorously mixed with a Vortex mixer for 2 min and sonicated for 15 min under nitrogen atmosphere using a Branson Sonifier Model 450 (450 W, 20 kHz) to convert it into small unilamellar vesicles. The final concentration was as follows: L-α-phosphatidylcholine, 10.0 mM; spin label agent, 0.1 mM; AMVN, 10.0 mM; antioxidant (TAK-218 or α-tocopherol), 0.1 mM.

4) When AMVN was used as a radical initiator, liposomal suspensions containing AMVN were incubated at 50 °C in air. The reaction mixture was taken into the quartz flat cell to measure the ESR spectra of the liposome at various time intervals.

5) When AAPH (final concentration; 20.0 mM) was used in experiments, it was added to the solution at the beginning of the measurement, and oxidation was carried out at 40 °C or 45 °C.

Interaction between TAK-218 and Liposomal Membranes: Measurement of the Membrane Fluidity The spin label, 16DSA was incorporated into the liposomal membranes using the above thin-film method. Final concentration was as follows; L-α-phosphatidylcholine (from egg yolk), 10.0 mM; 16DSA, 0.1 mM; TAK-218 or α-tocopherol, 1.0—5.0 mM (molar ratio to lipid, 10—50%); phosphate buffer solution (60.0 mM KH_2PO_4 +6.67 mM Na_2HPO_4, pH=7.8). The additive effect of the antioxidants on the membrane fluidity was monitored by the correlation time (τ_R) calculated from the following equation[15—17]:

$$\tau_R=7.1\times10^{-10}\delta H(0)\{R(-1)+R(+1)-2\} \quad (1)$$

where $R(-1)$, $R(+1)$ are given by

$$R(m)=\delta H(m)/\delta H(0)=\sqrt{I(0)/I(m)} \quad (m=-1,0,1) \quad (2)$$

where $I(m)$ and $\delta H(m)$ are the peak intensity and line width of the ESR signal, respectively.

ESR Spectra of TAK-218 Radical Since it became clear from our studies that TAK-218 radicals play an important role in the radical scavenging activity, we measured the ESR spectrum of this radical. The radical was obtained by the oxidation of TAK-218 with H_2O_2 at 40—80 °C. The reaction mixture consisted of TAK-218 (50.0 mM, 0.1 ml) and H_2O_2 (1.5%wt, 0.1 ml) dissolved in distilled water (H_2O) or deuterium oxide (D_2O) under air.

ESR Measurements ESR spectra were recorded on an X-band JEOL JES-RE3X spectrometer (JEOL Co., Ltd., Tokyo) operating at 9.20 GHz with a 100 KHz high-frequency modulation amplitude ranging from 0.5 G to 1.0 G. The specification of this spectrometer was as follows: magnetic field, 327 mT; microwave power, 4.0 mW; sweep width, ±5.0 mT; sweep time, 5—10 mT/min; response time, 0.03 s; and receiver gain, ×100—×2000. JEOL digital variable temperature accessories and an X-band universal cavity (cylindrical TE011 mode) were used. For the ESR measurement, the temperature was monitored and controlled using a copper–constantan thermocouple that was placed in the sample just below the active volume of the cavity.

Results and Discussion

Radical Scavenging Activities Stable Radical Scavenging Activity: TAK-218 reacted with stable radicals, galvi-

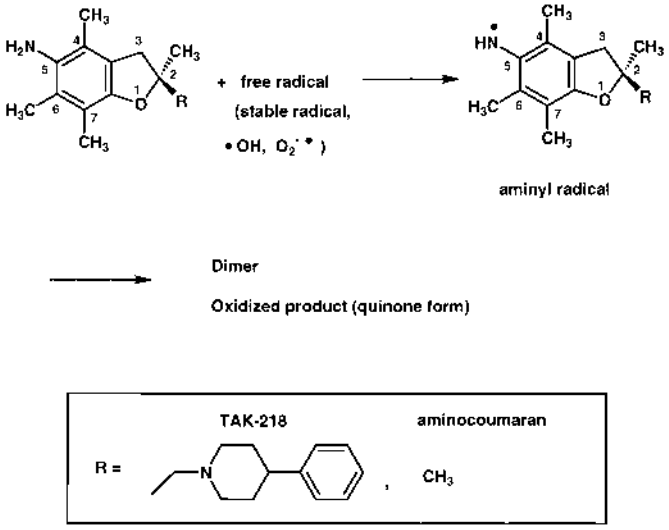


Fig. 2. Mechanism of the Free Radical Scavenging Reaction of TAK-218

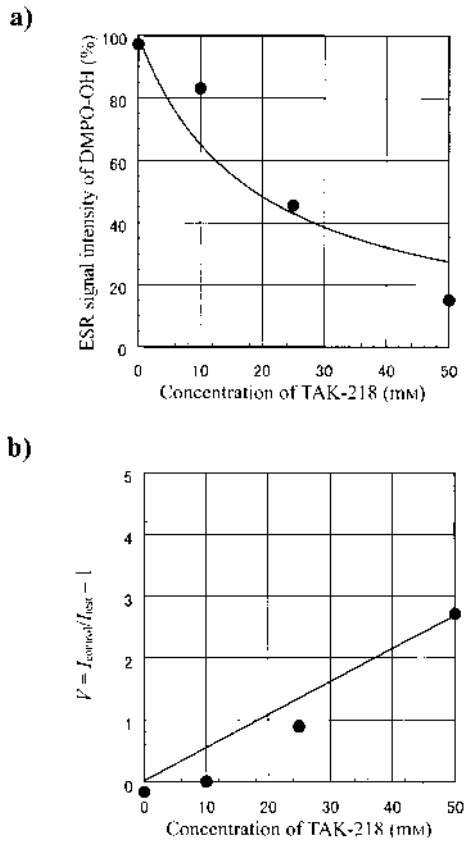


Fig. 3. Hydroxyl Radical Scavenging Activities of TAK-218 Obtained by the Spin Trapping Method in the ADP–Fe(II)–H_2O_2–DMPO System

a) The ESR signal intensity (%) of DMPO–OH adduct as plotted against the concentration of the added TAK-218. The function used for curve fitting is $y=100/(5.33\times10^{-2}x+1)$.

b) The same as shown in a) but linearized by the least-squares method in order to calculate IC_{50} values. The function used for curve fitting is $y=5.33\times10^{-2}x$, $R^2=0.97$.

noxyl and DPPH to rapidly quench their ESR signals. TAK-218 exhibited radical scavenging activity towards both substances, which are model radicals for the lipid radicals generated during lipid peroxidation.

When α-tocopherol reacted with galvinoxyl, the ESR signal of α-tocopheroxyl radical was observed, but the aminyl radical derived from TAK-218 was not detected by ESR measurement. By analogy with α-tocopherol,[18,19] it was presumed that TAK-218 reacted rapidly with stable radicals to give a quinone as a two-electron oxidized product (Fig. 2).

Table 1. Hydroxyl Radical Scavenging Activities Obtained by the Spin Trapping Method in the ADP–Fe(II)–H_2O_2–DMPO System

Compound	IC_{50} (mM)
Ascorbic acid	0.26
TAK-218	19
Aminocoumaran	38
DMSO	>100
Mannitol	>100

Hydroxyl Radical Scavenging Activity: Addition of TAK-218 to the ADP+Fe(II)+H_2O_2+DMPO system significantly reduced the ESR signal of DMPO–OH in proportion to the amount of TAK-218 added (Fig. 3). The 50% inhibition concentration (IC_{50}) of TAK-218 to the DMPO–OH is 19 mM. IC_{50} values of the other antioxidants are shown in Table 1; as indicated, TAK-218 exhibited stronger scavenging activity towards the hydroxyl radical than did the well-known hydroxyl radical scavengers, mannitol and dimethylsulfoxide (DMSO).

Superoxide Radical Scavenging Activity: Addition of TAK-218 to the XOD+HPX+DMPO system reduced the ESR signal of DMPO–O_2^- in proportion to the added TAK-218 (Fig. 4). Nevertheless, even if the concentration of TAK-218 was increased, its effect tended to show a saturation-like curve, *i.e.*, it could not achieve complete inhibition of the formation of DMPO–O_2^- adduct. The same phenomenon was observed, when we used another antioxidants, superoxide dismutase (SOD).[10] The IC_{50} of TAK-218 to the DMPO–O_2^- is 6.9 mM. IC_{50} values of the other antioxidants are shown in Table 2. Towards the superoxide radical, TAK-218 showed equal potency to glutathione.

Inhibition of Lipid Peroxidation Lipid Peroxidation of Liposomal Membrane Initiated by an Azo Compound: In the absence of a radical initiator, the rate of autooxidation of phosphatidylcholine was quite slow, but the addition of an azo compound as an initiator could induce accelerative oxidation. AAPH and AMVN were used as water-soluble and lipid-soluble initiators, respectively. These azo compounds enable us to simulate the attack of free radicals from outside or inside the membrane.

Oxidation of lipids initiated by the pyrolysis of an azo compound, A–N=N–A, can be represented by the following scheme.[6]

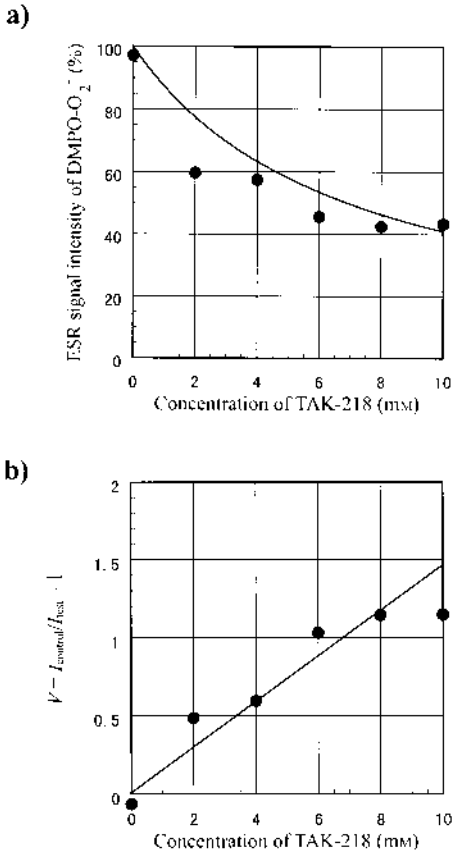
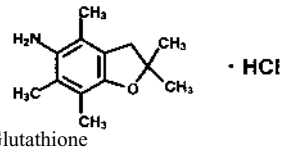
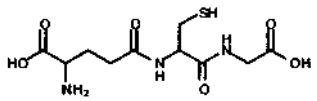
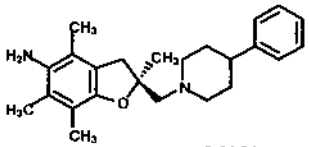


Fig. 4. Superoxide Radical Scavenging Activities of TAK-218 Obtained by the Spin Trapping Method in the XOD–HPX–DMPO System

a) The ESR signal intensity (%) of DMPO–O_2^- adduct as plotted against the concentration of the added TAK-218. The function used for curve fitting is $y=100/(1.46\times10^{-1}x+1)$.

b) The same as shown in a) but linearized by the least-squares method in order to calculate IC_{50} values. The function used for curve fitting is $y=1.46\times10^{-1}x$, $R^2=0.90$.

Table 2. Superoxide Radical Scavenging Activities Obtained by the Spin Trapping Method in the XOD–HPX–DMPO System

Compound	IC_{50} (mM)	Rate consatant k ($\times10^2$ M^{-1} s^{-1})
Aminocoumaran	4.9	7.3
 · HCl		
Glutathione	6.4	5.6
		
TAK-218	6.9	5.2
 · 2 HCl		

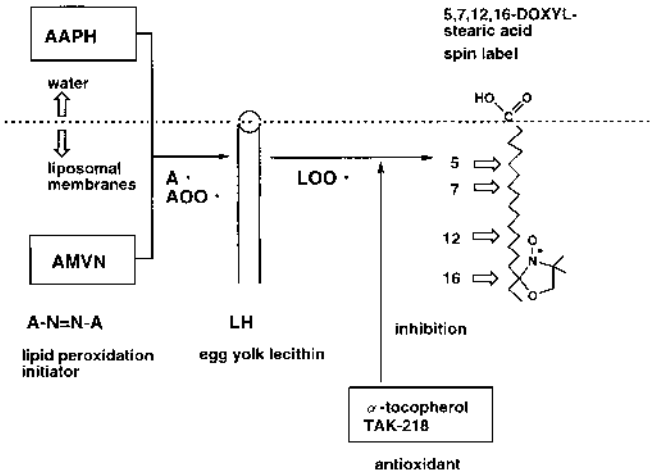


Fig. 5. Evaluation of the Inhibition of the Lipid Peroxidation by TAK-218 Using the ESR Spin-Label Technique

Schematic diagram of the experiment shows the reaction in the liposomal membranes.

Initiation:

$$A\text{–}N{=}N\text{–}A \rightarrow (1-e)A\text{–}A+2eA^{\cdot}+N_2$$
$$A^{\cdot}+O_2 \rightarrow AOO^{\cdot}$$
$$AOO^{\cdot}+LH \xrightarrow{O_2} AOOH+LOO^{\cdot}$$

Propagation:

$$LOO^{\cdot}+LH \rightarrow LOOH+L^{\cdot}$$
$$L^{\cdot}+O_2 \rightarrow LOO^{\cdot}$$

Termination:

$$LOO^{\cdot}+IH \rightarrow LOOH+I^{\cdot}$$
$$(n-1)LOO^{\cdot}+I^{\cdot} \rightarrow \text{nonradical products}$$

In this scheme, "LH" represents lipid, "IH" is antioxidant, "L˙" and "LOO˙" are alkyl and alkylperoxy radicals, respectively, "*e*" is efficiency of radical production and "*n*" is the stoichiometric number of radicals trapped by each antioxidant.

Free radicals generated in liposomal membranes react with spin labels in the membranes to reduce their ESR signals with the passage of time. If antioxidants co-exist with spin labels in the membrane, they partially scavenge the free radicals due to lipid peroxidation and decrease the rate of the consumption of the spin labels.

Figure 5 is a schematic diagram of the assay system used in this study to evaluate the inhibition of the lipid peroxidation by the antioxidant, showing the reaction in the liposomal membranes.

Figure 6 shows the ESR spectra of the four spin labels 5DSA,7DSA,12DSA and 16DSA, incorporated into the liposomes. The ESR spectra show an apparent large anisotropy of g and hyperfine splitting values in the order 16DSA$<$12DSA$<$7DSA$<$5DSA, suggesting a decrease in the molecular mobility of the spin label. In this study, a half-life period, $T_{1/2}$, represents the rates of consumption of the spin label. Increase in the $T_{1/2}$ of the spin label co-existing with the antioxidant would suggest that this antioxidant could inhibit lipid peroxidation.

Attack of Free Radicals Induced by AAPH from the Aqueous Region: AAPH was used as a water-soluble radical ini-

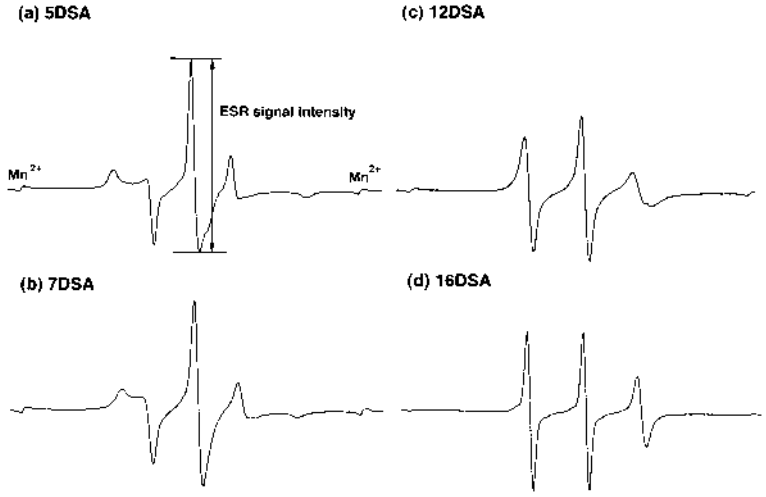


Fig. 6. ESR Spectra of 5DSA, 7DSA, 12DSA, 16DSA Spin Labels Incorporated into the Membranes at Room Temperature (T=20 °C)

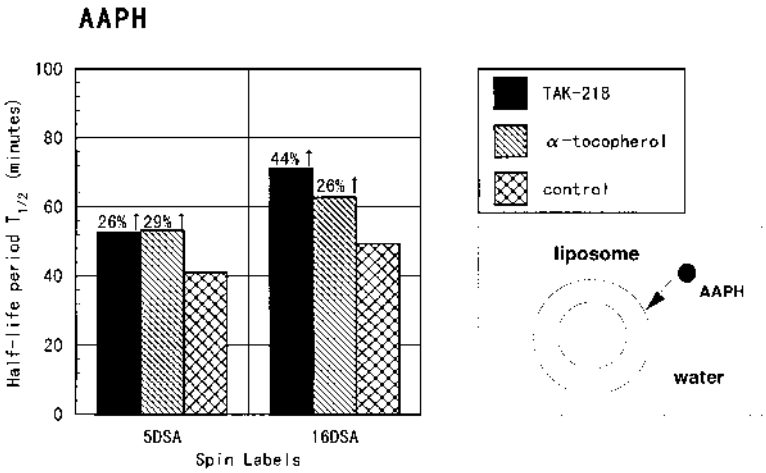


Fig. 7. $T_{1/2}$ of Spin Labels (5DSA, 16DSA) Incorporated into the Liposomes in the Presence of Antioxidants (TAK-218 or α-Tocopherol)
The oxidation was initiated with AAPH at 45 °C.

tiator to simulate radical attack from outside of the membrane. 5DSA and 16DSA were adopted as the spin labels. When the oxidation was initiated with AAPH at 40 °C, both α-tocopherol and TAK-218 completely suppressed the decrease of ESR signal of 5DSA and 16DSA during 5-hours of incubation (data not shown). This indicated that both antioxidants completely inhibited the lipid peroxidation by radicals generated in an aqueous phase using AAPH.

At a higher incubation temperature (45 °C), addition of α-tocopherol exhibited slightly longer $T_{1/2}$ than did the addition of TAK-218 for 5DSA as a spin label, while addition of TAK-218 exhibited longer $T_{1/2}$ than did the addition of α-tocopherol for 16DSA as a spin label (Fig. 7). This result showed that α-tocopherol scavenged the free radicals produced in aqueous regions near the membrane's surface. In contrast, TAK-218 could scavenge the radicals more effectively in the interior of the membrane than α-tocopherol. The difference between TAK-218 and α-tocopherol in radical scavenging activity in the membrane system is due to the different mobility of these compounds in the liposomal membrane. Although the phytyl side chain of α-tocopherol is required for retention by the biomembranes, it appears that this side chain reduces the mobility of α-tocopherol within the membranes.[12—14] The benzpiperidine group in TAK-218, however, does not prevent the mobility in the biomembranes, and thus TAK-218 can move through the membrane freely and scavenge the radical in the membrane interior.

Attack of Free Radicals Induced by AMVN in the Membranes: AMVN was used as a lipid-soluble radical initiator to simulate radical attack inside the membrane. Figure 8 shows the half-life period ($T_{1/2}$) of spin labels (5DSA, 7DSA, 12DSA, 16DSA) incorporated into the liposomes in the absence or presence of antioxidants (TAK-218 or α-tocopherol), when the oxidation was initiated by AMVN at 50 °C under air. Calculations of the $T_{1/2}$ in the case of 5DSA and 16DSA are demonstrated in Figs. 9 and 10. The ESR signal intensities used for the calculation of the $T_{1/2}$ values were those with the best R^2 values obtained from duplicate measurements.

Interestingly, it was found that the increment rate of 5DSA spin label's $T_{1/2}$, when co-existing with α-tocopherol, was the greatest among all of the spin labels (see Fig. 8), which suggests that α-tocopherol scavenges radicals near the surface more efficiently than it does in the inner region of the mem-

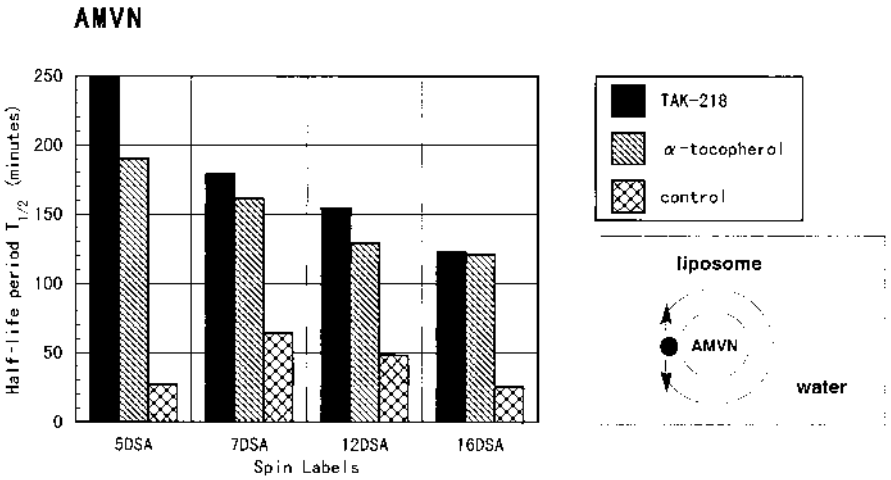


Fig. 8. $T_{1/2}$ of Spin Labels (5DSA, 7DSA, 12DSA, 16DSA) Incorporated into the Liposomes in the Presence of Antioxidants (TAK-218 or α-Tocopherol)

The oxidation was initiated with AMVN at 50 °C. The increment rates of the $T_{1/2}$ co-existing with TAK-218 or α-tocopherol to the $T_{1/2}$ in the control system are: 920% (5DSA), 290% (7DSA), 330% (12DSA) and 460% (16DSA) co-existing with TAK-218, or 700% (5DSA), 260% (7DSA), 270% (12DSA) and 450% (16DSA) co-existing with α-tocopherol.

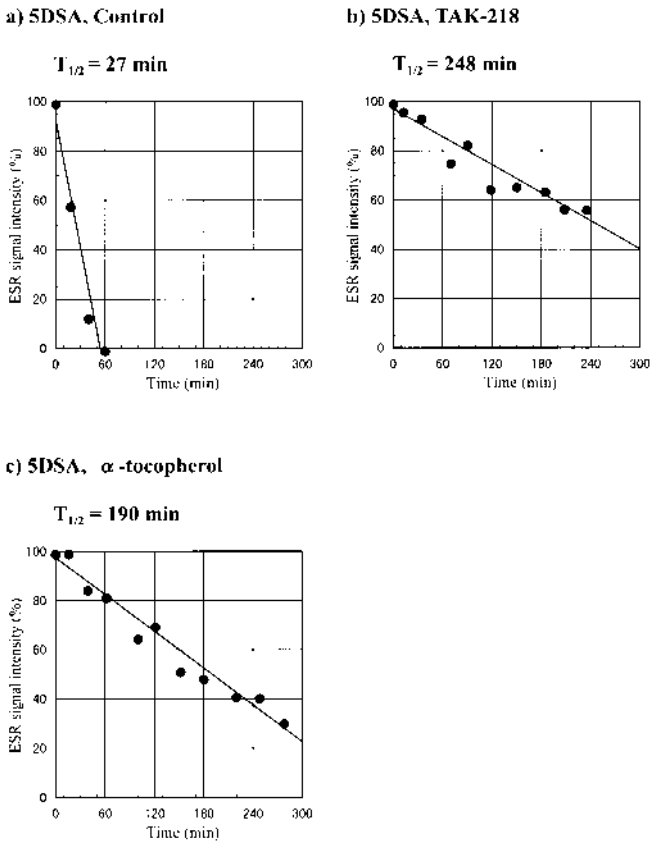


Fig. 9. Decrease of ESR Signal Intensities of 5DSA Spin Label Incorporated into the Liposomes in the Presence of Antioxidants (TAK-218 or α-Tocopherol)

The oxidation was initiated with AMVN at 50 °C. After we plotted ESR signal intensities, we linearized them using the least-squares method. Calculated R^2 values are as follows: a) R^2=0.95, b) R^2=0.91 and c) R^2=0.96.

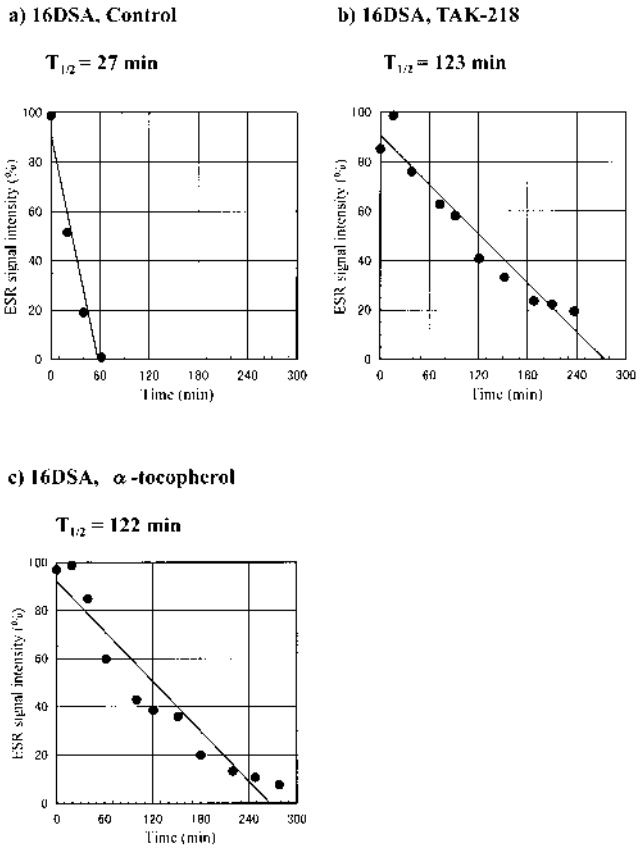


Fig. 10. Decrease of ESR Signal Intensities of 16DSA Spin Label Incorporated into the Liposomes in the Presence of Antioxidants (TAK-218 or α-Tocopherol)

The oxidation was initiated with AMVN at 50 °C. After we plotted ESR signal intensities, we linearized them using the least-squares method. Calculated R^2 values are as follows: a) R^2=0.95, b) R^2=0.94 and c) R^2=0.92.

branes. This result is in accord with Niki's reports.[12—14] The $T_{1/2}$ of spin labels containing TAK-218 also decreased in the order 5DSA≫16DSA>12DSA, 7DSA, in a similar manner to α-tocopherol. TAK-218, however, exhibited a longer $T_{1/2}$ value than α-tocopherol with all the spin labels. Comparing these results, it can be said that TAK-218 scavenges radicals more easily within the membranes than α-tocopherol.

Interaction between TAK-218 and Liposomal Membranes. Measurement of the Membrane Fluidity The result of the liposomal experiment clearly shows that the inhibition of lipid peroxidation by TAK-218 is more effective than that by α-tocopherol. It is assumed that this is mainly

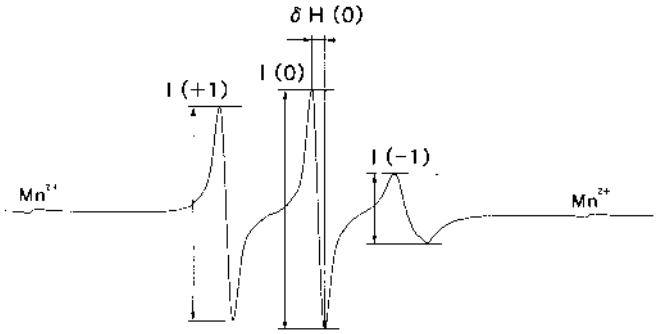


Fig. 11. Calculation of the Correlation Time (τ_R) of Rotational Diffusion Using ESR Spectrum of 16DSA in L-α-Phosphatidylcholine Liposomal Membranes

Temperature=20 °C.

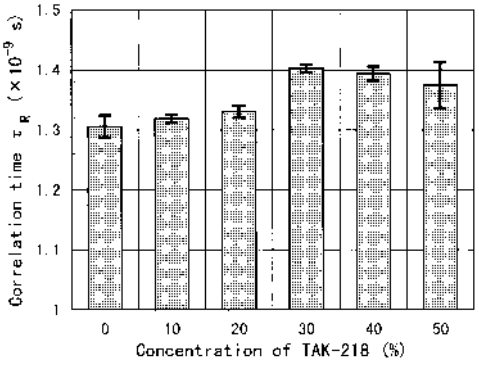


Fig. 12. The Effect of TAK-218 on the ESR Correlation Time (τ_R) of 16DSA in the Liposomal Membranes of L-α-Phosphatidylcholine

Temperature=20 °C.

because TAK-218 can easily move through the membrane and scavenge the radicals in the interior. However, the C log P value (calculated partition coefficient between aqueous and membrane regions) for α-tocopherol (C log P=12.1) shows it to be more hydrophobic than TAK-218 (C log P=5.58), which would generally suggest that the total distribution of TAK-218 in the membrane region will be smaller than that of α-tocopherol. To confirm that TAK-218 can penetrate to the membrane interior, we examined the interaction between TAK-218 and the membrane.

The ESR spectra of 16DSA in the membrane, which is used for the calculation of τ_R, is shown in Fig. 11. The effect of TAK-218 and α-tocopherol on the correlation time, τ_R, of 16DSA in the L-α-phosphatidylcholine liposomal membranes is shown in Figs. 12 and 13, respectively. In this experiment, ESR spectra were measured at room temperature (T=20 °C). Since the phase transition temperature of this liposome is about 0 °C, τ_R was measured at above the phase transition temperature (liquid-crystal region). The τ_R of 16DSA in the liposomes increased with an increase in the additive concentration of TAK-218 and α-tocopherol to the liposomes, indicating that the membranes exhibited rigidity and reduced fluidity. The effect of α-tocopherol on reducing τ_R is more remarkable than that of TAK-218, when compared at the same additive concentration.

These results suggest that the phytyl side chain of α-tocopherol causes the stronger interaction with the membrane.

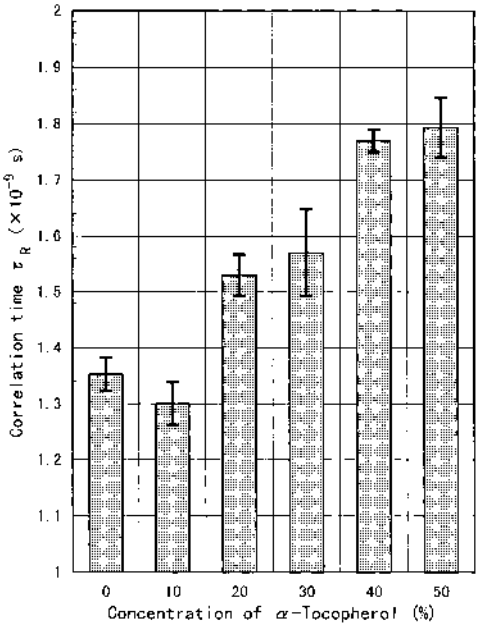


Fig. 13. The Effect of α-Tocopherol on the ESR Correlation Time (τ_R) of 16DSA in the Liposomal Membranes of L-α-Phosphatidylcholine

Temperature=20 °C.

We have confirmed that TAK-218 can reside in the interior of the membrane, since it has an effect on the τ_R of 16DSA in the interior.

ESR Spectra of TAK-218 Radical The experimental results described above definitely indicate: a) that TAK-218 exhibits strong radical scavenging activity in a homogeneous solution and b) demonstrates the remarkable inhibition of lipid peroxidation in liposomal membrane. Though the radical scavenging reaction mechanism of TAK-218, shown in Fig. 2, is proposed, it has not been confirmed experimentally.

It is assumed that the aminyl radical is derived from TAK-218, when TAK-218 scavenges free radicals such as the model radicals (DPPH, galvinoxyl), hydroxyl radical and superoxide radical, as shown in Fig. 2. Since the aminyl radicals play an important role in the realization of the radical scavenging activity of TAK-218, we determined the hyperfine splitting constant of the aminyl radicals in well-resolved ESR spectra produced by several methods.

As soon as the TAK-218 solution was mixed with H_2O_2 at room temperature, the ESR signal of the radicals derived from TAK-218 was observed at the minimum detection level. When the temperature of the mixture was raised from 40 °C to 80 °C, the ESR signal of the TAK-218 radical exhibited a stronger intensity. The concentration of the generating radicals at 40 °C and 80 °C was about 1.80×10^{-7} and 5.50×10^{-7} M, respectively. The ESR spectra of the TAK-218 radical, obtained by oxidation using H_2O_2 at 80 °C, is shown in Fig. 14. When TAK-218 was reacted with H_2O_2 in D_2O solution, the NH group in the TAK-218 radical underwent a change to ND (D: deuterium). The ESR spectrum of the TAK-218 radical in D_2O solution is shown in Fig. 15; it was measured at high resolution with a modulation width of 0.5 G. The ESR spectrum of the TAK-218 radical consists of twenty-seven peaks, whose hyperfine structure (hfs) is 1.82 G. The ESR spectrum changes in hfs (0.97 G), when the hydrogen in the amino group is substituted by deuterium,

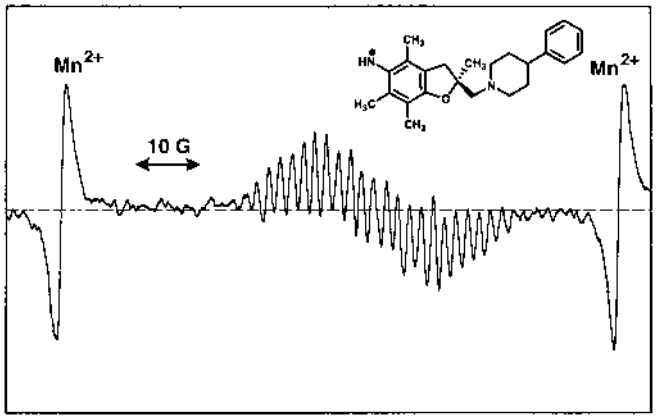


Fig. 14. ESR Spectrum of TAK-218 Radical at 80 °C

The reaction mixture contained 25 mM TAK-218 and 0.5% H_2O_2 in H_2O solution. The specification of this spectrometer was as follows: sweep width, 100 G; mod. width 1.0 G; sweep time, 2.0 min; time constant, 0.3 s; and receiver gain, ×2000.

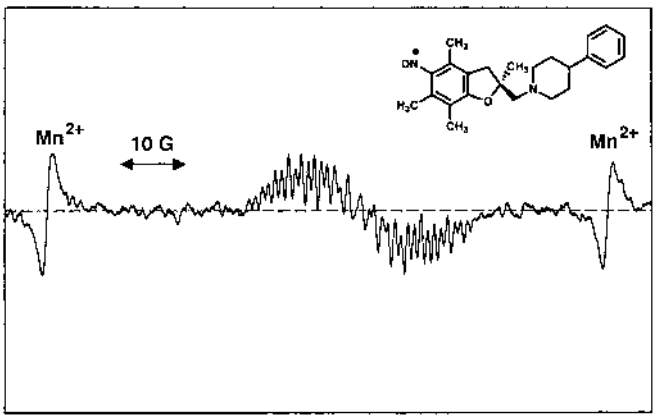


Fig. 15. ESR Spectrum of TAK-218 Radical at 80 °C

The reaction mixture contained 25 mM TAK-218 and 0.5% H_2O_2 in D_2O solution. The specification of this spectrometer was as follows: sweep width, 100 G; mod. width 0.50 G; sweep time, 2.0 min; time constant, 0.3 s; and receiver gain, ×2000.

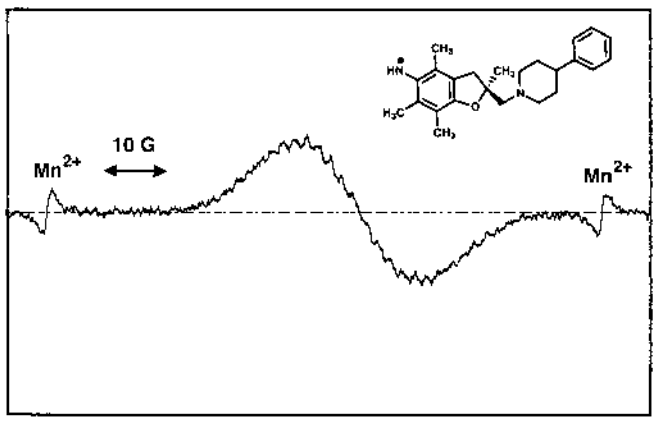


Fig. 16. ESR Spectrum of TAK-218 Radical at 20 °C

The reaction mixture contained 0.1 M TAK-218 and PbO_2 in CH_3OH solution. The specification of this spectrometer was as follows: sweep width, 100 G; mod. width 1.0 G; sweep time, 2.0 min; time constant, 0.1 s; and receiver gain, ×1000.

which indicates that the TAK-218 radical has an exchangeable hydrogen in its molecule. The structure of the aminyl radical is shown in Fig. 2 and the same aminyl radical was observed using PbO_2 as an oxidation reagent, as shown in Fig. 16. However, the spectra did not exhibit the hyperfine structure due to the presence of atmospheric oxygen in the solvent.

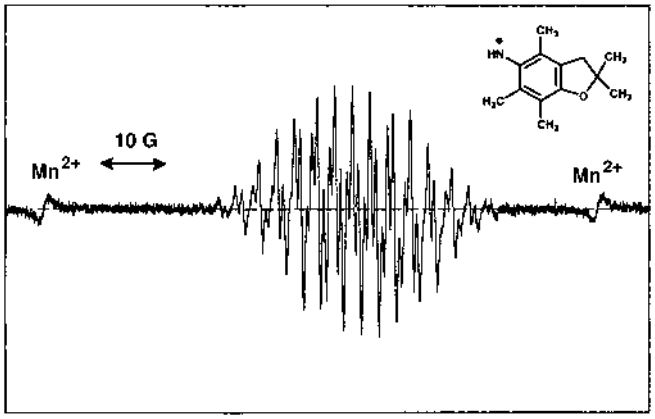


Fig. 17. ESR Spectrum of Aminocoumaran Radical at 50 °C

The reaction mixture contained 25 mM aminocoumaran and 0.5% H_2O_2 in H_2O solution. The specification of this spectrometer was as follows: sweep width, 100 G; mod. width 0.50 G; sweep time, 4.0 min; time constant, 0.03 s; and receiver gain, ×630.

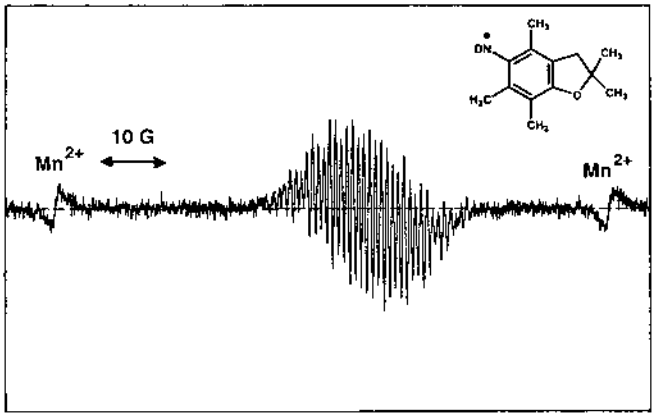


Fig. 18. ESR Spectrum of Aminocoumaran Radical at 80 °C

The reaction mixture contained 25 mM aminocoumaran and 0.5% H_2O_2 in D_2O solution. The specification of this spectrometer was as follows: sweep width, 100 G; mod. width 0.50 G; sweep time, 2.0 min; time constant, 0.03 s; and receiver gain, ×1000.

ESR spectra of the other aminocoumaran compound, whose chemical structure is shown in Fig. 2, are shown in Figs. 17 and 18. The hyperfine structure of the spectra arise from the coupling with four groups of protons: with coupling constants of a_{N5}=8.96 G (5-position NH_2, nitrogen), a_{H5}=5.97 G (5-position NH_2 proton), a_{H4}=2.99 G (4-position CH_3 proton) and a_{H6}=2.99 G (6-position CH_3 proton). The hyperfine structure was assigned by referring to other aminyl radicals in the literature.[18—20] We have thus identified the aminyl radicals derived from TAK-218 by the direct measurement of ESR spectra.

By analogy with α-tocopherol, TAK-218's radical must be a stable radical in order for it to exhibit the excellent antioxidant activity in the radical chain reaction which occurs in the membrane during lipid peroxidation. According to the hfs of the aminyl radicals, the spin density is observed not only at the 5-position nitrogen atom but also at the two ortho carbon atoms, at the 4- and 6-position of the benzofuran ring. These results suggest that the TAK-218 radical is a stable radical with delocalization of the unpaired π-electron.

Conclusion

The radical scavenging activities of TAK-218, which are very important for its protective effects against CNS trauma and ischemia, were studied using ESR spectroscopy and the following conclusions were deduced.

First, towards the hydroxyl radical in homogeneous solution, TAK-218 exhibited stronger scavenging activity than did the well-known scavengers, mannitol and DMSO. Towards the superoxide radicals, TAK-218 was comparable in activity to glutathione.

Second, the inhibition of lipid peroxidation by α-tocopherol and TAK-218 in the liposomal membranes was studied using an ESR spin-label technique. When the lipid peroxidation was initiated by radicals generated in an aqueous region using a water-soluble radical initiator (AAPH), both α-tocopherol and TAK-218 completely suppressed the lipid peroxidation in the model membranes. When the amount of radicals generated from AAPH was increased by raising the incubation temperature, α-tocopherol scavenged the radicals more effectively than TAK-218 near the surface of the membranes, while TAK-218 scavenged radicals more effectively in the interior of the membrane. The difference between TAK-218 and α-tocopherol in radical scavenging activity in the model membrane system is due to the different distribution pattern of these compounds. When the lipid peroxidation was initiated using a lipid-soluble radical initiator (AMVN), both compounds inhibited the lipid peroxidation, however, TAK-218 exhibited more potent inhibitory activity than α-tocopherol.

Further, the additive effect of TAK-218 and α-tocopherol on the liposomes was examined by monitoring the correlation time of spin labels (16DSA). Since the effect of α-tocopherol on the correlation time was greater than that of TAK-218, it was presumed that α-tocopherol exhibited stronger interaction with the lipid in the model membranes.

Based on these experimental results, TAK-218 is believed to be beneficial not only at the initial stage of an injury, but also the progressing stages of an injury, in which the injury progresses into the interior of the membrane.

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