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FORMATION OF STABLE FREE RADICALS FROM KAMPO MEDICINES TJ-9, TJ-15, TJ-23, TJ-96, TJ-114 AND THEIR ANTIOXIDANT EFFECT ON LOW DENSITY LIPOPROTEINS

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The Japanese herbal Kampo medicines TJ-9 (A), TJ-15 (B), TJ-23 (C), TJ-114 (D) and TJ-96 (E) were effective (2–5*x* less than α-tocopherol) in inhibiting a copper-induced peroxidation of low density lipoprotein. Kampo medicines dissolved in *n*-butanol formed stable free radical(s), detected by EPR spectroscopy as a single asymmetric line with *g*-value $g = 2.005$. The radical concentration increased in the order: $C < D \approx A \approx E < B$. When the Kampo medicines were oxidized in *n*-butanol by excess of PbO_2 their radical concentration increased 7–15 fold and was in the order $C < D < A \approx E \ll B$. A relationship between the potency of the medicines to inhibit peroxidation of LDL and their ability to form stable free radicals upon oxidation was observed. The medicine which formed more radicals was more efficient in inhibiting peroxidation of LDL. In order to study whether Kampo medicines can reduce α-tocopherol radical, the α-tocopherol radical was generated by the reaction of α-tocopherol with UV irradiated di-*tert*-butylperoxide and by autooxidation of α-tocopherol in *n*-butanol (25 $\mu l\,ml^{-1}$) in air. In both systems vitamin-C > Kampo B decreased the concentration of the α-tocopherol radical and the EPR spectrum of Kampo B stable radical(s) appeared. The effect of other Kampo medicines was not clearly seen since their EPR spectra were superimposed with the spectrum of the α-tocopherol radical. The results indicate that Kampo medicines possess electron donor properties and ability to form stable radical(s). The results may contribute to understanding beneficial effects of Kampo medicines in diseases in which free radical damage is suggested.

KEY WORDS: Kampo medicines, free radical scavenger, α-tocopherol, EPR spectroscopy, low density lipoprotein.

ABBREVIATIONS: LDL, low density lipoprotein; α-TR˙, α-Tocopherol radical; A, TJ-9 (Sho-saiko-to); B, TJ-15 (Oren-gedoku-to); C, TJ-23 (Toki-shakuyaku-san); D, TJ-114 (Sairei-to); E, TJ-96 (Saiboku-to); DTBP, di-*tert*-Butylperoxide; Vit-C, Vitamin C.

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INTRODUCTION

Traditional Kampo prescriptions, TJ-9, TJ-15, TJ-23, TJ-96 and TJ-114 have been used for the treatment of various clinical symptoms, such as bronchial asthma, allergic reactions, viral hepatitis, nephritis and others.[1,2] For example TJ-15 was reported to improve various clinical symptoms associated with cerebral apoplexy,[3] it has a hypotensive action on the cardiovascular circulation[4] and positive effects in chronic cerebral ischemia in experimental animals.[5] Also, administration of TJ-15 to spontaneously hypertensive rats has been reported to prevent the onset of stroke and to prolong survival.[6] TJ-15 reduced blood pressure in stroke prone spontaneously hypertensive rats.[7]

Free radicals have been proposed to induce cellular damage which may play a role in heart diseases, rheumatoid arthritis, cancer, inflammatory disorders, toxicity as well as in aging processes.[8] Scavengers of free radicals provide significant improvement in models for treatment of "free-radical diseases".[9,10] The Japanese herbal Kampo medicines were shown to exhibit beneficial effects in various models of diseases in which the deleterious role of free radicals and lipid peroxidation may be involved.

Oxidized low density lipoprotein (LDL) is taken up by the scavenger receptor of macrophages,[11,12] which is believed to play a role in the atherogenic process.[13,15] Therefore, drugs which can protect LDL against oxidation may possess anti-atherogenic potency as was found for probucol,[16] 17 beta estradiol,[17] phenothiazines[18] and the known antioxidant butylated hydroxytoluene.[19]

In our previous studies[20,21] we found that Kampo medicines TJ-9, TJ-15, TJ-23, TJ-96 and TJ-114 scavenged $OH^{\cdot}$ radical produced during Fenton type reactions and inhibited peroxidation of lipid liposomes.

The beneficial effects of Kampo medicines in diseases and the mechanism of the antioxidant effect of the Kampo medicines is not fully understood. Therefore in the present work we used EPR spectroscopy to study the formation of free radicals on oxidation of Kampo medicines, interaction of Kampo medicines with α-$TR^{\cdot}$ and their effect on oxidation of low density lipoprotein.

MATERIALS AND METHODS

Chemicals and their Origin

Kampo medicines TJ-9 (A), TJ-15 (B), TJ-23 (C), TJ-114 (D) and TJ-96 (E) were from Tsumura & Co. (Japan). α-Tocopherol was from Sigma (USA). Ascorbic acid (Vit-C) was from Lachema (CSFR) and di-*tert*-butylperoxide (DTBP) from Fluka (Switzerland).

Kampo Medicine Radicals

Kampo medicine (20 mg) was added to 300 μl of *n*-butanol without or with excess of PbO_2. The mixture was vortexed for 30 s, and 2 min after mixing the EPR spectrum was recorded at 25°C by a BRUKER 200 D-SRC spectrometer, in the *x* band region with central field 3357 G, sweep width 50 G, modulation amplitude was 2.5 G. Relative free radical concentration was estimated from the intensity of the EPR spectra.

α-Tocopherol Radical - Kampo Interaction

Two model systems were used to generate α-tocopherol radical (α-$TR^{\cdot}$) and to study

its interaction with Kampo medicines. The first system consisted of α-TR˙ and DTBP in organic solution. α-Tocopherol (100 μl), benzene (2 ml) and DTBP (250 μl) were mixed. The solution was irradiated by UV light in quartz cuvettes for 4 min under a xenon lamp. After the irradiation, 2 ml of *n*-butanol and 4 ml of benzene was added to the solution. For EPR measurement, 300 μl of the sample was taken and vortexed for 30 s with 10 μl of Vit-C solution in ethanol or with 20 mg of Kampo medicines. The EPR spectra were measured in plastic tubes (300 μl) 1–3 min after vortexing the sample as described above.

In the second system the α-TR˙ was formed by autooxidation of α-tocopherol in *n*-butanol (7.5 μl/300 μl) and by incubation in a glass vial with or without 20 mg of Kampo (or 1 mg Vit-C) under air at day light condition at room temperature. The EPR spectrum of α-TR˙ was measured at various times as described above. In another type of experiment α-tocopherol was dissolved in *n*-butanol (100 μl/4 ml) and incubated as described above. At different times 300 μl of the solution was mixed with 20 mg of Kampo medicine (or 1 mg Vit-C) for 30 s and the EPR spectrum was measured as described above. The relative concentration of α-TR was estimated from the intensity of the second line of the EPR spectrum of α-TR˙ as given in Figure 5a.

LDL Peroxidation

LDL was prepared from fresh normal human blood by ultracentrifugation (1.024–1.050 fraction) as described.[22] Isolated LDL was dialysed 24 h against buffer (in mmol/l): NaCl 150, 5 Tris-HCl, pH 7.4.

Samples for LDL peroxidation was prepared as follows: LDL (0.028 mg) in 250 μl of the buffer was incubated at 37°C for 5 h without and with Kampo medicines in the buffer solution. α-Tocopherol was added to LDL solution in ethanol (0.1–2% ethanol final concentration). Peroxidation of the samples was induced by $CuSO_4$ (5 μmol/l), which was added 5 min after the drugs.

TBA Test

The extent of lipid peroxidation was assessed by measurement of formation of TBA reactive products, mainly of the TBA-malondialdehyde (TBA-MDA) complex, according to a slightly modified method[23] of Haenan and Bast.[24] The incubated samples (0.2 ml) were added to 0.15 ml of BHT (1.53 mg BHT/1 ml ethanol) to prevent further peroxidation. Then 1.5 ml of TBA solution (2.1 g TBA, 84 g trichloroacetic acid, 3.57 ml of 37% HCl diluted with H_2O to 500 ml) was added and the samples were incubated at 80°C for 25 min. The samples were cooled at 10°C in a water bath and centrifuged at 1600 × *g* for 5 min. The supernatant was analyzed spectroscopically, where the absorption at [534 nm − (500 nm–576 nm)/2] was taken as a relative value of lipid peroxidation.

RESULTS

Kampo Medicine Radicals

Kampo medicines mixed with *n*-butanol formed stable free radicals. Thus the EPR spectrum of the Kampo B free radical is shown in Figure 1a. The EPR signal of the radicals was a single asymmetric line with *g*-value $g = 2.005$ and was stable for more

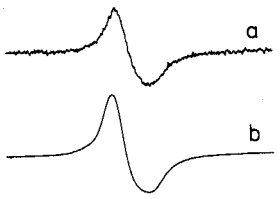

FIGURE 1 EPR spectra of Kampo medicine TJ-15 in *n*-butanol (20 mg/300 μl) without (a) or with excess of PbO_2 (b). Spectral width 5 mT. Spectrum b is 10 × attenuated.

than 60 min. All other Kampo medicines formed similar stable free radicals, but they had different intensities, as shown in Figure 2. The concentration of the stable free radicals formed by Kampo medicines increased in the order: C < D ≈ A ≈ E < B.

To study the electron donating properties of the Kampo medicines, the medicines were oxidized in *n*-butanol by adding excess PbO_2. The medicines showed different susceptibilities to be oxidized by PbO_2. The EPR signal intensity of the so formed stable free radicals increased 7–15 fold (Figure 1b). The highest susceptibility was found for Kampo B. The order of the radical concentration of the oxidized medicines was C < D < A ≈ E ≪ B (Figure 3).

α-Tocopherol Radical – Kampo Interaction

Under UV irradiation, DTBP in benzene decomposes and produces geminate radicals. These radicals abstract hydrogen from α-tocopherol and produce α-tocopherol radicals. The formation of α-TR$^{\cdot}$ after UV irradiation was detected by EPR spectroscopy. A typical 7-line EPR spectrum of α-TR$^{\cdot}$ is shown in Figure 4a. The halftime decay of the EPR signal was >40 min and was not studied in detail. The EPR signal of the α-TR$^{\cdot}$ decreased when Kampo B was added to the samples (Figure 4b), with subsequent appearance of a Kampo B stable radical. The signal of α-TR$^{\cdot}$ was abolished totally when Vit-C was added (Figure 4c). The spectrum b is a superposition of the α-TR$^{\cdot}$ and of Kampo B radical spectrum.

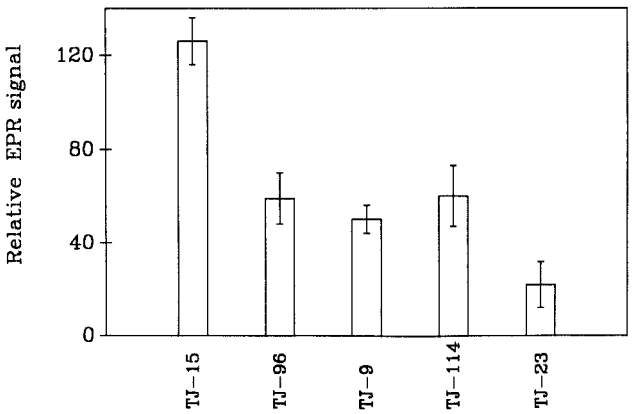

FIGURE 2 Comparison of EPR signal intensity of the Kampo medicine stable radicals in *n*-butanol (20 mg/300 μl). Results are mean ± SD, $n = 4$.

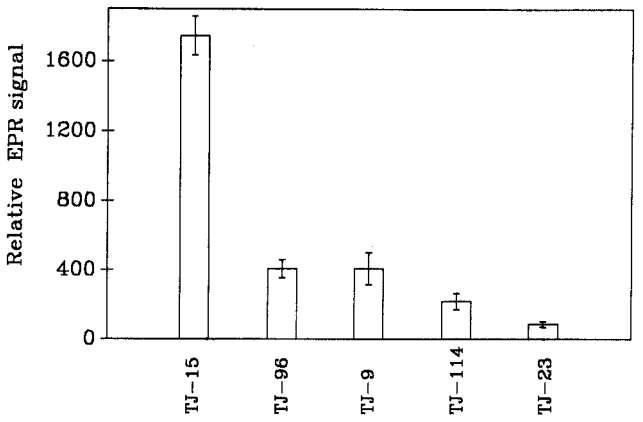

FIGURE 3 Comparison of EPR signal intensity of the Kampo medicines stable radicals in *n*-butanol (20 mg/300 μl) in excess of PbO_2. Results are mean $\pm$ SD, $n = 4$.

In the second system, the α-TR˙ was formed by autooxidation of α-tocopherol in *n*-butanol under air. An EPR spectrum of α-TR˙ with or without Kampo B or Vit-C is shown in Figure 5. The results were similar to those obtained in the first system. The concentration of the α-TR˙ in the control sample increased gradually (Figure 6) while the intensity of the EPR signal of α-TR˙ (Figure 5a) decreased when the solution contained Kampo B (Figure 5b) with subsequent appearance of Kampo B stable radical(s). The EPR signal of α-TR˙ disappeared when samples contained Vit-C (Figure 5c). The effects of the other Kampo medicines were several times smaller, possibly nonsignificant, yet they could not be measured properly, since the EPR spectra of the medicines were superimposed on the EPR spectrum of the α-tocopherol radical.

Vit-C inhibited the formation of α-TR˙ more than Kampo-B in *n*-butanol measured during 170 min as shown in Figure 6. In another experiment, Kampo B was added to the α-TR˙ solution after 60 min of incubation (Figure 7). Kampo B decreased the concentration of α-TR˙ measured during 120 min.

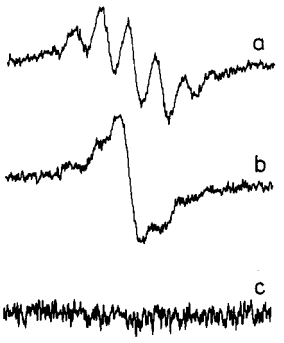

FIGURE 4 EPR spectra of a α-TR˙ formed by UV decomposition of DTBP in a solution of benzene: *n*-butanol = 1 : 3 without (a) or with 20 mg/300 μl of Kampo B (b) or with 1 mg/300 μl Vit-C (c). Spectral width 5 mT. Spectrum c is 3 fold amplifield.

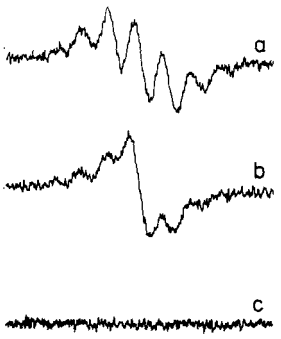

FIGURE 5 EPR spectra of α-TR˙ formed by autooxidation of α-tocopherol in *n*-butanol without (a) or with 20 mg/300 μl of Kampo B (b) or with 1 mg/300 μl Vit-C (c). Spectral width 5 mT.

Kampo Medicines and Oxidation of LDL

Kampo medicines inhibited oxidation of LDL. A comparison of the antioxidant effect of Kampo medicines and α-tocopherol on oxidation of LDL induced by 5 μmol/l $CuSO_4$ is shown in Figure 8. Kampo medicines protected the LDL against oxidation, measured as formation of TBA-reactive products, with the order of potency: C < D ≈ E ≈ A < B ⩽ α-tocopherol.

It was of interest to compare the potency of the medicines to inhibit peroxidation of LDL with their ability to form stable free radicals upon oxidation. The relationship is shown in Figure 9. The medicines which formed more radicals were more efficient in inhibiting peroxidation of LDL.

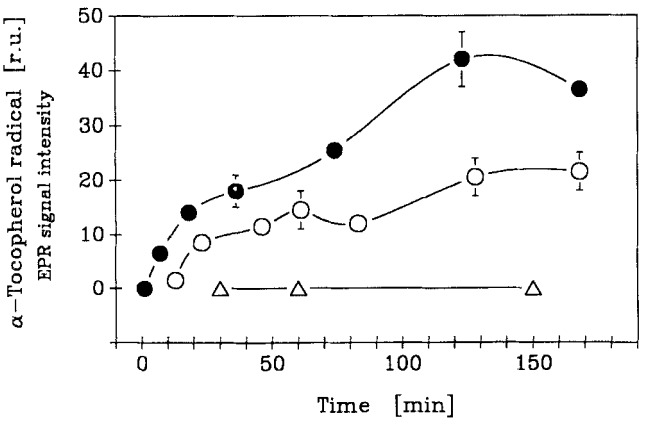

FIGURE 6 Time dependence of EPR spectral intensity of α-TR˙ formed by disolving α-tocopherol in *n*-butanol (7.5 μl/300 μl) and incubated in glass vial without (filled circles) or with 20 mg of Kampo (open circles) or 1 mg Vit-C (triangles).

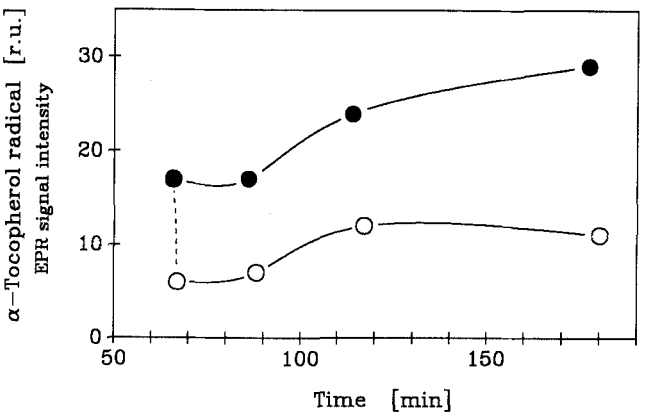

FIGURE 7 Time dependence of EPR spectral intensity of α-TR· formed by dissolving of α-tocopherol in *n*-butanol (7.5 μl/300 μl) and incubated in glass vial (filled circles). After 64 min of the incubation 20 mg of Kampo was added (open circles).

DISCUSSION

Kampo Medicine Radicals

Each Kampo medicine is a mixture of molecular components from different herbs. They already show stable radicals when dissolved in organic solution. The concentration of the radicals increased when the medicines were mixed with the oxidizing agent PbO_2. These results indicate that the medicines contain molecular components which are able to donate an electron and function as antioxidants. The medicines had different potencies to form free radicals. We found some relationship between the potency of the medicines to inhibit peroxidation of LDL and their ability to form

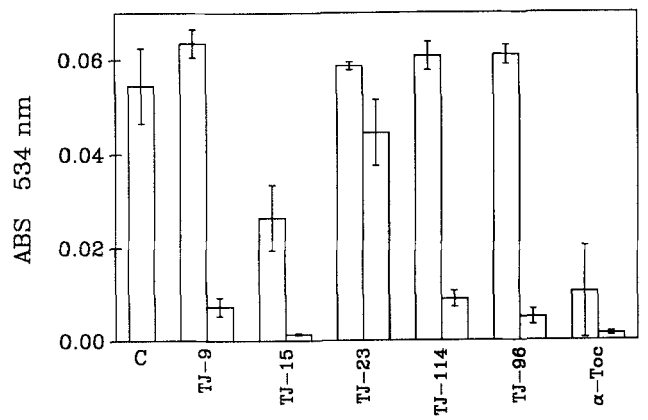

FIGURE 8 Relative absorption (at 534 nm) of TBA-reactive products of LDL (1.4 mg LDL/ml) induced by $CuSO_4$ (5 μmol/l) after incubation for 5 h at 37°. C-control. Concentrations of Kampo medicines and α-tocopherol (α-toc) were 20 μg/ml (left column) and 80 μg/ml (right column). Results are mean ± SD, $n = 3$.

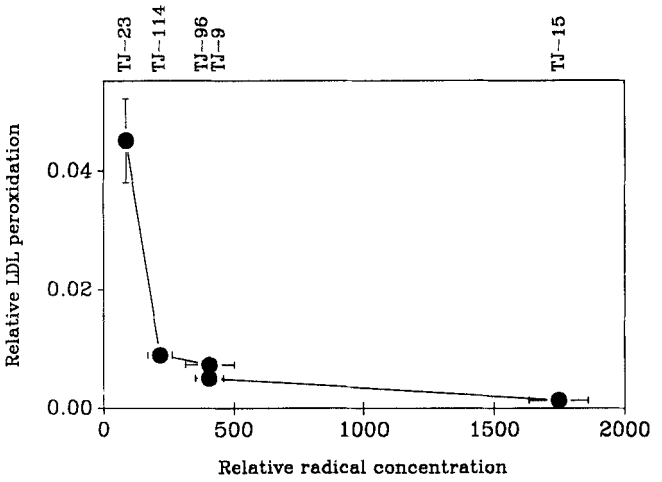

FIGURE 9 Relationships between the ability of the Kampo medicines to form free radicals upon oxidation by PbO_2 (data taken from Figure 3) and their potency to inhibit peroxidation of LDL at concentrations of 80 μg/ml (data taken from Figure 8).

stable free radicals upon oxidation (Figure 9). The medicines which formed more radicals were more efficient in inhibiting peroxidation of LDL.

In our previous studies[20,21] we found that the Kampo medicines TJ-9, TJ-15, TJ-23, TJ-96 and TJ-114 scavenged $OH^{\cdot}$ radical and inhibited lipid peroxidation with different potencies. Peroxidation of phosphatidylcholine liposomes was induced by addition of $FeSO_4$ and H_2O_2 and the extent of lipid peroxidation in the samples was estimated by a thiobarbituric acid test.[21] Therefore it was interesting to compare the potency of the medicines to scavenge $OH^{\cdot}$ radical and to inhibit lipid peroxidation in phosphatidylcholine liposomes with their ability to form stable free radicals upon oxidation. A relationship was found between the ability of the medicines to form free radicals and their established potency[21] to inhibit lipid peroxidation in the liposomes (Figure 10). A similar relationship was found between the potency of the medicines to inhibit peroxidation of LDL and their ability to form stable free radicals upon oxidation (Figure 9). The medicines which formed more radicals were more efficient in inhibiting lipid peroxidation. However there was no correlation between the effect of the medicines to form free radicals and their established potency[20] to scavenge $OH^{\cdot}$ radical. The results indicate that the property of compounds to form stable free radical(s) upon PbO_2 oxidation may be linked to their ability to inhibit peroxidation in the LDL and liposomes.

α-Tocopherol Radical — Kampo Interaction

α-Tocopherol, a potent chain-breaking antioxidant in biomembranes, can quench radicals and generate α-$TR^{\cdot}$. During this process the α-tocopherol concentration decreases, since it is not synthesized *in vivo*. In the presence of reducing agents, α-$TR^{\cdot}$ might be reduced to regenerate α-tocopherol.[25] Accordingly, α-tocopherol may scavenge more radicals than expected, if the reducing agents are present. Therefore the compounds which can reduce α-$TR^{\cdot}$ may be potentially beneficial in diseases in

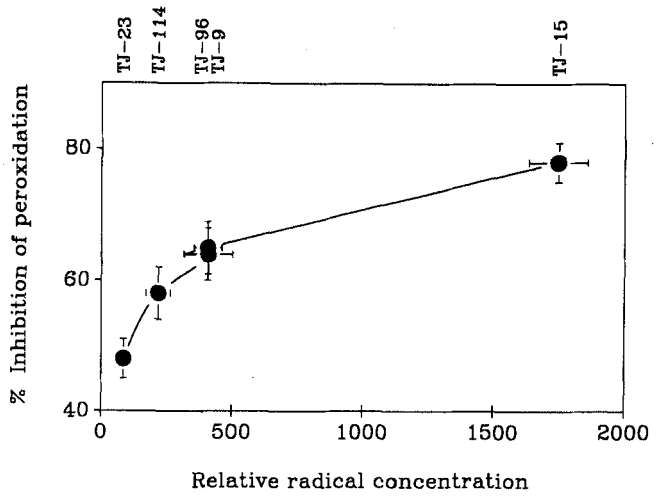

FIGURE 10 Relationship between the ability of the Kampo medicines to form free radicals upon oxidation by PbO_2 (data taken from Figure 3) and their published potency[21] to inhibit lipid peroxidation in liposomes at concentration of 50 mg/1 g lipid.

which free radicals are involved. It was found that α-TR˙ can be reduced to α-tocopherol by Vit-C, cysteine and possibly by glutathione.[25–27] Recently Hiramatsu *et al.*[28] reported that Kampo medicine TJ-960 reduced α-TR˙ in rat liver membranes.

To study the interaction of Kampo medicines with α-TR˙, their solution in *n*-butanol was used as a model for a lipid membrane environment. The α-TR˙ concentration formed by autooxidation of α-tocopherol in *n*-butanol was due to the presence of oxygen, since the concentration of the α-TR˙ was lower when the solution was degassed by argon. The EPR spectra of α-TR˙ (Figures 4 and 5) are similar to those found for α-TR˙ in other systems.[26,28] We found that TJ-15 decreased the concentration of α-TR˙, probably by reducing α-TR˙ or by interfering with autooxidation of α-tocopherol. Vit-C, which is known to reduce α-TR˙,[26] diminished the concentration of α-TR˙ in parallel experiments. It is known that higher plants contain several kinds of compounds which have antioxidant activity, among them flavonoids, phenolic acids, alkaloids, chlorophyll derivatives, and carotenoids.[29,30] Further study is needed, to establish which compounds can reduce α-TR˙.

Kampo and LDL Oxidation

Lipid peroxidation in LDL was found to be linked with its vitamin E content and it was suggested that oxidation of LDL is preceeded by a destruction of vitamin E.[15] Since we found that Kampo B decreased the concentration of α-TR˙ in a model system, and was the most efficient in inhibiting LDL oxidation, it may be suggested that it can regenerate α-TR˙ in LDL.

In conclusion, Kampo medicines possess electron donor properties with subsequent formation of stable free radicals, and Kampo B probably reduces α-TR˙. These properties may account for their antioxidant activities. They protect LDL against lipid peroxidation induced by $CuSO_4$. These properties of Kampo medicines may contribute to their beneficial biological effects.

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