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EVALUATION OF SUPEROXIDE SCAVENGING ACTIVITY OF OPC-14117 BY ELECTRON SPIN RESONANCE TECHNIQUE

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OPC-14117 is a potent drug which has both brain function activating effect and protective effect against cerebral ischemia. Occurrences of these effects might be expected due to superoxide dismutase-like activity of OPC-14117. The present study has been conducted to evaluate the active oxygen scavenging activity of OPC-14117 and to explain the mechanisms of its pharmacological activities. The reaction of OPC-14117 and superoxide anion, generated in potassium superoxide, was examined by electron spin resonance technique at both liquid nitrogen (77 K) and room (22°C) temperatures. OPC-14117 showed a higher superoxide scavenging activity than that of α-tocopherol in an aprotic solvent system. The active moiety of OPC-14117 to provide the scavenging effect was found due to the phenolic hydroxyl group of its indan skeleton.

KEY WORDS: OPC-14117, α-tocopherol, superoxide scavenging activity, potassium superoxide, spin-trapping.

INTRODUCTION

OPC-14117 (7-hydroxy-1-(4-(3-methoxyphenyl)-1-piperazinyl)acetylamino-2,2,4,6-tetramethylindan) (Figure 1a) was synthesized by Oshiro *et al.* in 1984[1] as a new type of brain function improving agent and was found to distribute in brain at high concentrations.[2] OPC-14117 was experimentally demonstrated by Kikuchi *et al.* to be a novel drug which has both brain function activating effect and protective effect against cerebral ischemia in mice and rats.[3] The most serious damage to the organism is now well known to be an oxidative destruction of membrane of tissues by active oxygen species after reperfusion of blood flow.[4,5] OPC-14117 is recognized to have a superoxide dismutase (SOD)-like activity in the brain. We, therefore, have investigated the reaction of OPC-14117 and superoxide anion radicals ($O_2^{\cdot-}$), one of the active oxygen species, to evaluate chemically its anti-cerebral ischemia or antioxidant effect and to explain the mode of actions in the brain.

To evaluate the SOD-like activity of a drug, certain active oxygen species are added in a reaction system. One of the currently used methods for this purpose is an electron spin resonance (ESR) measurement which employs the combinations of xanthine–xanthine oxidase system as a superoxide generating system and the spin trapping technique to know the scavenging activity of the superoxide anion in the presence of

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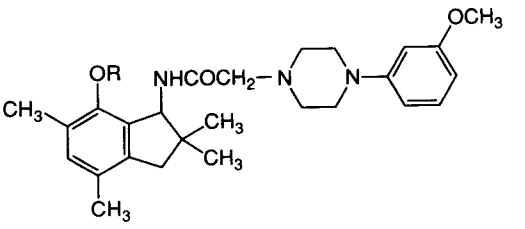

FIGURE 1 Structures of OPC-14117 and its methoxy derivative. (a) R = H, (OPC-14117); (b) R = CH_3, (OPC-14230).

a drug.[6–8] This method, however, seems to be unsuitable for the evaluation of SOD-like activity of OPC-14117 because this drug is practically insoluble in aqueous solution. When we think over that OPC-14117 is a potent drug improving brain functions, the SOD-like activity of this compound should be evaluated in non-aqueous medium, because brain is considered to be in high hydrophobic environments with various types of lipids. Thus we used the superoxide anions generated with potassium superoxide (KO_2) and crown-ether in an aprotic solvent system.[9] Further, ESR detections of the superoxide anions were performed in two methods, the one is the direct detection of $O_2^{\cdot-}$ by freezing at liquid nitrogen temperature (77 K) and the other is the indirect spin-trapping using 5,5-dimethyl-1-pyrolline-1-oxide (DMPO) as a spin trapping reagent at room temperature (22°C). The obtained SOD-like activity of OPC-14117 was compared with that of α-tocopherol as a natural antioxidant.

EXPERIMENTAL

Apparatus

ESR spectra were measured at liquid nitrogen temperature (77 K) or room temperature (22°C) by a JEOL JES-FE1XG X-band ESR spectrometer (9.1 GHz) with 100 KHz field modulation at modulation amplitude of 0.63 mT and microwave power of 5 mW which was calibrated with a Takeda Riken frequency counter, TR5212. A standard quartz cell (5-mm, i.d.) and a quartz-glass dewar were used for the measurements at 77 K, and a flat cell (10 mm × 50 mm, 200 μl in volume, LABOTEC Co.) was used for the spin trapping measurements at room temperature. The magnetic field strength was corrected by the hyperfine coupling constant (8.69 mT) of Mn(II) ions doped in magnesium oxide powder, and the *g*-values of the observed ESR spectra were estimated based on the *g*-value of lithium tetracyanoquinodimethane radicals (Li-TCNQ, $g = 2.00252$) as a standard.

Reagents

OPC-14117 and its methoxy derivative OPC-14230 (Figure 1b) were synthesized according to the methods of Oshiro *et al.*[1] α-Tocopherol (vitamin E) was supplied by Wako Pure Chemicals Co. DMPO and 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TANOL) were obtained from LABOTEC Co. KO_2 and dibenzo 18-crown-6 ether (DB18C6) were purchased from Aldrich Chemical Co.

Dimethylsulfoxide (DMSO) and dimethylformamide (DMF) as the solvents, were of guaranteed reagent grade and were used after drying with molecular sieves (Wako Pure Chemical Co., mesh size = 3 Å, 4 Å and 5 Å) and filtration. Other reagents were also of guaranteed reagent grade but used without further purification.

Preparation of Superoxide Anion Solution

Accurately weighed 3.17 mg (0.045 mmol) of KO_2 and 27.8 mg (0.077 mmol) of DB18C6 were transferred into a test tube and a 5 ml of a mixed solvent of toluene-DMSO (3 : 2) was added. The mixture was purged with argon gas for 60 sec and sealed with septum, followed by sonication for 60 sec. The supernatant solution was used for the ESR measurements.

The superoxide anion generated in this system was identified by both the *g*-values of its ESR spectrum measured at 77 K[9] and ESR parameters of O_2^--DMPO spin adduct measured at room temperature.[9,10] The concentration of the O_2^- in the solution was determined by the peak area of its ESR spectrum at 77 K, based on the calibration curve obtained from the known amounts of TANOL at the same temperature.

ESR Measurements

a. *Direct/freezing method.* To a 200 μl portion of O_2^- solution taken into the ESR standard cell, a 100 μl of drug solution of DMF was added. After mixing for 60 sec, the solution was frozen at liquid nitrogen temperature and ESR spectrum was recorded.

b. *Indirect/spin-trapping method.* To a 200 μl portion of O_2^- solution taken into the micro test tube, a 100 μl of drug solution of DMF was added and the solution was mixed. After 30 sec, a 10 μl of DMPO was added and mixed well. The solution was transferred into a flat-cell and the ESR spectrum was recorded after 60 sec of addition of DMPO.

RESULTS

Superoxide Scavenging Activity of OPC-14117 Estimated by Direct Freezing Method

ESR spectrum (77 K) of the frozen O_2^- solution without OPC-14117 showed characteristic signals at $g_\perp = 2.005$ (strong signal) and at $g_\parallel = 2.121$ (weak signal).[9] (Figure 2a) When a 100 μl of 1 mM OPC-14117 solution was added to the O_2^- solution at room temperature and the solution was frozen, the signals due to the superoxide anion disappeared completely (Figure 2b). Thus the various concentrations of OPC-14117 solution was added to the O_2^- solution to evaluate the SOD-like activity of this compound quantitatively. Figure 3 shows dose-dependent O_2^- scavenging rate of OPC-14117 together with that of α-tocopherol. The scavenging rate was calculated from the signal intensities due to O_2^- in the solution comparing with that in control solution without OPC-14117. This result indicated that OPC-14117 scavenged superoxide anion in the organic solvent. The superoxide scavenging activity of OPC-14117 depended on the concentration of the added drug, and a 0.4 mM of OPC-14117 solution scavenged superoxide anion completely.

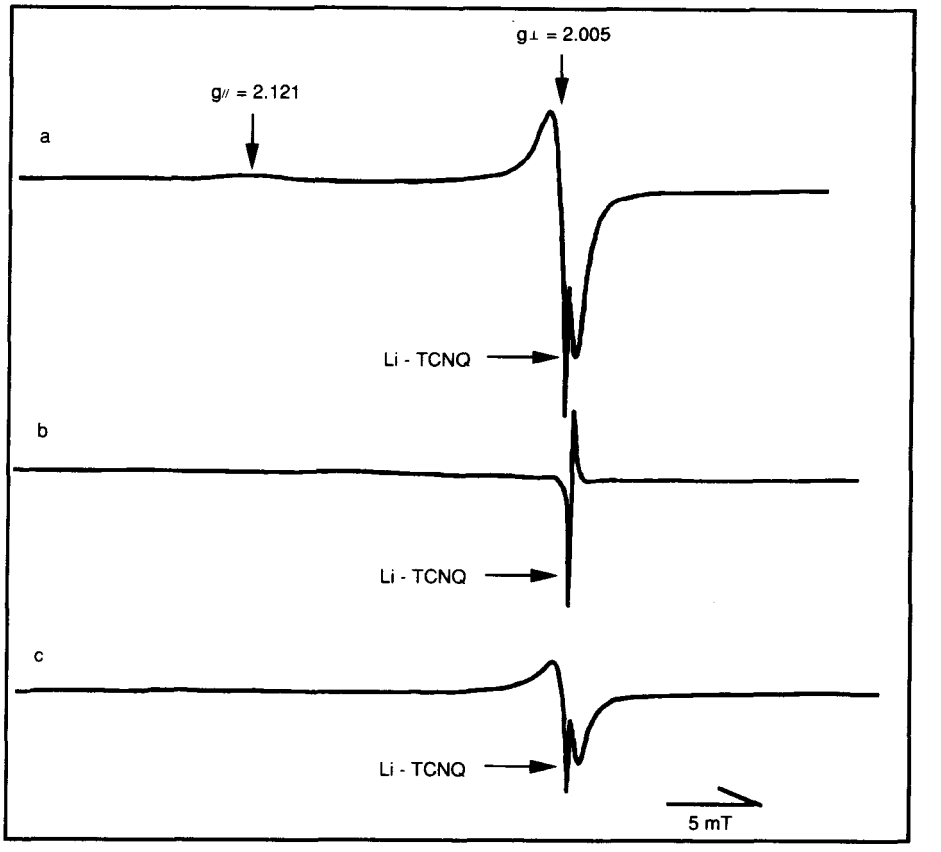

FIGURE 2 ESR spectra showing superoxide scavenging activity of OPC-14117 and α-tocopherol observed at 77 K. (a) control; (b) with OPC-14117 (1 mM); (c) with α-tocopherol (1 mM).

Similar experiments were performed using a natural antioxidant α-tocopherol. α-Tocopherol also showed the dose-dependent O_2^- scavenging activity like that of OPC-14117 (Figure 3). The activity of α-tocopherol was found to be approximately equal with that of OPC-14117 in concentration of 1.5 mM of the compound, however, OPC-14117 was more active in low concentration range of 0.1–0.3 mM. The concentration to scavenge 50% of the O_2^- (SC_{50}) in the direct method was estimated to be about 0.1 mM for OPC-14117 and about 0.2 mM for α-tocopherol (Figure 3).

The antioxidant activity in terms of SOD-like activity of α-tocopherol is ascribable to the phenolic hydroxy group. OPC-14117 has also a hydroxy group at the 7th position of indan skeleton. Therefore, the superoxide scavenging activity of the 7-methoxy derivative (OPC-14230) of OPC-14117 (Figure 1b), was evaluated in a same manner. The scavenging rate at the concentration of 1.4 mM was 97 ± 3% for OPC-14117 and 14 ± 14% for 7-methoxy derivative. Thus the scavenging activity of OPC-14117 was concluded due to the phenolic hydroxy group of indan skeleton.

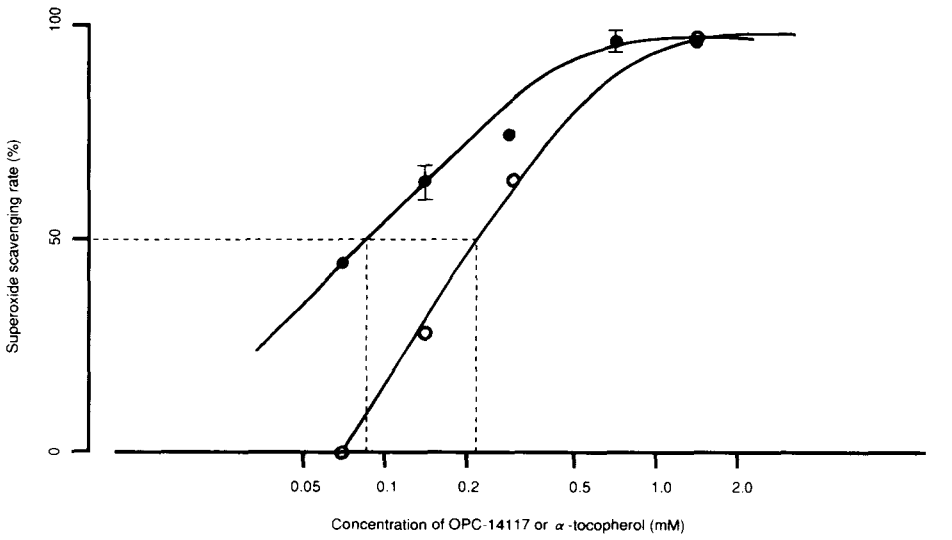

FIGURE 3 Dose dependent superoxide scavenging activity of OPC-14117 and α-tocopherol, estimated by freezing method. (Superoxide scavenging rates of OPC-14117 at the concentration of 0.14 mM and 1.4 mM were indicated as the mean value ± S.D. (n = 3).)

Superoxide Scavenging Activity of OPC-14117 Estimated by Spin Trapping Method

Signal due to superoxide anions detected by ESR spin trapping technique was typically depicted in Figure 4a. The spectrum was identified as superoxide-DMPO spin adduct by the coupling constants a_N = 1.29 mT, a_β^H = 1.07 mT, and a_γ^H = 0.14 mT of its simulated hyperfine structure (Figure 4). These signals due to O_2^- disappeared by adding OPC-14117 solution (Figure 4b) depending on the concentration of OPC-14117 as shown in Figure 5. The concentration of O_2^- was calculated based on the concentrations of the known amounts of TANOL. The SC_{50} of OPC-14117 in this system was thus estimated about 0.04 mM. This result was in good agreement with that of the freezing method described before.

DISCUSSION

When OPC-14117 was given orally to mice or rats at the dose of 30 mg/kg, the concentrations of OPC-14117 in brains were found to present approximately 4.5- or 5.0-fold higher than those in the plasma, respectively, as determined by high performance liquid chromatography.[2] The brain is known in highly hydrophobic environments with various types of lipids. Since OPC-14117 has experimentally been observed to be effective in improving experimental cerebral dysfunctions and protecting the brain against ischemia and reperfusion of blood flow,[3] in which the generation of active oxygen species like superoxide anions has been proposed so far, we studied from a view point of physico-chemistry whether OPC-14117 has an activity to scavenge the superoxide anions in an aprotic solvent system, which might be

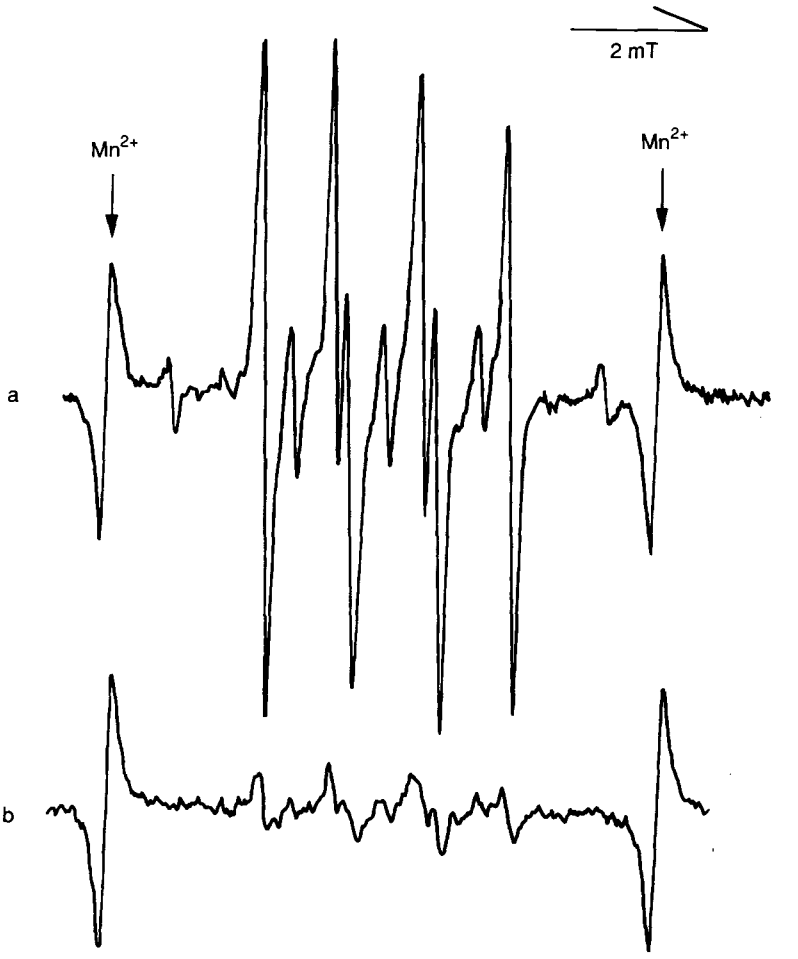

FIGURE 4 ESR spin trapping of superoxide anion radicals generated by KO_2^- crown ether system (a) and that in the presence of OPC-14117 (0.17 mM) (b).

analogous to the brain hydrophobic environments. Thus, OPC-14117 has been demonstrated to have the O_2^- scavenging activity in an aprotic solvent system on the basis of the both ESR results of the direct superoxide anion detection at 77 K and the ESR trapping method at room temperature (Figures 3 and 5). The activity was found to be approximately two times higher than that of α-tocopherol as evaluated by SC_{50} values, and it was due to its phenolic hydroxy group. The active oxygen scavenging effect of OPC-14117 seemed to be one of the important physico-chemical characters to understand the brain function improving effects of this drug.

In recent years, the spin trapping technique using the xanthine–xanthine oxidase system as a O_2^- generating system has been established as a useful evaluating method of SOD-like activity of a compound. However, the evaluating system using aprotic

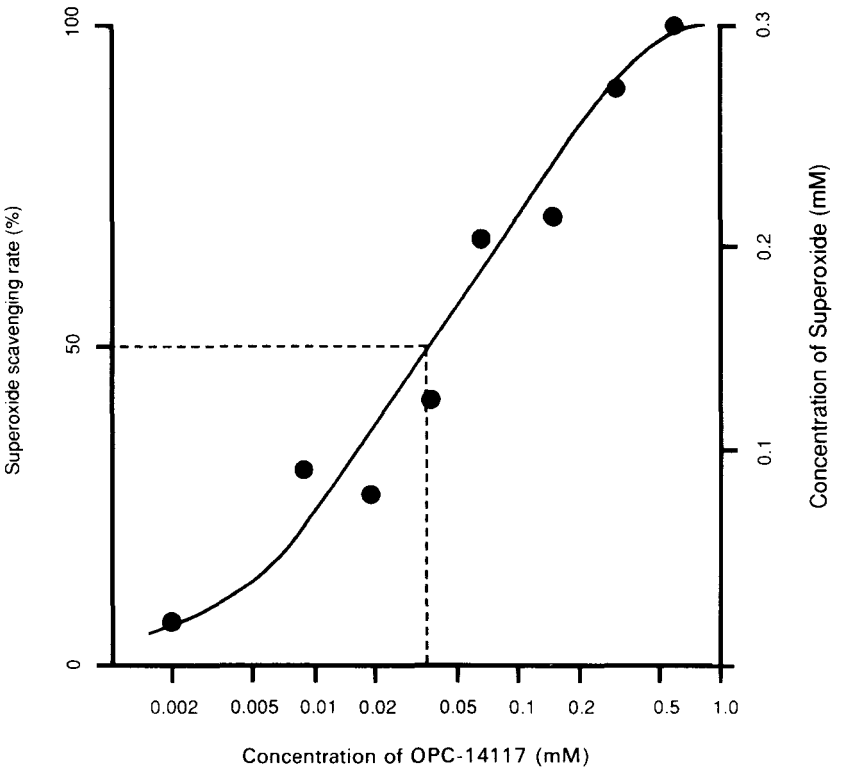

FIGURE 5 Dose-dependent superoxide scavenging activity of OPC-14117, estimated by spin trapping method.

solvents was not reported as far as we know. The present direct method performed at 77 K is needed for the evaluation of the drugs which practically insoluble in water such as OPC-14117. The direct or indirect determination of O_2^- scavenging activity in aprotic solvent systems proposed here, therefore, might be a useful technique for the evaluation of SOD-like activity of a drug.

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