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Kinobeon A, purified from cultured safflower cells, is a novel and potent singlet oxygen quencher*

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We recently reported that kinobeon A, produced from safflower cells, suppressed the free radical-induced damage of cell and microsomal membranes. In the present study, we investigated whether kinobeon A quenches singlet oxygen, another important active oxygen species. Kinobeon A inhibited the singlet oxygen-induced oxidation of squalene. The second-order rate constant between singlet oxygen and kinobeon A was 1.15×10^{10} $M^{-1}s^{-1}$ in methanol containing 10% dimethyl sulfoxide at 37°C. Those of α-tocopherol and β-carotene, which are known potent singlet oxygen quenchers, were 4.45×10^{8} $M^{-1}s^{-1}$ and 1.26×10^{10} $M^{-1}s^{-1}$, respectively. When kinobeon A was incubated with a thermolytic singlet oxygen generator, its concentration decreased. However, this change was extremely small compared to the amount of singlet oxygen formed and the inhibitory effect of kinobeon A on squalene oxidation by singlet oxygen. In conclusion, kinobeon A was a strong singlet oxygen quencher. It reacted chemically with singlet oxygen, but it was physical quenching that was mainly responsible for the elimination of singlet oxygen by kinobeon A. Kinobeon A is expected to have a preventive effect on singlet oxygen-related diseases of the skin or eyes.

Keywords: kinobeon A, singlet oxygen, antioxidant, quencher, endoperoxide, safflower

Active oxygen species, such as free radicals and singlet oxygen ($^1O_2$), induce cellular injury *via* the accumulation of oxidative damage to DNA, lipids, and protein, and/or by the induction of uncontrolled signal transduction (Halliwell & Gutteridge, 1999; Klotz *et al.*, 2000). Therefore, oxidative stress is suggested to be a cause of various diseases (Halliwell & Gutteridge, 1999). On the other hand, antioxidants, such as α-tocopherol, ascorbic acid and ubiquinol, protect biological systems from oxidative stress (Halliwell & Gutteridge, 1999). We recently found a new antioxidant, kinobeon A (Kanehira *et al.*, 2003). Kinobeon A is a unique red compound produced from safflower (*Carthamus tinctorius* L.) cells cultured under specific conditions (Wakayama *et al.*, 1994) and has not been found in natural safflowers, other plants, animals or microorganisms. Safflower is a valuable plant used as an edible fat, as a Chinese medicine, in cosmetics, and in foodstuffs as a colorant. Kinobeon A inhibited the oxidation of rat liver microsomal membrane induced by the Fe^{2+}-ADP/NADPH system, protected bovine kidney cell cultures from oxidative stress (hydrogen peroxide, *tert*-butyl hydroperoxide), and scavenged the superoxide anion produced in the hypoxanthine/xanthine oxidase system (Kanehira *et al.*, 2003). However, the quenching of $^1O_2$ by kinobeon A is still not well established. *In vivo*, $^1O_2$ is produced by exposure to sunlight and from neutrophils and eosinophils. Neutrophils use $^1O_2$ when they kill bacteria to protect biological systems (Nakano *et al.*, 1998; Tatsuzawa *et al.*, 1998; 1999; 2000; Arisawa *et*

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Abbreviations: AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile); IC_{50}, half inhibitory concentration; k_Q, second order rate constant for quenching; NEPO, 3-(4′-methyl-1′-naphthyl)-propionic acid, 1′,4′-endoperoxide; $^1O_2$, singlet oxygen.

al., 2003). On the contrast, $^1O_2$ is also considered to be a causative factor of various skin diseases and eye diseases (Halliwell & Gutteridge, 1999). Therefore, the search for a novel effective $^1O_2$ quencher is important. $^1O_2$ is consumed by an antioxidant *via* a chemical addition reaction and/or physical quenching, such as electron transfer. Kinobeon A has many double bonds, which may be able to react with $^1O_2$. In the present study, we investigated the potential of kinobeon A as $^1O_2$ quencher.

MATERIALS AND METHODS

Materials. Squalene was purchased from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). α-Tocopherol, β-carotene, isoluminol and microperoxidase were obtained from Sigma Chemical Co. (St. Louis, MO, USA). 2,2′-Azobis(2,4-dimethylvaleronitrile) (AMVN) was purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Kinobeon A was prepared as described previously (Wakayama *et al.*, 1994). Solvents and other reagents were of the highest grade commercially available. 3-(4′-Methyl-1′-naphthyl)-propionic acid, 1′,4′-endoperoxide (NEPO) was from Dr. Ken Fujimori (University of Tsukuba, Japan).

Effect of kinobeon A, α-tocopherol or β-carotene on $^1O_2$-induced squalene oxidation. Squalene was purified by HPLC using a CAPCELLPAK C18 column (20 × 250 mm, 5 μm, Shiseido Co. Ltd, Tokyo, Japan) and methanol as the mobile phase (flow rate: 10 ml/min), as reported previously (Nakano *et al.*, 1998). Purified squalene was dissolved in chloroform and stored at –80°C prior to use. Kinobeon A was dissolved in dimethyl sulfoxide. The concentration of kinobeon A was calculated by using its molar absorption coefficient at 520 nm (1.95×10^5 $M^{-1}cm^{-1}$; our data). α-Tocopherol and β-carotene were dissolved in methanol. The concentration of NEPO was calculated as previously reported (Nakano *et al.*, 1998). After the solvent was removed from the chloroform solution of squalene under reduced pressure, methanol was added. The reaction was started by the addition of a NEPO solution (final concentration: 100 μM) to the mixture containing 1 mM squalene and 1–100 μM kinobeon A, 10–1000 μM α-tocopherol or 1–13 μM β-carotene in methanol/dimethyl sulfoxide (9:1, v/v; total volume: 1.0 ml) at 37°C. An aliquot of the reaction mixture (50 μl) was removed every 30 min for 90 min and injected into the HPLC using a CAPCELLPAK C18 (4.6 × 250 mm, 5 μm, Shiseido Co., Ltd, Tokyo, Japan) as an analytical column and methanol as the mobile phase (flow rate: 2 ml/min). Squalene hydroperoxide was detected in a hydroperoxide-specific assay using the chemiluminescence of isoluminol (Yamamoto *et al.*, 1987; Nakano *et al.*, 1998).

Reaction between kinobeon A and $^1O_2$ or AMVN-derived radical. Kinobeon A (10 μM) was incubated with 1 mM NEPO in methanol containing 10% dimethyl sulfoxide at 37°C. AMVN (1 mM) was also used instead of NEPO to compare the $^1O_2$-induced oxidation with the radical-induced oxidation of kinobeon A. An aliquot of the reaction mixture (20 μl) was removed every 15 min for 75 min and injected into the HPLC system. A CAPCELLPAK C18 (4.6 × 250 mm, 5 μm) and methanol/water (3:2, v/v) were used as a column and the mobile phase, respectively. The flow rate was 1.0 ml/min. Kinobeon A was detected at 520 nm.

Spectrophotometry. Kinobeon A (10 μM) was incubated with 5 mM NEPO in methanol containing 10% dimethyl sulfoxide at 37°C. The UV-VIS absorption spectrum was measured (260–600 nm) every 10 min for 60 min using a spectrophotometer (U-3210, Hitachi Ltd, Tokyo, Japan).

RESULTS

Kinetic analysis of NEPO thermolysis in methanol containing 10% dimethyl sulfoxide at 37°C

NEPO, a thermolytic $^1O_2$ generator, was used in the present study. The kinetics of the decomposition of NEPO in methanol containing 10% dimethyl sulfoxide at 37°C was examined first, as described previously (Nakano *et al.*, 1998), to clarify the total amount of $^1O_2$ formed by the thermal decomposition of NEPO in each experiment. The wavelength of the maximum absorbance (λ_{max}), molar absorption coefficient at λ_{max} of 3-(4′-methyl-1′-naphthyl)-propionic acid (a molecule remained after $^1O_2$ was produced from NEPO) and the first order rate constant of NEPO thermolysis were obtained as 287.4 ± 0.6 nm, 7800 ± 70 $M^{-1}cm^{-1}$, and $(1.91 \pm 0.09) \times 10^{-4}$ s^{-1} (mean ± S.D., n = 3), respectively. Since the thermolysis of NEPO is a first order reaction, the total amount of $^1O_2$ formed can be calculated using eqn. 1.

$$[^1O_2]_{total} = [NEPO]_0 \times (1 - \exp(-1.91 \times 10^{-4} \times t)\ [M] \quad (1)$$

where t (in seconds) stands for reaction time.

Suppression of $^1O_2$-induced squalene oxidation by kinobeon A

The effect of kinobeon A on the $^1O_2$-induced oxidation of squalene in organic solvent was investigated. Squalene was used since it is one of the most vulnerable lipids to $^1O_2$ (Nakano *et al.*, 1998). Kinobeon A inhibited the $^1O_2$-induced oxidation of squalene dose-dependently (Fig. 1A). α-Tocopherol and β-carotene, known as strong $^1O_2$ quenchers (Di

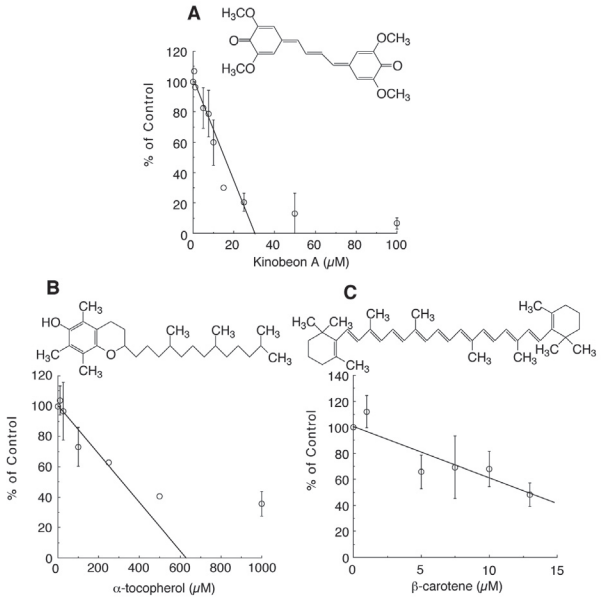


Figure 1. Inhibition of $^1O_2$-induced squalene oxidation by each quencher.
(A) Kinobeon A, (B) α-tocopherol, and (C) β-carotene. Squalene (1 mM) was incubated with 100 μM NEPO and an $^1O_2$ quencher in methanol containing 10% dimethyl sulfoxide for 90 min at 37°C. The ratio of squalene hydroperoxide formed in the presence of the quencher to that in its absence (control) is shown. Results are expressed as means ± S.D. (n = 3). On some points, error bars are not seen since they are very small. The structure of each quencher is also shown.

Mascio *et al.*, 1989; Kaiser *et al.*, 1990; Tatsuzawa *et al.*, 2000), were used to verify the quenching abilities of kinobeon A. They also inhibited the $^1O_2$-induced oxidation of squalene in a dose-dependent manner (Fig. 1B and C). The half inhibitory concentrations (IC_{50}s) of α-tocopherol and β-carotene to $^1O_2$-induced oxidation of squalene in the present experimental system were 316 μM and 13 μM, respectively (Table 1). That of kinobeon A was 15 μM (Table 1). Thus, the IC_{50} of kinobeon A was similar to that of β-carotene and much smaller than that of α-tocopherol. Judging from these results, kinobeon A can act as a potent $^1O_2$ quencher.

Kinetic analysis of reaction between each quencher and $^1O_2$

Moreover, second-order rate constants between $^1O_2$ and each quencher in methanol involving 10% dimethyl sulfoxide were roughly calculated using eqn. 2 (Young *et al.*, 1971; Kohno *et al.*, 1995).

$$S_0/S_Q = 1 + (k_Q/k_d)[Q] \quad (2)$$

where S_0 and S_Q represent slopes of the formation of squalene hydroperoxide plotted as a function of time in the absence and presence of each quencher, respectively. k_d denotes the first-order rate constant of $^1O_2$ decay. In the present study, 1.8×10^5 s^{-1} (k_d in methanol) (Young *et al.*, 1971) was used as the k_d for the calculation of second-order rate constant, since k_d is slightly lower in dimethyl sulfoxide ($3.3–5.2 \times 10^4$ s^{-1}) than in methanol ($0.9–2.0 \times 10^5$ s^{-1}) (Bellus, 1978). [Q] and k_Q represent the initial concentration of each quencher and second-order rate constant of the reaction between $^1O_2$ and the quencher involving physical quenching and a chemical reaction, respectively. k_Q values in the present study were shown in Table 1. Reported k_Q values of α-tocopherol and β-carotene were 2.5×10^8 $M^{-1}s^{-1}$ in n-butanol at 35°C

Table 1. Second-order rate constants (k_Q) and IC_{50} between singlet oxygen and quencher in methanol containing 10% dimethyl sulfoxide at 37°C

Quencher	IC_{50} (μM)	k_Q ($M^{-1}s^{-1}$)
Kinobeon A	15	1.15×10^{10}
α-Tocopherol	316	4.45×10^8
β-Carotene	13	1.26×10^{10}

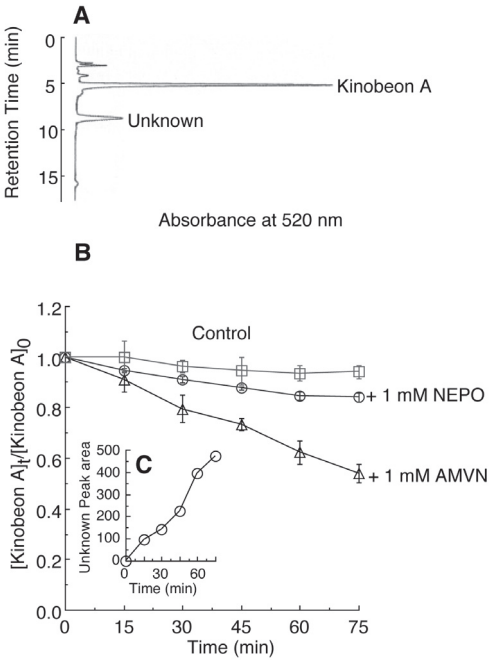


Figure 2. Reaction of kinobeon A with $^1O_2$ or peroxyl radical.
(A) Representative chromatogram from the kinobeon A analysis. Kinobeon A (10 μM) was incubated with AMVN (1 mM) in methanol containing 10% dimethyl sulfoxide at 37°C. (B) Change in kinobeon A concentration during the incubation with 1 mM NEPO or 1 mM AMVN in methanol containing 10% dimethyl sulfoxide at 37°C. A control experiment without NEPO or AMVN was also performed. Results are expressed as the ratio to the initial concentration of kinobeon A and as means ± S.D. (n = 3). Squares: control; circles: 1 mM NEPO; triangles: 1 mM AMVN. (C) Change in the unknown peak area during the reaction between kinobeon A and 1 mM AMVN. The initial concentration of kinobeon A was 10 μM.

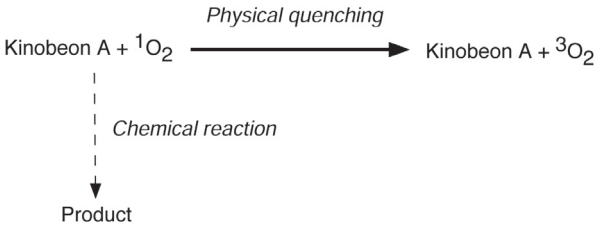
(Kohno *et al.*, 1995) and 1.4×10^{10} $M^{-1}s^{-1}$ in ethanol/chloroform/water (50:50:1, by vol.) (Di Mascio *et al.*, 1989), respectively. These values were comparable to those obtained in the present study, although a different kind of organic solvent was used. These data also showed the high potential of kinobeon A as an $^1O_2$ quencher.

Reaction of kinobeon A with $^1O_2$

The change in the concentration of kinobeon A was followed using HPLC to elucidate the mechanism by which kinobeon A eliminates $^1O_2$ (Fig. 2A). The concentration of kinobeon A (retention time: 5.3 min) decreased slightly during the incubation of 10 μM kinobeon A with 1 mM NEPO in methanol containing 10% dimethyl sulfoxide at 37°C. This decrease was slightly greater than that in the control system without NEPO. The new peak, however, did not appear on the chromatograms. The concentration of kinobeon A was also decreased by 1 mM AMVN, known as an oil-soluble radical initiator (Niki, 1990), in the same solvent at 37°C. In this case, an unknown peak (Fig. 2A; retention time: 9 min) was observed and its area slightly increased time-dependently (Fig. 2C). It might be an oxidation product of kinobeon A. The reaction between kinobeon A (10 μM) and $^1O_2$ (5 mM NEPO) was also examined spectrophotometrically. Only a micromolar amount of kinobeon A was lost and new absorbance did not appear, although 2.49 mM $^1O_2$ had formed for 60 min at 37°C (not shown).

DISCUSSION

In the present study, we showed the potential of kinobeon A as an $^1O_2$ quencher. The total amount of $^1O_2$ formed for 90 min was 64.3 μM (value obtained by calculation using eqn. 1) when 1 mM squalene was incubated with 100 μM NEPO in methanol containing 10% dimethyl sulfoxide at 37°C. On the other hand, 1.02 ± 0.28 μM (mean ±S.D., n = 9) of squalene hydroperoxide was formed in 90 min. Approximately 1.59% of the $^1O_2$ formed contributed to the oxidation of squalene. Thus, the decay of $^1O_2$ was significantly faster than the rate of oxidation product formation from squalene by $^1O_2$. It was reported that the rate constants of $^1O_2$ decay were $0.9–2.0 \times 10^5$ s^{-1} and $3.3–5.2 \times 10^4$ s^{-1} in methanol and dimethyl sulfoxide, respectively (Bellus, 1978). The k_Q of squalene was reported to be 2.66×10^6 $M^{-1}s^{-1}$ in n-butanol at 35°C (Kohno *et al.*, 1995). Since 1 mM squalene was used in the present study, the $k_Q[Q]$ was 2.66×10^3 s^{-1}. These values were consistent with the present results, although different solvents were used in each system.



Scheme 1. Proposed pathway of scavenging of singlet oxygen by kinobeon A.

The total amount of $^1O_2$ formed from 1 mM NEPO in 75 min in this experimental system was 577 μM. The loss of kinobeon A within 75 min in this system was 0.99 μM. If $^1O_2$ reacts with kinobeon A at a ratio of 1:1, 0.17% of the $^1O_2$ formed was consumed by kinobeon A. This value was extremely small in comparison with the inhibitory efficiency of kinobeon A on the $^1O_2$-induced oxidation of squalene, even if most $^1O_2$ was lost *via* decay (quenching by organic solvent). Therefore, most of the $^1O_2$ consumed by kinobeon A would be physically quenched without a chemical reaction under the present conditions.

On the other hand, a 46.1% decrease in kinobeon A was observed in methanol containing 10% dimethyl sulfoxide during the incubation of 10 μM kinobeon A with 1 mM AMVN for 75 min at 37°C. The initial rate of peroxyl radical formation from AMVN was calculated as follows; $R_i = 2ek_d[AMVN]$; the initial rate of the thermal decomposition of AMVN was estimated to be approximately linear, since AMVN has a long half-life (2.8 days, calculated from reference (Niki *et al.*, 1986)). R_i, e and k_d stand for the initial rate of peroxyl radical formation by AMVN thermolysis, the efficiency of free radical generation from AMVN and the rate constant of decomposition of AMVN, respectively. The ek_d in benzene at 37°C is 2×10^{-6} s^{-1} (Niki *et al.*, 1986). Therefore, R_i is calculated from the equation; $4 \times 10^{-6} \times [AMVN]$ (Ms^{-1}). Eighteen micromolar peroxyl radical is formed from 1 mM AMVN in benzene for 75 min at 37°C. This value would not be significantly different from that obtained in methanol containing 10% dimethyl sulfoxide. The total amount of peroxyl radicals formed in the AMVN system would be much smaller than that of $^1O_2$ formed in the NEPO system, even if the kinetic chain length was about 10. On the basis of the above result, kinobeon A can trap (peroxyl) radicals efficiently. This is consistent with the finding that kinobeon A inhibited the oxidative damage to rat liver microsomal membrane induced by Fe^{2+}-ADP/NADPH (Kanehira *et al.*, 2003). These results show that kinobeon A chemically reacts efficiently with the peroxyl radical, but only slightly with $^1O_2$.

The present study shows that kinobeon A can react chemically with $^1O_2$, but mainly quenches

$^1O_2$ *via* an energy transfer or electron transfer mechanism (physical quenching; Scheme 1). In conclusion, kinobeon A is a potent $^1O_2$ quencher. Therefore, it is expected to be a valuable reagent for $^1O_2$-related disorders, such as skin and eye disease.

Acknowledgements

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