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Kinetic Radical-scavenging Activity of Colchicine and Tropolone

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Abstract. *The kinetics of radical-scavenging activities for colchicine and tropolone remain unknown. Their antioxidant activities were determined by the induction period (IP) method in the polymerization of methyl methacrylate initiated by thermal decomposition of 2,2'-azobisisobutyronitrile (AIBN, R·) or benzoyl peroxide (BPO, PhCOO·) using differential scanning calorimetry (DSC) under nearly anaerobic conditions. The IPs for colchicine and tropolone were very short despite the addition of a high concentration of these compounds relative to initiators; the stoichiometric factor (n, the number of moles of PhCOO· trapped by the antioxidant) was approximately 0.03 and 0.04 for colchicine and tropolone, respectively. The n value of these compounds for R· was less than that for PhCOO·. The rate constant of inhibition to that of propagation (k_{inh}/k_p) for both compounds was 23-27, and the difference between them was considerably small. Both compounds had weak antioxidant properties at very high concentrations.*

Colchicine, the major alkaloid of *Colchicum autumnal*, is an ancient drug used in medicine for its antimitotic, anti-inflammatory and antineoplastic effects (1-2). It inhibits cell mitosis by binding to the protein tubulin in the mitotic spindle and preventing its polymerization into microtubules. Thus, microtubules are among the most successful targets for anticancer therapy. Colchicine contains a tropolone moiety (C ring), in addition to a trimethoxybenzene ring (A ring) and a seven-membered ring (B ring) (Figure 1). From the chemical point of view, the most intriguing portion of the molecule is the tropolone ring, on which a large number of reactions can take place, such as singlet oxygen addition (3) and photochemical rearrangement (4). This suggests the occurrence of free-radical addition to the tropolone ring found in colchicines and tropolone. The antioxidant activity of colchicines has been reported: it acted as an antioxidant and a protective agent against lipid peroxidation in rat models of liver injury (5, 6). However, the antioxidant activity implied by these studies appeared to be remarkably high when considering the microgram quantities used in the experiments and accounting for possible accumulation and bioavailability effects (7). Thus, direct evidence for the radical-scavenging activity and protective action of colchicine is lacking. Since *in vivo* experiments are too complex to be amenable to simple interpretation, colchicine and its metabolites were investigated in an *in vitro* cell culture model using human promyelocytic leukemia HL-60 cells, suggesting that colchicine metabolites with a nucleophilic hydroxy group on a carbon ring possess one of the prerequisites of antioxidants (8). However, it remains unclear from these findings whether colchicines itself, without an OH group, can prevent lipid peroxidation *via* direct antioxidant action.

Tropolone is a member of a family of naturally occurring compounds responsible for the durability of the heartwood of several cupressaceous trees. These compounds are well established, as possessing both metal-chelating and antioxidant properties (9). A study of the effects of colchicines and tropolone, both of which possess a tropolone moiety, on hydrogen peroxide-induced DNA damage in Jukat cells indicated a protective effect for the latter but not the former (9). Tropolone was reported to possess both weak antioxidant and weak radical-scavenging properties (10).

Kinetic studies of the radical-scavenging activity of these two compounds are lacking. We reported the use of differential scanning calorimetry (DSC) to evaluate the radical-scavenging activities of butylated hydroxytoluene-related quinones (11), β-carotenes (12), polyamines (13) and polyphenols (14) *via* the induction period method. The antioxidant activity of these compounds was well predicted by a model based on kinetic and thermodynamic data. Although induction period methods measuring O_2 consumption have also been used to evaluate the antioxidant activity of β-carotenes, quinones and secondary amines in the styrene-azo-initiator system (15) and the

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Key Words: Colchicine, tropolone, stoichiometric factor, inhibition rate constant, antioxidant.

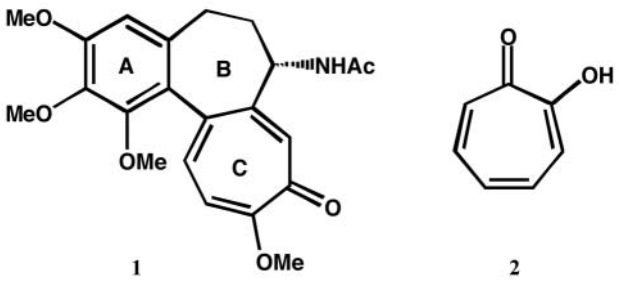

Figure 1. *Chemical structures of colchicine (**1**) and tropolone (**2**).*

aqueous linoleic acid system (16) at high oxygen tensions the values of k_{inh} were too small to be measured, and therefore these approaches appear to be unsuitable for our studies.

Consequently, to clarify whether compounds colchicine and tropolone act as antioxidants against lipid peroxidation, the induction period method used (11-14) to investigate their radical-scavenging activity in the polymerization of methyl methacrylate (MMA) initiated by AIBN or BPO under nearly anaerobic conditions.

Materials and Methods

Colchicine and tropolone (Figure 1) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). MMA (Tokyo Kasei Chem. Co., Tokyo, Japan) was purified by distillation. 2, 2'-azobisisobutyronitril (AIBN) and benzoyl peroxide (BPO) (Tokyo Kasei Chem. Co., Japan) were recrystallized from methanol and methanol/chloroform (1:1 v/v), respectively.

Induction period and initial rate of polymerization. The induction period (IP) and initial rate of polymerization in the presence (Rp_{inh}) or absence (Rp_{con}) of an antioxidant were determined using the method reported elsewhere (11-13). In brief, the experimental resin consisted of MMA and AIBN (or BPO) with or without additives. AIBN (or BPO) were added at 1.0 mol%, and the additives were used at 0, 0.1, 0.2, 0.5, 0.7 and 1.0 mol%. Approximately 10 μl of the experimental resin (MMA: 9.12-9.96 mg) were loaded into an aluminum sample container, which was sealed by applying pressure. The container was placed in a differential scanning calorimeter (model DSC 3100; MAC Science Co., Tokyo, Japan) kept at 70˚C, and the thermal changes induced by polymerization were recorded for the appropriate periods. The heat due to polymerization of MMA was 13.0 kcal/mole in this experiment. The conversion of all samples, as calculated from DSC thermograms, was 91-96%. Polymerization curves were derived from DSC thermograms using the integrated heat evoked by the polymerization of MMA. Polymerization curves break when an inhibitor is consumed (Figure 2). These breaks are sharp and provide a reliable measure of the IP of the inhibitor. The presence of oxygen retards polymerization because oxygen reacts with MMA radicals activated by the initiator and then subsequently produces a non-radical product. Thus, polymerization of the control was slightly inhibited, even though the reaction was carried out in a sealed DSC pan, because the pan contained a small amount of oxygen since it had been sealed in air. Tangents were drawn to polymerization curves at an early stage in the run. The IP of test compounds was determined from the length of time between the zero point on the abscissa and the point of intersection of tangents drawn to the early stage of polymerization. The IP was calculated from the difference between the induction period of specimens and that of controls. The initial rates of polymerization in the absence (Rp_{con}) and presence (Rp_{inh}) of colchicine and tropolone were calculated from the slope of the plots of the first linear line of the conversion rate of MMA polymerization (tangent drawn at the early polymerization stage).

Rate of initiation. The induction period method was used to determine the rate of initiation (R_i) due to the thermal decomposition of AIBN or BPO according to Eq. [**1**]:

$$R_i = n\,[IH]_0/[IP] \qquad [1]$$

where $[IH]_0$ is the concentration of the inhibitor at time zero and [IP] is the induction period. 2, 6-*tert*-butyl-4-methoxyphenol (DTBM) was used to determine R_i, since its stoichiometric factor, *n*, is known to be 2.00 (17). In the case of [MMA] = 9.4 M and [AIBN or BPO] = 0.1 M at 70˚C, the induction period method using DTBM gave the rate of initiation, Ri, at 70˚C. The R_i values of AIBN and BPO were 5.66×10^{-6} Ms^{-} and 2.28×10^{-6} Ms^{-1}, respectively.

Measurement of stoichiometric factor (n). The relative *n* value in Eq. [**2**] can be calculated from the induction period in the presence of inhibitors:

$$n = R_i[IP]/[IH] \qquad [2]$$

where [IP] is the induction period in the presence of an inhibitor. The number of moles of peroxy radicals trapped by the antioxidant is calculated with respect to 1 mole of inhibitor moiety unit.

Measurement of the inhibition rate constant (k_{inh}). When R_i is constant, *i.e.* when new chains are started at a constant rate, a steady-state treatment can be applied and the initial rate of polymerization of MMA is given by Eq. [**3**](11):

$$Rp_{con} = \{k_p\,[MMA]\,R_i^{1/2}\}/(2k_t)^{1/2} \qquad [3]$$

where MMA represents methyl methacrylate and k_p and k_t are the rate constants for chain propagation and termination, respectively. The $k_p/(2k_t)^{1/2}$ rate of polymerization of MMA (9.4 M) by AIBN (1 mol%) and BPO (1 mol%) at 70˚C was a constant value, 9.86×10^{-2} $M^{-1/2}$ $s^{-1/2}$ (13). The Rp_{inh} rates are determined by Eq. [**4**]:

$$Rp_{inh} = \{k_p\,[MMA]\,R_i\}/\{n\,k_{inh}\,[IH]\} \qquad [4]$$

in which Rp_{inh} is the initial rate of inhibited polymerization, [MMA], *n*, [IH] and k_p are defined above, and k_{inh} is the rate constant for scavenging (inhibiting) of MMA radicals by an antioxidant. From Eq. [**2**] and Eq. [**4**], k_{inh}/k_p can be calculated (Eq. [**5**]):

$$k_{inh}/k_p = [MMA]/\{[IP]x[Rp_{inh}]\} \qquad [5]$$

Computational detail. Theoretical calculations were carried out using the PM3 semiempirical MO method as implemented in the MOPAC program on a CAChe work system (Fujitsu, Ltd., Japan). The energy levels of the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO) for colchicine, tropolone and MMA, and also of the singly occupied molecular orbital (SOMO) for AIBN or BPO, were calculated. As an example, the reactivity of BPO for colchicine or MMA is shown in Figure 1.

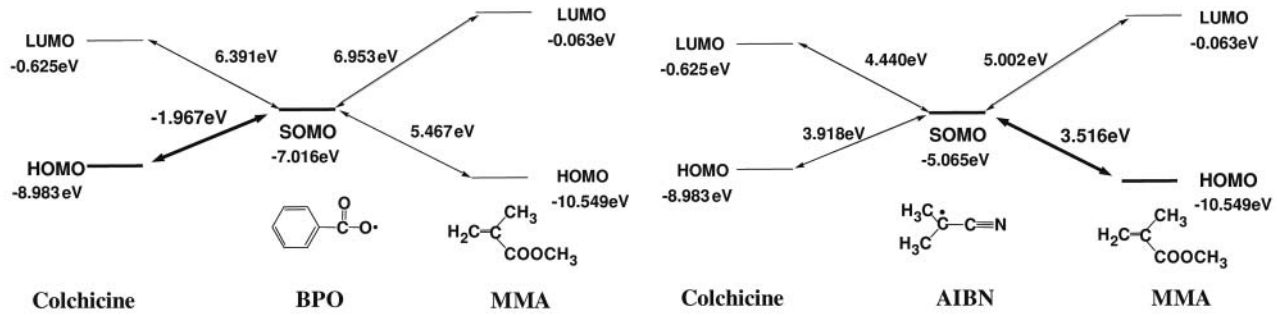

Figure 2. *The energy levels of the SOMO of BPO and of the HOMO and LUMO of colchicine.*

Results

Induction period (IP). Time-exothermic (top) and time-conversion (bottom) curves for colchicine (A) and tropolone (B) for the AIBN and BPO systems are shown in Figure 3A and B. Plots of IP *vs.* inhibitor concentration for compounds colchicine and tropolone in the AIBN-MMA and BPO-MMA systems are shown in Figures 4A and 5A, respectively. The IP for colchicine in the BPO system increased linearly in a dose-dependent manner, whereas the IP in the AIBN system increased slightly but was not remarkably different from that of the control. The Rp_{inh}/Rp_{con} value for colchicine in the BPO system decreased parabolically as the inhibitor concentration increased, whereas Rp_{inh}/Rp_{con} in the AIBN system did not change (Figure 4). Together, these findings indicate that colchicines had not effect on polymerization of MMA initiated by AIBN, but inhibited polymerization initiated by BPO. The parabolic relationship between Rp_{inh}/Rp_{con} and concentration found in the BPO system may be explained by the low solubility of colchicine in MMA, because mixtures of 0.7-1.0 mol% colchicine with MMA became cloudy. On the other hand, the IP for tropolone in both systems increased linearly as the inhibitor concentration was increased, with a steeper increase, in the BPO system compared with the AIBN system (Figure 5). The Rp_{inh}/Rp_{con} value for tropolone in both systems decreased linearly with inhibitor concentration, with a steeper decrease in the BPO system compared with the AIBN system. Therefore, tropolone was much more effective on BPO-initiated polymerization of MMA than on AIBN-initiated polymerization.

Stoichiometric factor (n) and k_{inh}/k_p. We then, examined the *n* and k_{inh}/k_p values calculated from Eq. [**2**] and Eq. [**5**], respectively. Relative *n* values and k_{inh}/k_p values for 0.2 mol% colchicine and tropolone in the BPO system were calculated, yielding values of 0.03-0.04 and 23-27, respectively, for both compounds. The small *n* values suggest that both were weak inhibitors. The *n* value for colchicine was greater than that of tropolone. The marked and dose-dependent reduction of Rp_{inh}/Rp_{con} by colchicines and tropolone indicated that these compounds acted as retarders of polymerization. The k_{inh} for colchicines and tropolone was estimated to be $9.3x10^4$ $M^{-1}s^{-1}$ –$10.9x10^4$ $M^{-1}s^{-1}$. (Note: the k_p of MMA at 70˚C, 405 $M^{-1}s^{-1}$, was extrapolated from values of 143 $M^{-1}s^{-1}$ at 30˚C and 367 $M^{-1}s^{-1}$ at 60˚C (18).

Discussion

Both colchicine and tropolone scavenged much more PhCOO· radical derived from BPO than R· radical from AIBN. The molecular orbital (MO) theory is a useful tool to predict reactivity (12), and the radical-scavenging activity of both compounds is in accordance with HOMO-SOMO considerations of their interaction with alkyl or peroxyl radicals. The energy difference between the SOMO of PhCOO· or R· and the HOMO of compounds colchicines and tropolone were calculated using the PM3 semiempirical method. A typical case is shown in Figure 1. The energy difference between the SOMO of PhCOO· and the HOMO of colchicines was 1.967 eV, whereas the difference between the SOMO of PhCOO· and the HOMO of MMA was 3.533 eV. This energy difference suggests that PhCOO· reacts preferentially with colchicine rather than with MMA. Similarly, in the AIBN system, R. reacts with colchicine, but the energy difference (3.918 eV) between the SOMO of R· (5.065 eV) and the HOMO of colchicine is greater than that (1.967 eV) between the SOMO of PhCOO· and HOMO of colchicine in the BPO system described above. The difference in transition energies between the radical systems suggests the preferential reaction of colchicine with PhCOO· from the BPO system, which is supported by observations. The transition energy for tropolone was calculated. The energy difference between the SOMO of PhCOO· and the HOMO of tropolone was 1.988 eV, and this value is smaller than the energy difference between the SOMO of PhCOO· and the HOMO of MMA (3.533 eV). Similarly, the energy difference between the SOMO of R· and the HOMO of tropolone was

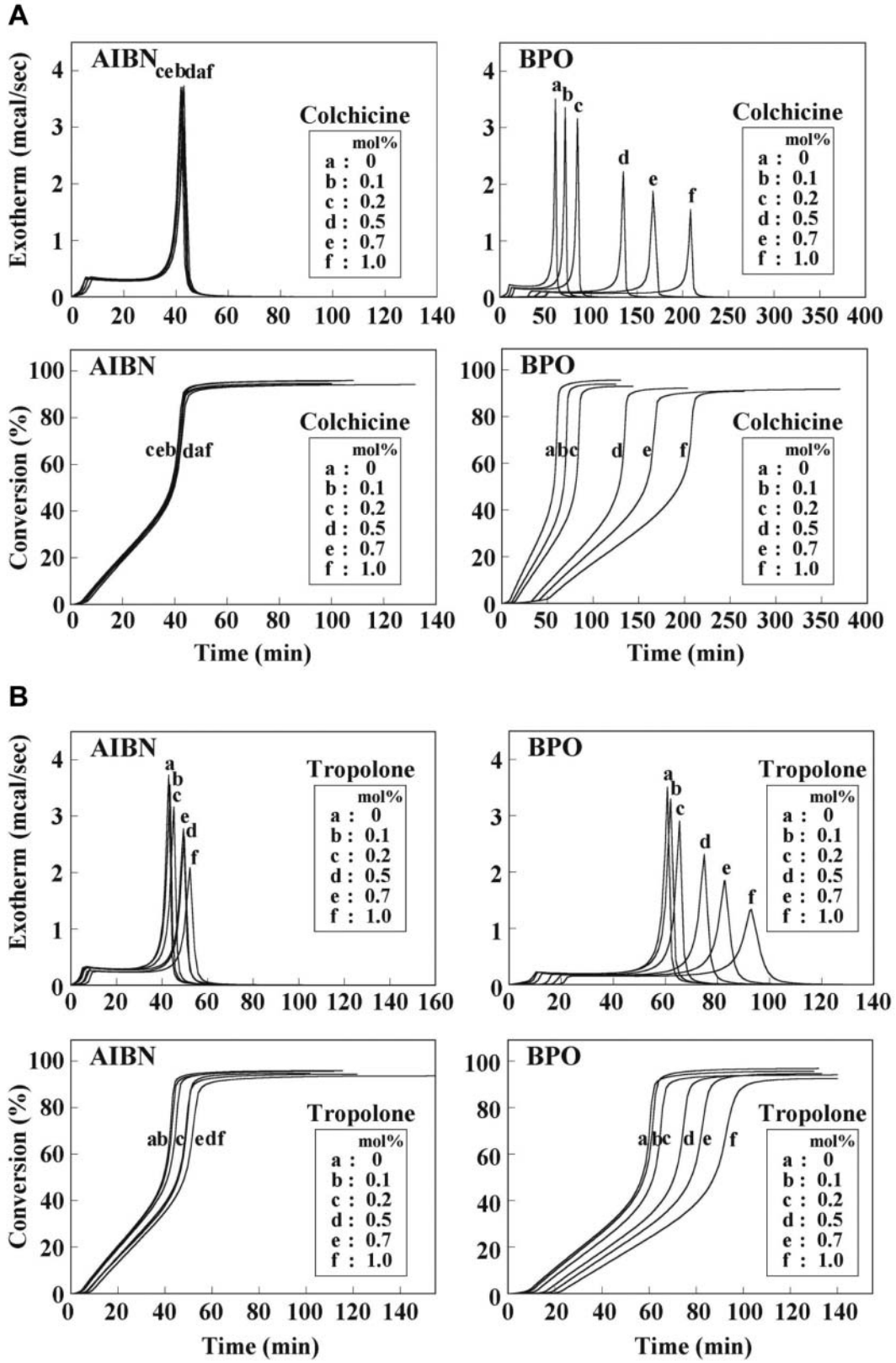

Figure 3. *Time-exotherm (top) and time-conversion curves (bottom) for AIBN and BPO. A) colchocine, B) tropolone. The procedures are described in the Materials and Methods.*

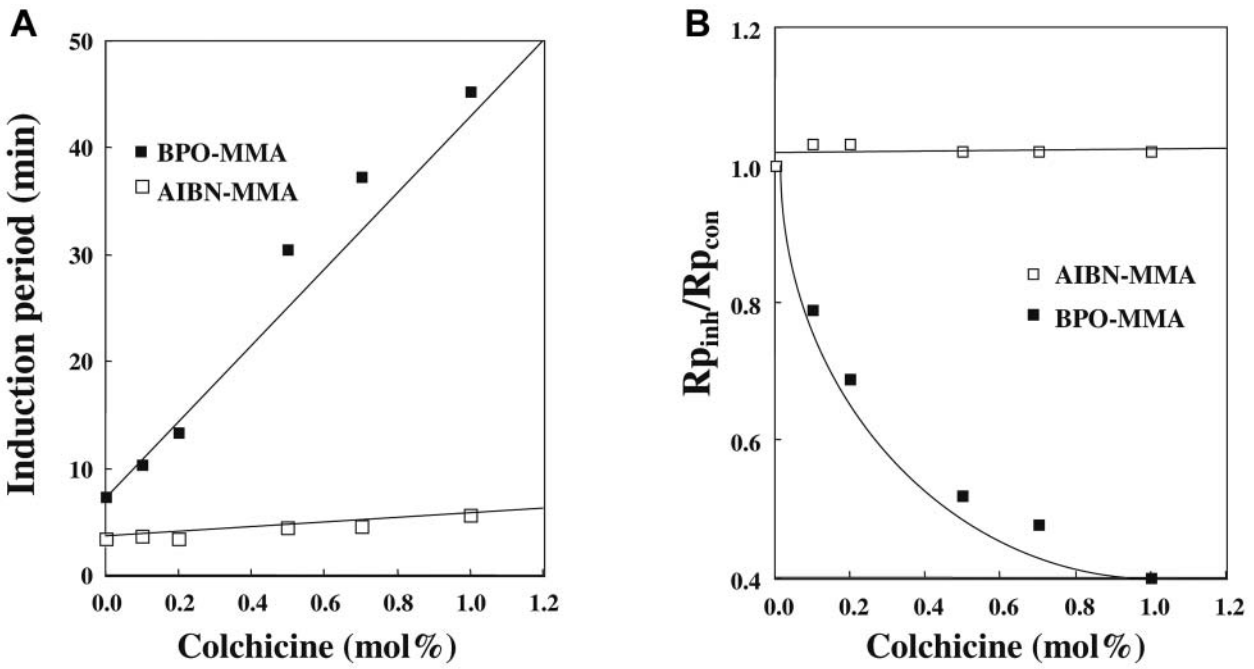

Figure 4. *Relationships between the induction period (A) or Rp_{inh}/Rp_{con} (B) and concentrations of colchicine.*

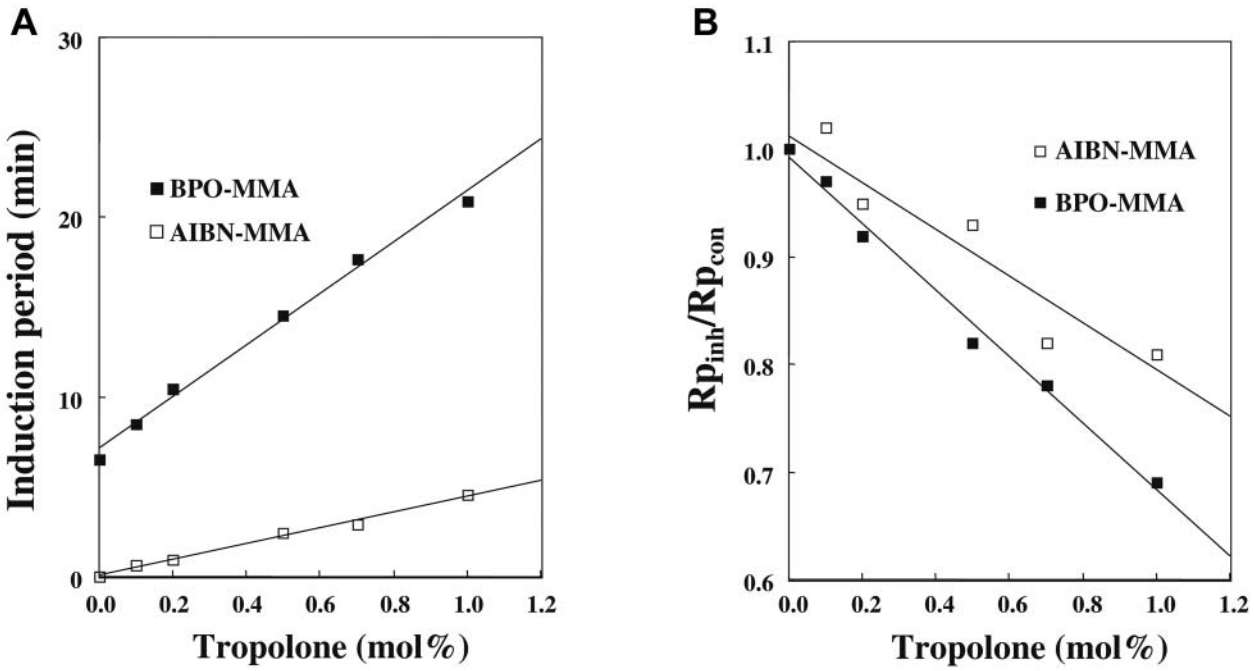

Figure 5. *Relationships between the induction period (A) or Rp_{inh}/Rp_{con} (B) and concentrations of tropolone.*

3.949 eV, which is also smaller than the energy difference between the SOMO of R· and the HOMO of MMA (5.484 eV). The difference in transition energy between the radical systems suggests that PhCOO· addition to tropolone was the preferred reaction, which is supported by our observations.

Comparing the k_{inh}/k_p of colchicine with that of tropolone in both systems, the two compounds showed similar activity. This suggests that the radical susceptible moiety in the tropolone ring (C ring). Singlet oxygen addition to colchicine was previously reported to give a different modification of

the C ring (3). Colchicine was oxidized by BPO for 7 h in ethyl acetate on refluxing at 70˚C. The reaction products found a complex mixture (about 85% of starting materials were recovered), which were separated into several fractions on a silica gel chromatography. The pmr spectra of the aromatic protons of these fractions were observed at around 7.5-8.2 ppm, which were 1-1.8 ppm downfield from the tropolone ring in colchicines (6.9-7.4 ppm). Hence the reaction might proceed with the ring contraction of the seven-member tropolone ring to benzene ring.

In the present study, colchicine was poor radical scavenger in the system initiated by AIBN. It was found to exert no antioxidant activity against phospholipid peroxidation induced by azo-initiators in the presence of molecular oxygen in HL-60 (human promyelocytic leukemia) cells (8). Tropolone, but not colchicine, was an effective initiator of hydrogen peroxide-induced DNA damage and apoptosis in Jurkat cells (9). In a model system of oxidation of methyl linoleate (ML) in the presence of an azo-initiator, ML oxidation as measured by oxygen was only weakly inhibited by tropolone (10). Tropolone was also a poor radical scavenger against 2, 2-diphenyl-1-picrylhydrazyl free radical (DPPH·) (10). Taken together, these findings suggests that the radical-scavenging activity of colchicine and tropolone may be dependent on the radical species; colchicine is clearly insensitive to R·. In the present study, colchicine and tropolone showed relatively large k_{inh}/k_p values, and the calculated kinh of about $1x10^5$ $M^{-1}s^{-1}$ is of a similar magnitude to that of β-carotene, a polyene (12). The IP for β-carotene was very short despite the addition of a high concentration of this compound relative to the initiators, and *n* was near to 0 (12). The antioxidant properties of colchicine and tropolone were similar to that of β-carotene with conjugated double bonds. For radical addition to polyenes such as β-carotene, radicals produced in the allyl group have a well-known degradation chain transfer action (12, 15, 19). However, both colchicine and tropolone showed about ten-fold lower *n* values than those of β-carotene. The radical-scavenging mechanism of colchicine and tropolone still remain unknown. Both possess weak antioxidant properties at very high concentrations.

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