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Cytotoxicity, ROS-generation Activity and Radical-scavenging Activity of Curcumin and Related Compounds

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Abstract. *The cytotoxicity, ROS (reactive oxygen species)-generation activity and radical-scavenging activity of curcumin and related compounds such as eugenol, eugenol orthodimer (bis-eugenol; 3,3'-dimethoxy-5,5'-di-2-propenyl-1,1'-biphenyl-2,2'-diol) and isoeugenol were investigated. Their cytotoxicity against a human submandibular gland adenocarcinoma cell line (HSG) declined in the order curcumin > isoeugenol > bis-eugenol > eugenol. Since the hydrophobicity (log P) of curcumin, isoeugenol and eugenol is about 2.5, whereas that of bis-eugenol is 4.8, there was no relationship between cytotoxicity and log P. Generation of intracellular ROS in HSG cells was observed for curcumin alone in an assay using 5-(and -6)-carboxy-2',7'-dichlorofluorescein diacetate (CDFH-DA). The cytotoxicity of, and ROS generation by, curcumin were reduced by the addition of N-acetyl-L-cysteine (NAC) and glutathione, suggesting a possible link between cytotoxicity and ROS. The radical-scavenging (antioxidant) activity of curcumin and related compounds was determined quantitatively by the induction period method for polymerization of methyl methacrylate (MMA) initiated by peroxy radicals derived from benzoyl peroxide (BPO) under nearly anaerobic conditions. The length of the induction (inhibition) period for curcumin was significantly greater than that of the other compounds. This suggests that curcumin is an efficient scavenger of peroxy radicals. The curcumin radical possibly reacts with itself or with other radicals to yield polymeric stable products such as curcumin dimer. Such polyphenolic behavior of curcumin was considerably different from that of bis-eugenol, which, like curcumin, has two hydroxy groups, or of other compounds with one hydroxy group. The radical-scavenging activity was also investigated with 2,2-diphenyl-1-picrylhydrazyl (DPPH). Curcumin scavenged approximately one DPPH free radical, suggesting the formation of curcumin dimer. The possible formation of curcumin dimer was explored with a PM3 semiempirical molecular orbital method. A molecular mechanism of cancer prevention by curcumin is proposed, based on its high reactivity with peroxy radicals at low oxygen pressure and on ROS generation induced by curcumin radicals.*

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O-Methoxyphenols such as curcumin (1,7-bis-(4-hydroxy-3-methoxyphenol)-1,6-heptadiene-3,5-dione), eugenol (2-methoxy-4-allylphenol) and isoeugenol (4-propenyl-2-methoxyphenol) are components of flavors and are used in the food industry. These compounds, particularly curcumin, are of interest because of their anti-inflammatory and chemopreventive activities (1).

We have previously reported the radical-production and radical-scavenging activities, as well as the cytotoxicity, of o-methoxyphenols, demonstrating that these compounds act not only as antioxidants but also as prooxidants (2). Thus, the biological activities of these compounds are multiple rather than simple. When eugenol or isoeugenol are oxidized, these compounds can produce dimers as a result of the *ortho*-coupling reaction or other reactions (3). In general, *ortho*-substitution with an electron donor, such as the methoxy groups of curcumin or eugenol, increases the antioxidant activity of phenols by enhancing the stability of the phenoxyl radical by an inductive effect. Through an antioxidant process, *o*-methoxyphenols such as curcumin are oxidized and consequently become semiquinone radicals. In particular, such semiquinone radicals become long-lived stable radicals at low oxygen pressure. The oxygen tension under a 15 torr oxygen atmosphere is similar to that in many tissues (4), suggesting that oxygen in living cells is sparse. Previous quantitative *in vitro* studies of radical-scavenging activity were carried out under aerobic conditions (5-7) and we believe that the efficiency of antioxidants may be

considerably different under anaerobic conditions. Thus, we have previously used induction methods to investigate the radical-scavenging efficiency of polyphenols under nearly anaerobic conditions, and this method proved to be reliable in evaluating the radical-scavenging activity of natural and synthesized antioxidants (8, 9).

In the present study, the cytotoxicity of, and ROS generation by, curcumin, eugenol, isoeugenol and bis-eugenol were investigated in a human submandibular gland adenocarcinoma cell line (HSG). We also investigated the radical-scavenging activity of these compounds by two methods: the induction period method for polymerization of MMA initiated by BPO and a DPPH method. The radical-scavenging (antioxidant) activity of the *o*-methoxyphenols was evaluated from the length of the induction period and from the inhibition rate constant. Cytotoxicity, ROS generation and reactivity with peroxy radicals are discussed from an SAR (structure-activity relationship) perspective, with calculations by a PM3 semiempirical molecular orbital method.

Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies. Curcumin, eugenol and isoeugenol were used without further purification (Tokyo Kasei Chemical Co., Tokyo, Japan). Bis-eugenol was synthesized as previously described (10). MMA (Tokyo Kasei Chemical Co.) was purified by distillation. BPO (Tokyo Kasei Chemical Co.) was recrystallized from methanol/chloroform (1:1 v/v).

Cells and cell culture. HSG cells (supplied by Prof. Satoh, Tokushima University, Japan) were grown in MEM supplemented with 10% NBCS, detached from the culture dishes with 0.01% trypsin-0.04 M EDTA and subcultured once a week at a split ratio of 1:20 in a humidified 5% CO_2 atmosphere.

Cytotoxicity. HSG cells were seeded in 96-microwell plates at a density of 5 x 10^3 cells/well in 0.1 ml of MEM or α-MEM with 10% FBS and were cultured at 37˚C for two days. Before the addition of test materials, the cells were washed twice with serum-free medium. A stock solution of 100 mM test compound was prepared in DMSO and was diluted with DMSO. The test compounds were added to the wells at a 1/100 volume. The curcumin-related compounds were incubated with the cells at 37˚C for 24 hours. DMSO (1%)-treated cells were used as the control. After each well had been washed with fresh medium, 25 μl of Cell Titer 96 Aqueous One Solution was added to each well, and the cells were incubated for a further 3-6 hours; then, the absorbance was measured at 492 nm with a microplate reader (Biochromatic, Helsinki, Finland). The relative viable cell number was expressed as the percentage of the number of the experimental wells to that of the control (without compound) wells. Values were expressed as means±SD (n=8). Statistical analyses were performed by Student's *t*-test. The 50% cytotoxic concentration (CC_{50}) was determined from the dose-response curve in the concentration range 0-1 mM (*i.e.* 0, 0.01, 0.1 and 1 mM).

Detection of ROS production. The method for detection of intracellular ROS production with 5- (and -6)-carboxy-2',7'-dichlorofluorescein diacetate (CDFH-DA) was modified from that given in our previous report (8, 9). HSG cells were seeded at 1-2 x 10^5 cells per well and were cultured for 2 days to reach semiconfluence. The cells were then washed twice with HBS (145 mM NaCl, 5 mM KCl, 1mM $CaCl_2$, 0.5 mM $MgSO_4$, 5 mM glucose, 10mM Hepes; pH 7.4) 60 min before the assay. Each test compound was added to the well at a concentration of 10 μM to 1 mM and incubated for 30 min, and then DCFH-DA (10 μM) was added to the well and incubated for a further 30 min. The cells were detached with trypsin solution and washed with HBS. The cells (2-4 x 10^5) were suspended in 0.5 mM, HBS and the size (forward scatter), granularity (side scatter) and green fluorescence intensity at 520-530 nm of 10,000 individual cells were measured with a FACScan flow cytometer (EPICS ALTRA; Beckman Coulter, Miami, FL, USA).

DPPH radical-scavenging activity. The indicated compounds were diluted with ethanol at eight arbitrary concentrations. A portion (100 μl) of each test solution and 100 μl of 0.2 mM DPPH in ethanol solution were transferred to 96-well plates. After incubation for 5 min in the dark, the reduction of DPPH in test solution was determined at 540 nm in a microplate reader (Biochromatic, Helsinki, Finland). The 50% concentration (EC_{50}) of reduction was calculated from the DPPH reduction curves.

Determination of induction period (T_{inh}). The T_{inh} was determined by the method previously reported (9). In brief, the experimental resin consisted of MMA and BPO or AIBN with or without o-methoxyphenols, *i.e.* inhibitors. The concentration of BPO was 1 mol% and that of the inhibitors was 0.001-0.5 mol%. About 10 μl of the experimental resin (MMA) was loaded into an aluminum sample container and sealed by applying pressure. The container was placed in a differential scanning calorimeter (model DSC 3100; MAC Science Co., Tokyo, Japan) maintained at 70˚C. Thermal changes induced by polymerization of MMA were 13.0 kcal/mole in this experiment. Polymerization curves were derived from DSC thermograms using the integrated heat evoked by polymerization of MMA. The time-conversion curves in the absence (control) and the presence of the inhibitors (curcumin-related compounds), showed a break when the additives had been completely consumed (see Figure 2). These breaks were sharp and provided a reliable measure of the induction (inhibition) period and initial rate of polymerization. Such a break was detected in the absence of the inhibitor (control) because oxygen acts as an inhibitor. In the present study, the induction period for test compounds is presented as the difference from controls. Tangents were drawn to polymerization curves at an early stage in the run. The T_{inh} of test compounds was determined from the length of time between the zero point on the abscissa and the point of the intersection of tangents drawn to the early stage of polymerization. T_{inh} values were calculated from the difference in the induction periods between the test compounds and controls. The n values were calculated per unit of phenolic moiety by using Eq. (1):

$$T_{inh} = (n / R_i)[IH] \quad \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

in which T_{inh}, Ri and n represent the induction period, the rate of initiation (BPO=2.28 x 10^6 mole/(l x s) at 70˚C) and the stoichiometric factor (number of free radicals trapped by 1 mol of phenol), respectively. IH represents an inhibitor, *i.e.* o-methoxyphenol.

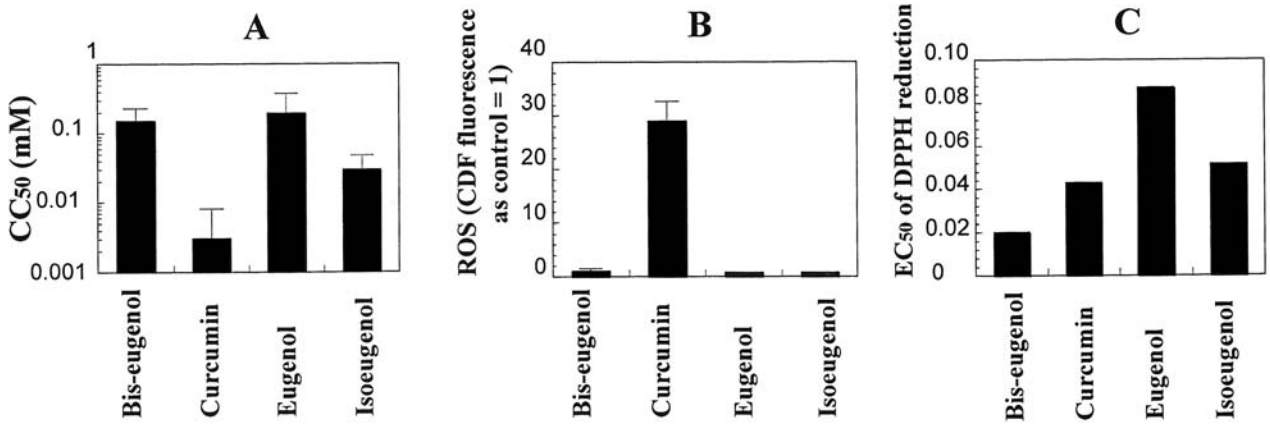
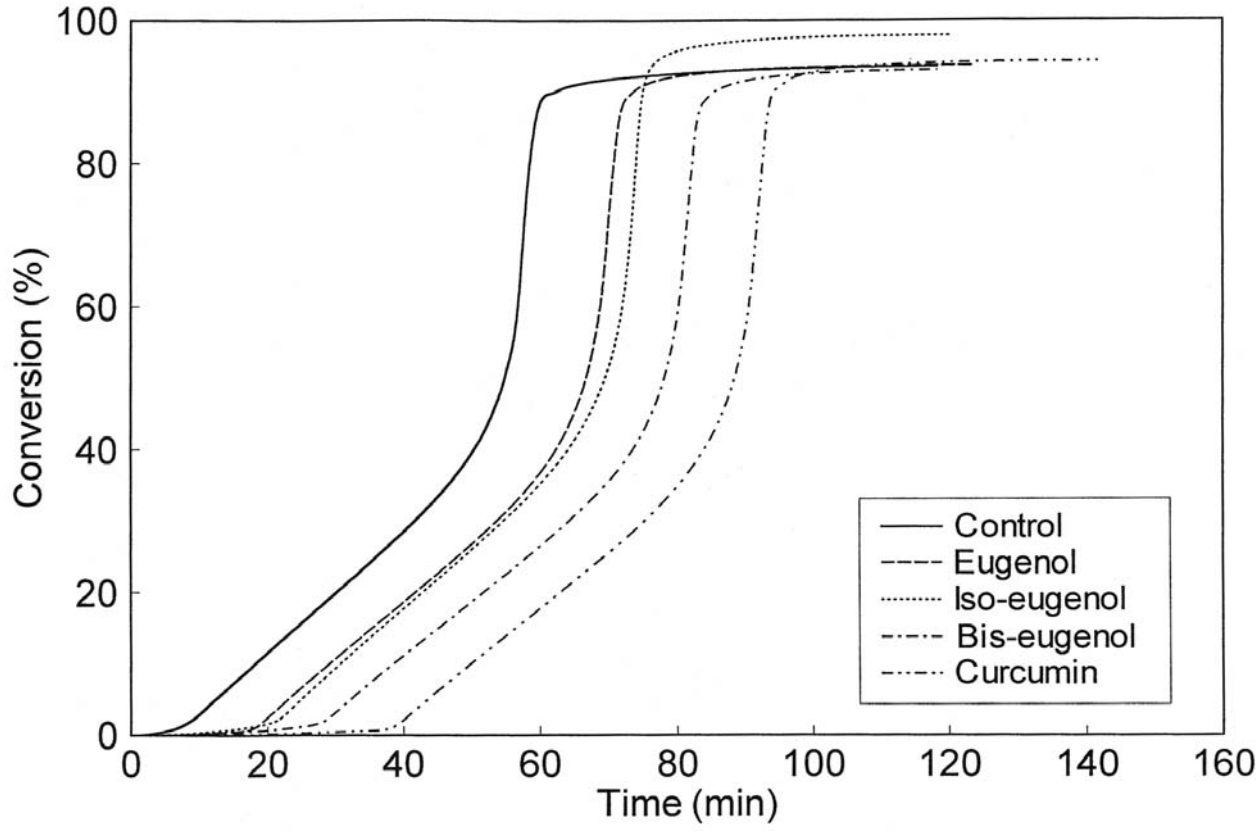


Figure 1. *The 50% cytotoxic concentration (CC_{50}) of curcumin-related compounds against HSG cells (A), the generation of ROS (B) and the 50% reduction concentration of DPPH (EC_{50}) (C). The bar (A, B) was the mean±SD of 8 different experiments. The significant difference between curcumin and bis-eugenol, eugenol or isoeugenol was found ($p<0.001$). The bar (C) was the mean of two separate experiments. For details, see Materials and Methods.*



Time-conversion curves for MMA-BPO (1mol%) polymerization at 70ÆC in the presence of 0.01 mol% eugenol derivatives.

Figure 2. *Time-conversion curves for polymerization of MMA initiated by BPO at 70°C. The method is described in the text. BPO, 0.1 mol/l; MMA, 9.4 mol/l.*

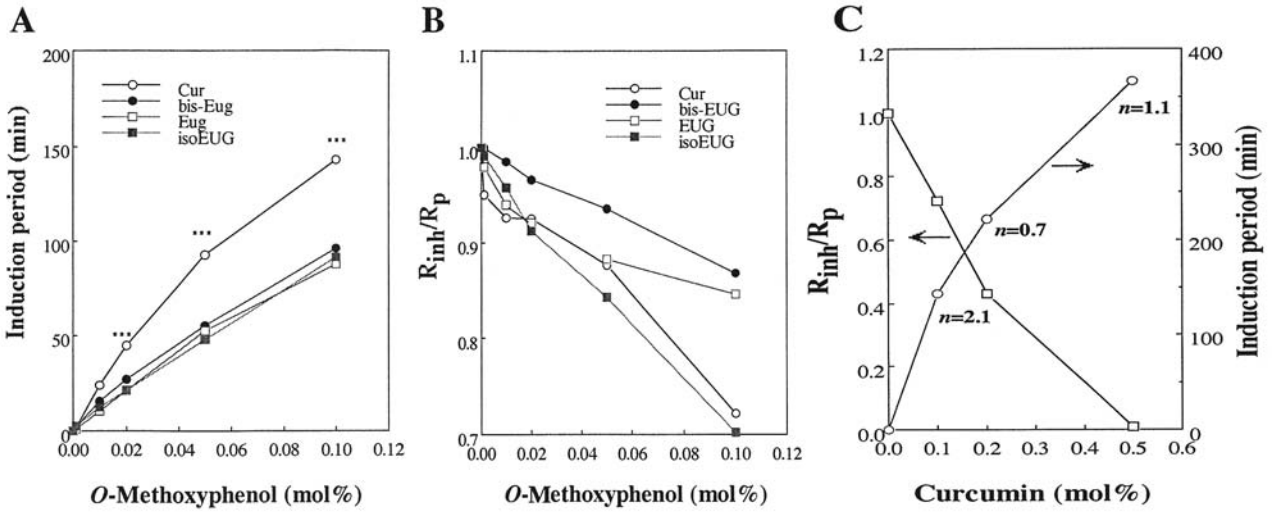


Figure 3. *Relationship between concentrations of o-methoxyphenols (curcumin-related compounds) and the induction period (A) or Ri_{nh}/R_p (B), and that between the induction period or n and the R_{inh}/R_p of curcumin (C). R_{inh} and R_p represent the initial rate of polymerization with an inhibitor and without an inhibitor (control), and n is the stoichiometric factor calculated from Eq. (1) in the text. The values were the mean of three different experiments. ***p<0.001.*

Initial rate of polymerization in the absence (R_p) or presence (R_{inh}) of inhibitor. The initial rate of polymerization inhibited by *o*-methoxyphenols was calculated from the slope of the first linear line of the conversion rate of polymerization of MMA (see Figure 2). The rate constant for inhibition (k_{inh}) was determined by the equation previously reported (5-7,9):

$$R_{inh}/R_p = \{(2kt\ Ri)^{1/2}\}/\{nk_{inh}\ [IH]\} \quad \ldots\ldots\ldots\ldots\ldots\ldots(2)$$

where k_t represents the rate of termination. k_t was estimated as $3.7 \times 10^7\ M^{-1}s^{-1}$ at 70°C (9).

Computational details. Theoretical calculations were performed by the PM3 semiempirical MO method as implemented in the MOPAC program on a Tektronix CAChe work system (Version 3.8). The hydrophobicity (log P) and the bond-dissociation enthalpy of phenolic O-H (ΔH) were obtained with COSMO (conductor-like-screening model).

Results

Cytotoxicity. The 50% cytotoxic concentrations of curcumin, bis-eugenol, eugenol and isoeugenol against HSG cells are shown in Figure 1A. The cytotoxicity against HSG cells declined in the order curcumin > iso-eugenol > bis-eugenol > eugenol. The octanol-water coefficient (log P) is also an important factor for cytotoxicity (11). The log P for curcumin, eugenol and isoeugenol is approximately the same, about 2.5, but curcumin was the most cytotoxic of these three compounds. Bis-eugenol (log P=4.8) was less cytotoxic.

ROS production. ROS production by *o*-methoxyphenol-treated HSG cells is shown in Figure 1B. Curcumin alone produced ROS. ROS production by curcumin was significantly reduced by the addition of NAC or glutathione (data not shown).

DPPH radical-scavenging activity. The stoichiometry was obtained from the EC_{50} of each methoxyphenol (Figure 1C). The stoichiometric factor (*n*) for curcumin and isoeugenol was about 1, suggesting that dimerization may occur between curcumin radicals or between isoeugenol radicals. The *n* for eugenol was about 0.5, also suggesting the formation of dimers. On the other hand, the *n* for bis-eugenol was close to 2. Dimerization of eugenol and isoeugenol was previously reported by a DPPH method (12).

Induction period. A typical time-conversion curve for curcumin-related compounds is shown in Figure 2. Curcumin showed the longest induction period. The induction periods for eugenol, bis-eugenol and isoeugenol were identical. The conversion for all compounds tested was about 95%. Curcumin and related compounds intercepted radicals, demonstrating that these compounds are chain-breaking antioxidants. The PM3 calculation based on the interaction between SOMO (Singly Occupied Molecular Orbital) and HOMO (Highest Occupied Molecular Orbital) or LUMO (Lowest Unoccupied Molecular Orbital) suggested that peroxy radicals derived

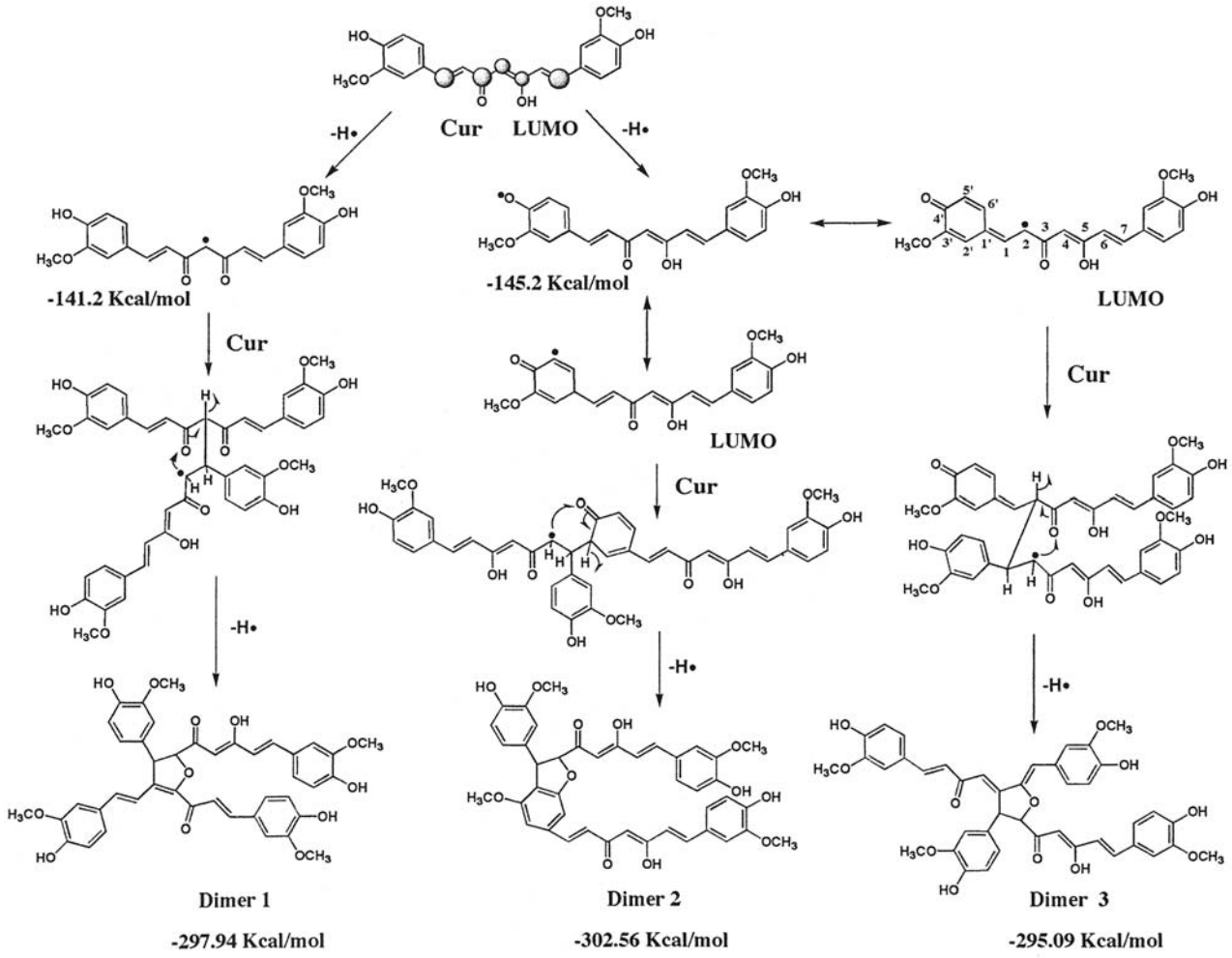


Figure 4. *Possible mechanism of formation of curcumin dimers by a PM3 method. Cur, curcumin; unit (kcal/mol), heat formation energy; LUMO, Lowest Unoccupied Molecular Orbital. Dark filled circles on the enol form of the β-diketone of curcumin represent the LUMO density of curcumin. The PM3 calculation for oxidized curcumins led preferably to dimer 3.*

from BPO react preferentially with curcumin and that polymerization of MMA will start when curcumin, an inhibitor, is completely consumed. Figure 3A shows the plots of induction period *vs* concentration. The induction period was enhanced in a dose-dependent manner. At a concentration of 0.1 mol%, the relative *n* value of curcumin was greater than 2.0, whereas that of eugenol and isoeugenol was about 1.5. The *n* value of bis-eugenol was 1.7. At higher concentrations of curcumin, the *n* values became about 1, possibly reflecting production of a large quantity of dimers in addition to curcumin-BPO radical coupling products (Figure 3C).

R_{inh}/R_p. Next, we examined the R_{inh}/R_p (Figure 2B). The curves for curcumin and isoeugenol were steeper than those for bis-eugenol. The R_{inh}/R_p for curcumin was studied at higher concentrations. The R_{inh}/R_p reached zero at 0.5 mol% and was in inverse proportion to the induction period. The k_{inh} for curcumin-related compounds at 0.10 mol% (0.01M) was calculated from Eq. (2), showing that the rates for curcumin, isoeugenol, eugenol and bis-eugenol were 0.85×10^3 $M^{-1}s^{-1}$, 1.37×10^3 $M^{-1}s^{-1}$, 1.25×10^3 $M^{-1}s^{-1}$ and 1.05×10^3 $M^{-1}s^{-1}$, respectively. The rate of superoxide radical scavenging by hesperatin, a polyphenol with methoxy groups, is $k=5.9 \times 10^3$ $M^{-1}s^{-1}$ (13). Despite the differences in the experimental systems, the rates in the present study were of similar magnitude. Curcumin-related compounds intercepted growing MMA radicals, resulting in the decrease in R_{inh}/R_p.

Discussion

Curcumin efficiently generated ROS and was the most cytotoxic compound. Recently, we found that curcumin-induced ROS generation and cytotoxicity against HSG cells was highly enhanced by visible-light (VL) irradiation (297, 400 LUX) at 400-700 nm (λ=470 nm) from a dental lamp used for curing resin restoration materials (data not shown). Since curcumin has a λ_{max} of approximately 440 nm, curcumin could be excited by VL-irradiation with a dental lamp. This suggested that curcumin could produce ROS by oxidation in the oral environment. VL-irradiated curcumin is biocidal against bacteria (14). The hyperproduction of ROS in curcumin-treated AK-5 cells has recently been reported (15), and redox signalling and caspase activation were suggested as the mechanisms responsible for the induction of curcumin-mediated apoptosis. Curcumin-induced ROS generation and induction of apoptosis is a well-studied phenomenon (16, 17). ROS are mainly generated at mitochondrial electron transport chains during normal cellular metabolism. In addition, various stimuli, including TNFα, Fas ligand and growth factors, rapidly provoke ROS accumulation in the target cells (18). Hansh *et al.* previously reported that induction of apoptosis by phenolic compounds was related to the hydrophobicity (octanol-water partition constant, log P) and the bond-dissociation enthalpy of the phenolic O-H (ΔH) (11), and remarked that the ΔH dependency is strong evidence for a radical reaction as the cause of the toxic effects. They assumed that the phenols are first oxidized by metabolically formed ROS to radicals that then attack DNA (12). The ΔH (kcal/mol) for curcumin-related compounds was calculated by a PM3 method, showing that the values for bis-eugenol, curcumin, eugenol and isoeugenol are 81.16, 80.41, 79.25 and 76.40, respectively. However, ROS generation by curcumin could not be explained by its value of ΔH alone, since the ΔH of butylated hydroxyanisole, a well-known apoptosis inducer, is 82.19 kcal/mol (19). In contrast, isoeugenol, with the smallest ΔH, possesses potent oxidative activity. Isoeugenol generates benzyl radicals, but not phenoxyl radicals (2). In the present study, isoeugenol was cytotoxic, possibly due to formation of benzyl radicals.

The radical-scavenging activity of curcumin has previously been studied by Hotta *et al.* using electrochemical methods (flow column electrolysis and cyclic methods) (20), indicating that the number of –OH moieties for curcumin appeared to be higher than 2 during its oxidation. This suggested that curcumin undergoes chemical reactions during its oxidation, possibly involving the formation of polymeric products. Toniolo *et al.* previously investigated the quenching of superoxide ions by curcumin in acetonitrile solvent, indicating that curcumin is able to react with 6 mol of such anion radicals (21). In addition, Masuda *et al.* previously reported that a relatively high concentration of curcumin produces dimers of curcumin as radical termination products in addition to the coupling products with curcumin and lipid peroxide, demonstrating radical-radical termination at the 2-position of curcumin (22). The curcumin radical could react with itself or with other radicals to yield stable polymeric products such as vanillin, ferulic acid and curcumin dimer (23). In the present study, the relative *n* values of curcumin were about 1 at higher concentrations, suggesting dimerization of curcumin. Thus, we used a PM3 method to re-evaluate the mechanism of formation of curcumin dimer previously proposed by Masuda *et al.* (22). As shown in Figure 4, curcumin can produce dimers. Such dimerization probably occurs in the presence of a large quantity of curcumin at low oxygen pressure. On the other hand, curcumin preferentially reacts with linoleate peroxy radicals produced from ethyl linoleate (24). Such alkyl peroxy radicals react readily with curcumin (25). Taking these findings together with those in the present study, we concluded that curcumin exhibits high reactivity with peroxy radicals. This suggests a strong interaction between curcumins and the unsaturated phospholipids in biological systems. Cancer chemoprevention by curcumin may be associated with the efficient reactivity of curcumin with peroxy radicals and, consequently, generation of ROS induced by curcumin radicals. We recently studied the anti-inflammatory activities of eugenol-related compounds, demonstrating their potent anti-inflammatory properties (26). A possible link between anti-inflammatory activity and the induction of apoptosis by anticancer drugs has been suggested from an SAR perspective. Further studies are required to define the sequence of the biological events from radical generation to cytotoxicity, apoptosis induction and/or anti-inflammatory activity.

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