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Review

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Dimerization, ROS formation, and biological activity of *o*-methoxyphenols

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Summary

o-Methoxyphenols are antioxidants widely used in the cosmetic and food industries. Dimers from **1**, **2**, or **3** were synthesized and their radical-scavenging and biological activities were compared with those of the original or other phenols. Radical-scavenging was evaluated from a kinetic induction period method (IPM). To simulate biomimetic thiol-cooxidation with antioxidants, the behavior of mixtures of **1**, **2**, **4**, or catechin with mercaptomethylimidazole (MMI), a thiol was investigated using IPM. Polyphenols **4** and catechin was accompanied by extensive oxygen uptake, suggesting the formation of thiyl radicals from MMI and their reaction with molecular oxygen. In contrast, **1** markedly enhanced radical-scavenging without oxygen uptake, probably because of the formation of EUGQM/MMI-conjugates. **2** showed relatively small oxygen uptake, probably resulting from the predominant formation of benzyl radicals. Intracellular reactive oxygen species (ROS) in cancer cells by **4**, but not by compounds **1**, **2**, **6**, **7**, **8**, **9**, and **10** was found, suggesting a possible link between physicochemical oxygen-uptake and intracellular ROS. The induction of apoptosis by **4** in HL-60 cells was accompanied by intracellular ROS. Dimers **6** and **7** inhibited nuclear factor (NF)-κB activation stimulated by lipopolysaccharide (LPS) in RAW 264.7 cells. Also, **6**, **7**, and **9** inhibited LPS-induced cyclooxygenase-2 expression in RAW 264.7 cells in a dose-dependent manner, whereas **1**, **2** and **3** did not. Dimerization of *o*-methoxyphenols may be a useful tool for the design of drugs to act as potent chemopreventive and anticancer agents.

Key words: ***o*-methoxyphenols • dimerization • stoichiometric factors • ROS formation • cooxidation of thiol • apoptosis • NF-κB • Cox-2**

Abbreviations: **AIBN** – 2,2'-azobisisobutyronitrile, **BDE** – bond dissociation energies, **BPO** – benzoic acid, **CDF-DA** – 5-(and-6)-carboxy-2',7'-dichlorofluorescin diacetate, **Cox-2** – cyclooxygenase-2, **DSC** – differential scanning calorimetry, **ESR** – electron spin resonance, **EUGQM** – eugenol quinone methide, **GSH** – glutathione, **HGF** – human gingival fibroblast, **HL** – 60 cells-human promyelocytic leukemia cell line, **HSG** – human submandibular gland carcinoma cell line, **IFN** – interferon, **iNOS** – inducible nitric oxide synthase, **IP** – induction period, **IPM** – induction period method, **log P** – octanol-water partition coefficient, **LPS** – lipopolysaccharide, **MMA** – methyl methacrylate, **MMI** – mercaptomethylimidazole, ***n*** – stoichiometric factor, **NAC** – *N*-acethyl-*L*-cysteine, **NF** – nuclear factor, **NO** – nitric oxide, **ROS** – reactive oxygen species.

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INTRODUCTION

o-Methoxyphenols, such as eugenol (2-methoxy-4--allylphenol) (**1**), isoeugenol (4-propenyl-2-methoxyphenol) (**2**), ferulic acid (4-hydroxy-3-methoxycinnamic acid) (**3**), and curcumin (1,7-bis-(4-hydroxy-3--methoxyphenol)-1,6-heptadiene-3,5-dione) (**4**), are components of flavors and are used in the food industry. These compounds are of interest because of their anti-inflammatory and chemopreventive properties, which result from their antioxidant activity[1, 44]. The term "antioxidant" has been used in a wide sense to mean mechanisms preventing the formation of reactive oxygen species (ROS), or in a narrow sense to mean mechanisms eliminating previously formed ROS by a chain-breaking action[9]. *o*-Methoxyphenols are prototypic chain-breaking antioxidants. Their chain-breaking action occurs during the autoxidation of polyunsaturated fatty acids in biological systems. Thus, the beneficial or adverse effects of *o*-methoxyphenols are initiated by abstraction of the hydrogen atom from the hydroxy group of the phenol. In eugenol (**1**), as a typical *o*-methoxyphenol, the free radicals formed react via their mesomeric forms, primarily in the *ortho* position, and then oxidative coupling of the phenols leads to dimerization[8, 13, 25, 48]. Oxidation of the phenolic compounds could give rise to similar radical species, semiquinone radicals, and the subsequent formation of EUGQM (2-methoxy-4-allylidene-2,5-cyclohexadien-1-one) (**5**) which could react with bioactive substances such as amino acids, peptides, proteins, or lipids either directly or via hydrogen abstraction from thiol groups followed by reaction of the thiyl radical with a second phenolic radical[68, 69]. In contrast, isoeugenol (**2**) exhibits both phenolic and benzyl coupling[5, 8]. *In vivo*, the formation of benzyl radicals would be predominant and they would undergo the same sort of reactions with bioactive substances as does eugenol. Thus, dimerization plays a crucial role in the biological activities of *o*-methoxyphenols *in vivo*.

Dimerization of *o*-methoxyphenols can reduce their prooxidant activity, and the biological activities of these compounds may be enhanced by increasing their antioxidant activity. Thus, we synthesized dimers from the corresponding monomers: *bis*-EUG (3,3'-dimethoxy-5,5'-di-2-propenyl-1,1'-biphenyl-2,2'--diol) (**6**) from eugenol, dehydrodiisoeugenol (2-(3-methoxy-4-hydroxyphenyl)-3-methyl-5-(1-propenyl)-7-methoxy-2,3-dihydrobenzofuran) (**7**) from isoeugenol, α-diisoeugenol (*R*-1-ethyl-5-hydroxy-*t*-3-(4-hydroxy--3-methoxyphenyl)-6-methoxy-c-2-methylindane) (**8**) from isoeugenol, and diFA (4-*cis*,8-*cis*-bis(4-hydroxy--3-methoxyphenyl)-3,7-dioxyabicyclo[3.3.0]octane--2,6-dione) (**9**) from ferulic acid (Fig. 1), and investigated their radical-scavenging activity, intracellular ROS formation and biological activities in comparison with those of the original monomers or other compounds[5, 20, 22, 45]. Here we show the results of our experiments and discuss the mechanisms of the prooxidant/antioxidant activity of *o*-methoxyphenols and their biological activities.

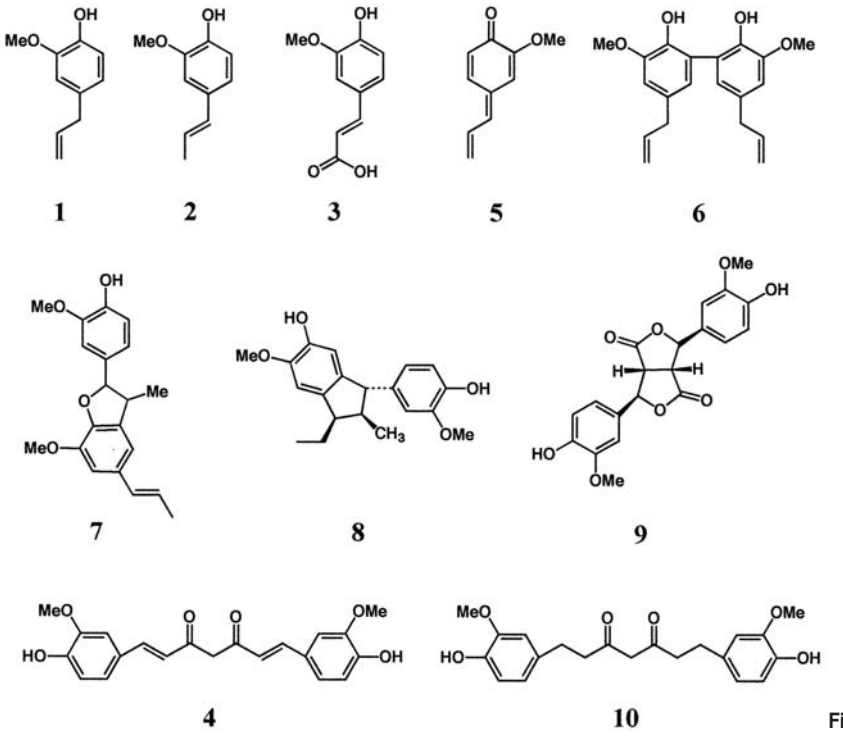

Figure 1. Structures of *o*-methoxyphenols

STOICHIOMETRIC FACTORS AND DIMERIZATION

Quantitative *in vitro* studies of autoxidation of styrene and the inhibition of autoxidation by synthetic and natural phenolic antioxidants have been performed[4, 15, 16]. Also, autoxidation of methyl linoleate or phospholipids and inhibition of autoxidation by antioxidants in micelles[56, 57], liposomes[36], or solutions in *tert*-butyl alcohol[46] have been investigated in order to determine the number of radicals trapped by each molecule of the phenol (the stoichiometric factor – *n*) and the rate constants for abstraction by peroxy radicals of the phenolic hydrogens from a number of phenols. Meaningful comparison between phenolic antioxidants requires that these factors can be individually evaluated. The radical-scavenging activities of phenolic compounds with high oxygen capacities may be affected by oxygen pressure and by the formation of ROS during the oxidation of phenols at high oxygen pressures[12]. Dual prooxidant and antioxidant activity can be found in *o*-methoxymonophenols as well as in polyphenols[20, 22]. The radical-scavenging activity of extracellular antioxidants for azo-initiator radicals (carbon-centered radicals) has previously been reported under anaerobic conditions, indicating that vitamin E and non-SH amino acids are ineffective scavengers and ascorbate is a poor scavenger of carbon-centered radicals, whereas cysteine and glutathione (GSH) are effective scavengers[62]. This result is in marked contrast to observations that vitamin E and ascorbate show high activity under high oxygen tension. The oxygen tension under a 15 torr oxygen atmosphere is similar to that in many tissues[16, 34], suggesting that oxygen in living cells is sparse. Previous quantitative *in vitro* studies of radical-scavenging activity have been carried out under aerobic conditions[4, 15, 36, 46, 56, 57], and we believe that the efficiency of antioxidants in this setting may be considerably different from that under nearly anaerobic conditions. Thus, we have previously used induction period methods to investigate the radical-scavenging efficiency of polyphenols[21], polyenes[23], and butylated hydroxytoluene (BHT)-related compounds[24] in the polymerization of methyl methacrylate (MMA) initiated by the thermal decomposition of 2,2'-azobisisobutyronitrile (AIBN) or benzoic acid (BPO) under nearly anaerobic conditions. This method has proved to be reliable in evaluating the radical-scavenging activity of natural and synthetic antioxidants. The *n* values for *o*-methoxyphenols have been previously determined by the induction period method[20–26, 31, 49].

In general, monophenolic compounds[31] show an *n* value of 2. An *n* value less than 2 suggests dimerization[31]. *o*-Methoxyphenols such as **1**, **2**, **3**, and 2-methoxy-4-methylphenol show *n* values of 1.2-1.5, suggesting dimerization. This reduction is probably related to the strong internal hydrogen bond between the phenolic OH group and the methoxy group[31]. The findings with these compounds are in good agreement with those of previous reports[13, 31, 49]. Hesperatin (3',5,7-trihydroxy-4-methoxyflavanone), a polyphenol, shows *n* values of about 1, resulting from dimerization[22]. Compound **7** shows an *n* of 1. The *n* value (about 1.8) for BHT suggests dimerization, which is a well-known *para-para* coupling reaction[31]. The *n* values for synthesized dimers such as **6**, **8**, *bis*-MMP (3,3'-di-methoxy-5,5'-dimethyl-1,1'--biphenyl-2,2'-diol), and *bis*-BHA (3,3'-di-*tert*-butyl-5,5'-di-methoxy-1,1'-biphenyl-2,2'-diol) are about 2. Compound **4** shows an *n* value of about 2. In contrast, the radical-scavenging activity of **4** has previously been studied by electrochemical methods (flow column electrolysis cyclic methods)[32], indicating that the number of –OH moieties for **4** appeared to be higher than 2 during its oxidation. This suggests that compound **4** undergoes chemical reactions during its oxidation, possibly involving the formation of polymeric products[72]. In a study of quenching of superoxide ions by **4** in acetonitrile solvent, 1 mol of **4** was able to react with 6 mol of such anion radicals[72]. This high value may be due to the methodology of the study, which was performed under aerobic conditions. A relatively high concentration of **4** produces dimers as radical termination products, demonstrating radical-radical termination at the 2-position of this compound[41]. Curcumin radicals could react with themselves or with other radicals to yield stable polymeric products such as vanillin, ferulic acid, and curcumin dimer[40]. Curcumin phenoxyl radicals have been shown by pulse radiolysis experiments to be generated by a one-electron oxidation[55]. Despite that, compound **4** showed an *n* value of 2 in the present study. This suggests that **4** scavenges one radical and subsequently undergoes dimerization or other reaction during the induction period. The dimer of **4** could scavenge one further radical, in sum scavenging two radicals. Thus, radical scavenging by this compound is complicated, possibly because of the formation of polymeric products[40]. PM 3 semi-empirical calculation for fully oxidized curcumin[22] suggests that the *n* value would be one.

PROOXIDANT/ANTIOXIDANT ACTIVITY

The prooxidant/antioxidant activity of phenolic compounds is dependent on such factors as metal-reducing potential, chelating behavior, pH, and solubility characteristics[19]. These factors, in addition to bioavailability and stability in tissues, should be considered when evaluating the potential bioactivity of phenolic antiox-

idants. Phenoxyl radical intensity for *o*-methoxyphenols has been measured under alkaline conditions by electron spin resonanse (ESR) spectroscopy[5, 47, 49]. Compounds **1**, **4**, **6**, **7**, and **8** produce radicals at pH 9.5 and many more radicals at pH>9.5, whereas compound **3** and phenol do not produce radicals[5, 49]. Phenoxyl radical formation by phenolic compounds is determined primarily by the homolytic bond dissociation energy (BDE) of the phenolic O-H bond, which can be estimated in part from the resonance stabilization of steric hindrance to abstraction of the phenolic hydrogen by free radicals such peroxy radicals[76]. The BDE (kcal/mol) calculated by the PM 3 semi-empirical method for compounds **7**, **8**, **4**, **1**, and **3,** and phenol is 83.76, 79.04, 80.41, 79.24, 84.30, and 87.10, respectively. Phenol and compound **3,** having BDEs greater than 84.0 kcal/mol, do not produce radicals even at pH values of 10–11. Also, none of the phenolic compounds described above produce radicals under physiological conditions, as judged by ESR spectroscopy[49].

Phenolic antioxidants undergo autoxidation catalyzed by transition metals such as Cu (II) and Fe (II) to produce superoxide anions. It is well known that superoxide anion dismutates to produce hydrogen peroxide and •OH radicals via the Fenton reaction[17]. However, transition metals *in vivo* and in plasma are complexed by proteins and probably do not catalyze the autoxidation of phenolic compounds to a significant extent[27]. Regardless of antioxidant defenses in biological systems, excessive production of $O_2^{\cdot-}$ and H_2O_2 can occur through secondary release of even a small percentage of metal ions from their physiological chelators such as ferritin[11].

As an alternative mechanism for the prooxidant activities of phenolic compounds, numerous peroxidases, such as plasma myeloperoxidase and cytochrome P-450, catalyze the production of prooxidant phenoxyl radicals. Intracellular phenoxyl radicals (redox-cycling phenols) are produced by myeloperoxidase-induced lipid peroxidation and cooxidize GSH to form thiyl radicals with concomitant oxygen activation[28]. Compound **4** with a phenol-containing ring significantly catalyzes oxygen uptake in the presence of GSH/HRP (horseradish peroxi-

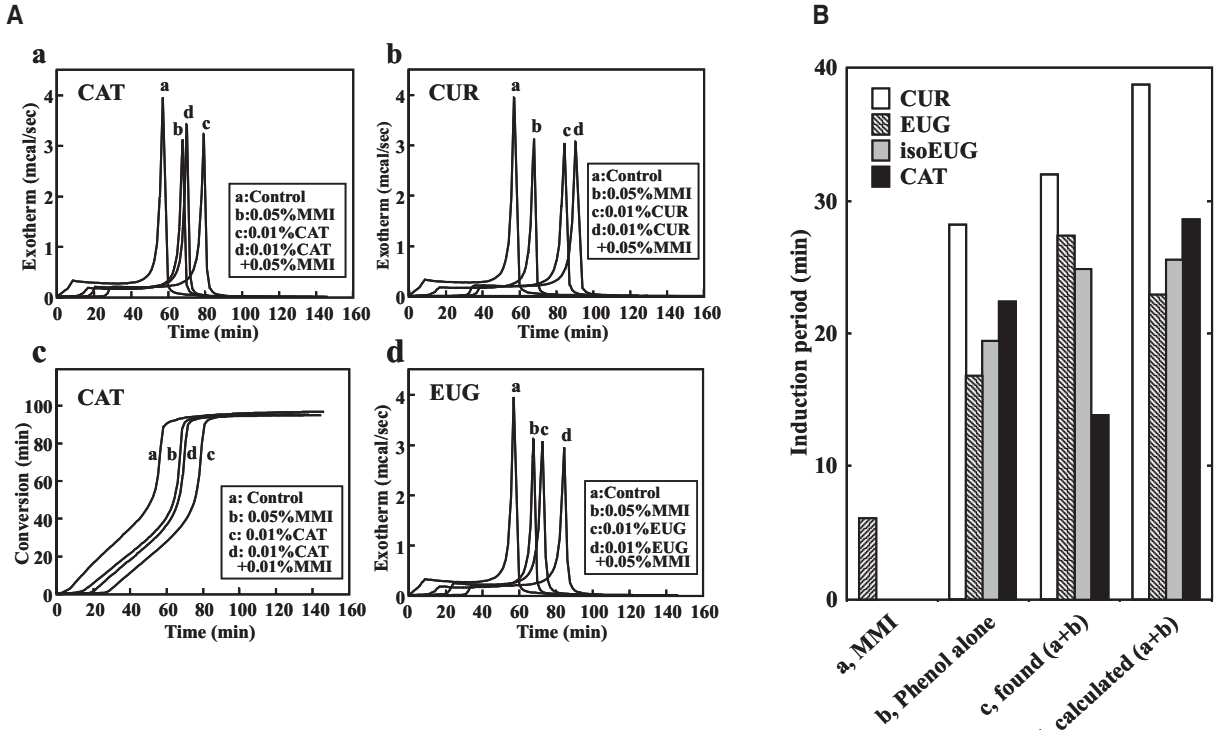

Figure 2. Exothermic curves and conversion curves (**A**) and induction period (**B**) for the polymerization of MMA-BPO in the presence of catechin (CAT), curcumin (CUR), isoeugenol (isoEUG), or eugenol (EUG) with or without mercaptomethylimidazole (MMI). Approximately 10 μl of the experimental resin (MMA 9.4 mol/l, BPO 0.1 mol/l) in the absence or presence of phenolic compounds (0.01 mol%) with or without MMI (0.05 mol%) was loaded into an aluminium sample container and sealed by applying pressure. The container was placed in a differential scanning calorimeter (model DSC3100; MAC Science Co., Tokyo, Japan) kept at 70°C and thermal changes induced by polymerization were recorded for the appropriate period (**Aa**, **b**, and **d**). The induction period (IP) was determined from the conversion curves (**Ac**). In panel **B**: the IP for controls (IP_{con}) was approximately 6.43 min. The concentration of molecular oxygen sealed in the DSC container was about 8.13×10^{-8} mol. The IP value for each sample was calculated from the equation $IP = IP_{test} - IP_{cont}$. The bar graphs present the means for three independent experiments (SD<15%). A significant difference between c and d was found for CAT, CUR and EUG ($p<0.05$).

dase)/H_2O_2. In contrast, none of the catechins (polyphenols with a catechol ring) cause oxygen uptake[27]. Fully oxidized catechins show an *n* value of 4, suggesting that a catechol (3',4'-dihydroxy) element in the B-ring is an important determinant[22].

To clarify the cooxidation mechanisms of thiols, we investigated the radical-scavenging activity of compounds **1**, **2**, and **4** as well as catechin (3,3',4',5,7-flavanpentol) in the presence of mercaptomethylimidazole (MMI), a thiol. MMI was used as a representative thiol, since attempts to use *N*-acetyl-cysteine or GSH were unsuccessful because of their limited solubility in MMA. Figure 2 shows typical time-exothermic (Aa, b, and d) and time-conversion (Ac) curves and the alteration of induction period (IP; Fig. 2B) for the phenolic compounds in the polymerization of MMA initiated by the thermal decomposition of BPO. The presence of oxygen retards polymerization of MMA because oxygen reacts with MMA radicals activated by PhCOO· radicals from BPO and subsequently produces non-radical products. The control shows an IP because the DSC pan contained a small amount of oxygen, since it was sealed in air. The IP for each compound was determined from time-conversion curves (Ac). The relative value of the IP declined in the order **4**>catechin>**2**>**1** (b in Fig. 2B). The *n* value for catechin is larger than that for curcumin[22], but compound **4** had the largest IP value, probably due to the formation of polymerized products acting as antioxidants[40]. For the polyphenols **4** and catechin, the IP value in c (found, a+b) was significantly smaller than that in d (calculated). The reduction in the IP value can probably be attributed to consumption of the oxygen in the DSC container. The hydrogen atom is taken from the SH group of MMI and a thiol RS radical is formed, which together with oxygen produces an oxo- or peroxo-sulfur radical, RSO· or RSOO··. In biological systems, the hydrogen atom may be abstracted from the -SH group of GSH (reduced form) through oxidation of **4** or catechin *in vivo* and, subsequently, the reaction of molecular oxygen with thiyl radicals could produce ROS. Pulse radiolysis studies[70] show that the disulfide radical anion rapidly reduces oxygen to form the superoxide radical anion and GSSG (GSH, oxidized form) with a rate constant of 0.16×10^9 $M^{-1}s^{-1}$ at pH 9. The rate constant for the disulfide radical anion is markedly greater than that for the reaction of methoxyphenols such as eugenol with superoxide ($\sim10^3$ $M^{-1}s^{-1}$)[66]. Decreased synthesis of GSH and increases in intracellular ROS and GSSG have been reported in human leukemia cells treated with catechin polyphenols[58].

The present finding of altered IP values suggests that thiol-sensitive intracellular oxidative events may occur in a physicochemical manner in biological systems. ROS and reactive sulfur species may act in unison to damage biomolecules such as phospholipids and DNA, possibly resulting in the induction of apoptosis or mutagenicity[75]. The relationships between DNA damage, apoptosis and mutagenicity have attracted considerable interest. Failure of cells to undergo apoptotic cell death might be involved in the pathogenesis of variety of human diseases, inducing cancer[67]. On the other hand, for compound **1** the found IP (c) was significantly greater than the calculated IP (d), which could result from an interaction between EUGQM (**5**) and the SH group in MMI and, subsequently, the formation of EUGQM/MMI conjugates. EUGQM/GSH conjugates derived from peroxidase-catalyzed oxidation of eugenols have previously been reported[69]. In contrast, compound **2** showed a relatively smaller found IP (c) compared with the calculated value (d), and the reduction of IP for **2** was markedly less than that for **4** or catechin (Fig. 2B). Interestingly, the MMI-cooxidation behavior of **2**, an isomer of **1,** was considerably different from that of **1**. This suggests that benzyl radical from **2** reacts poorly with MMI because of the high localizability in the benzene ring of this radical. Compound **2** preferentially undergoes dimerization due to the coupling reaction of benzyl radicals, or may undergo other reactions typical of benzyl radicals. Alternatively, benzyl radicals could react with nucleophilic substances, such as SH or NH_2 groups in proteins. We recently found decreases in cellular GSH in HSG cells treated with compound **2,** and especially with compound **4,** but not with compound **1** (data not shown). The alteration of IP for phenolic antioxidants with or without a thiol could help explain their effectiveness *in vivo* for inducing tumor cell apoptosis and for chemoprevention.

ROS FORMATION AND APOPTOSIS

ROS is a term that includes radical oxygen species such as O_2^- and ·OH as well as non-radical derivatives of molecular oxygen (O_2) such as H_2O_2. ROS are mainly generated at mitochondrial electron transport chains during normal cellular metabolism. In addition, various stimuli, including tumor necrosis factor (TNF)-α, Fas ligand, and growth factors, rapidly provoke ROS accumulation in their target cells[65]. ROS can combine with other atoms or larger molecules to form alkyl (R·) or peroxyl (ROO·) radicals in lipids. Phenolic compounds with antioxidant properties may enhance endogenous defense systems and reduce both initiation and propagation of ROS. The expression of antioxidant enzymes such as superoxide dismutase (SOD), catalase, and glutathione-S-transferase is regulated by ROS stress in a complex way[63].

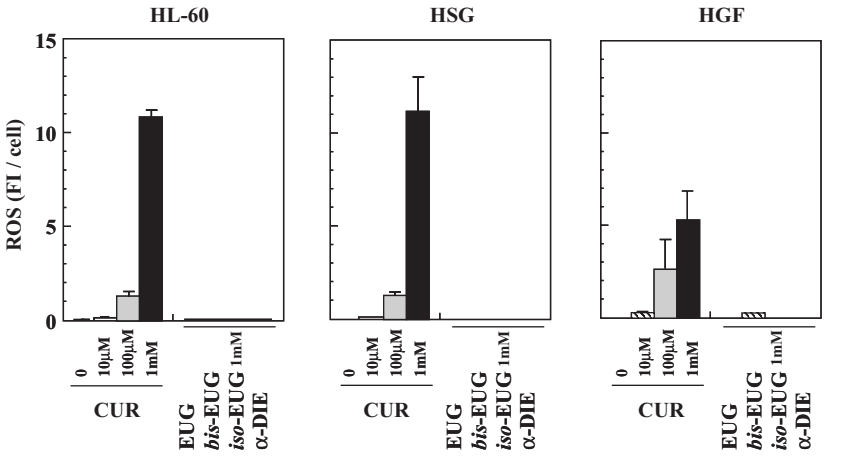

Figure 3. ROS formation in HL-60, HSG, and HGF cells treated with curcumin (CUR, **4**), *bis*-EU (**6**), EUG (**1**), *iso*-EUG (**2**) and α-DIE (**8**). Before each assay, HSG and HGF cells were incubated at a concentration of 4×10^4 cells in medium containing 10% FBS for 2 days in a 24-well plate, and then the cells were washed with HBS. HL-60 cells were washed with HBS, and 1×10^5 cells in HBS containing 1% FBS were used. Compound **4** (10 μM-1 mM) and compounds **6**, **8**, **2,** or **1** (1 mM) were added to the cells, which were then incubated in HBS for 30 min. Then 10 μM CDF-DA was added and incubation was continued for 30 min. Amounts of intracellular ROS were estimated by using a flow cytometer (Beckman EPICS) measuring green fluorescence (530-550 nm). Data are the means ±SD of three different experiments.

Despite the conventional dogma that ROS are harmful to cells, experimental evidence over the last decade has linked a low level of intracellular ROS to cellular proliferation and cell cycle progression. Recent studies have focused on the part played by a prooxidant intracellular milieu in the resistance of tumor cells to cell death signals delivered via the cell surface receptor or upon exposure to chemotherapeutic drugs[53, 54].

We investigated intracellular ROS formation by *o*-methoxyphenols in living cells by using the 5-(and 6-)-carboxy-2',7'-dichlorofluorescein diacetate (CDFH-DA) method (Fig. 3). Compound **4** generated much more ROS in both types of cancer cells tested (human promyelocytic leukemia HL-60 cell line and human submandibular gland tumor HSG cell line) than in normal primary cultured cells (HGF human gingival fibroblasts) at the cytotoxic higher concentration of 0.1–1 mM. In contrast, compounds **1**, **2**, **6**, and **8** generated considerably less ROS than did compound **4**, with ROS levels almost the same as those of controls. The intensity of compound **4**-induced ROS formation in HL-60 cells and HSG cells was similar, although HL-60 cells have endogenous enzymes such as myeloperoxidase that tightly regulate the level of cellular ROS[28]. Cancer cells exhibit increased intrinsic ROS stress, due in part to oncogenic stimulation, increased metabolic activity, and mitochondrial malfunction[53]. Analysis of leukemia cells shows a significant increase in ROS compared with normal lymphocytes, which have low cellular ROS[78]. Recently, we demonstrated that compound **4**-induced ROS formation in HSG cells is highly enhanced by visible-light (VL) irradiation at 400–700 nm (λ_{max}=470 nm)[20]; the absorbance of compound **4** has a λ_{max}=440 nm. Intracellular ROS formation in the presence of 0.5 μM curcumin was amplified by 10 min of VL irradiation to about 100 times that of a non-irradiated sample (fluorescence intensity (FI)/cell, about 0.1). VL-irradiated curcumin is biocidal against bacteria because of the hyperproduction of ROS[71]. Also, the cytotoxicity of eugenol in both HSG and HGF cells is activated by VL irradiation under weak alkaline conditions, followed by increasing ROS formation[20]. Curcumin-induced ROS formation in HL-60 or HSG cells was also enhanced by the addition of transition metal ions such as Cu (II) or Zn (II), although to a much lower level than by VL irradiation. Curcumin-induced ROS was suppressed by the addition of NAC, a precursor of GSH. GSH is significant as the substrate for glutathione peroxidase and transferase and other enzymes, as well as being a scavenger of free radicals in cells. Hyperproduction of ROS is found in curcumin-treated AK-5 cells[10], indicating that compound **4** is a ROS-generating anticancer agent. On the other hand, tetrahydroxycurcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)heptane-3,5-dione) (**10**) without the α, β-unsaturated carbonyl moiety does not produce intracellular ROS in HSG or HGF cells, even after VL irradiation or HRP/H_2O_2 treatment[6].

Compound **4**, but not compound **10**, causes a decrease in membrane mobility, probably resulting from the peroxidation by ROS of polyunsaturated phospholipids in cell membranes[6]. The prooxidant/antioxidant activity of **4** in polyunsaturated

lipids may occur by coupling reactions with lipids and, subsequently, an intermolecular Diels-Alder reaction[41]. These findings suggest that both the α, β-unsaturated carbonyl moiety of curcumin and the OH group are responsible for intercellular ROS formation. Curcumin depletes hepatocyte GSH to a greater extent than can be explained by GSSG formation, presumably because GSH transferases catalyze GSH conjugate formation with the α, β-unsaturated carbonyl moiety of curcumin[7]. However, our findings of a changed IP for curcumin in the presence of MMI suggest that the reduction of GSH may be attributable to the formation of thiyl radicals. The prooxidant activity of compound **4**, when metabolically activated by ROO· radicals, can lead to cooxidation of NADH (β-nicotinamide adenine dinucleotide) and ascorbate as well as GSH[27]. NADH is stoichiometrically oxidized without oxygen uptake, a reaction for which *o*-quinone metabolites as well as their *p*-quinone counterparts are responsible[27]. BHT--quinone is reduced by NADH to its semiquinone, which reacts with O_2 to form O_2^- and subsequently H_2O_2. Furthermore, the NAD· formed also reacts with O_2 to form NAD^+ and O_2^- at an almost diffusion-controlled rate[37]. Among the 2-*tert*-butyl phenolic compounds, BMP (2-*tert*-butyl-4-methylphenol), but not BHA (2-*tert*-butyl-4-methoxyphenol), shows enhanced cytotoxicity for HL-60 cells or human squamous carcinoma HSC-2 cells in the presence of NADH, a quinone reductase, possibly due to the formation of BMP *o*-quinone[59]. Ascorbate is cooxidized by phenoxyl radicals, but without oxygen activation[60]. ROS have been reported to be involved in tissue damage associated with a number of inflammatory diseases, including cancer, hepatic disease, diabetes, immunological disorders, and others[9]. The mechanisms of cancer prevention by compound **4** have been reviewed recently[1, 39]. Oxidative modification of redox-sensitive factors and intermediate signaling molecules is involved in ROS-mediated modulation of cell growth and cell survival characterized by the induction of apoptosis or necrosis.

Compound **4** is a well-known inducer of apoptosis. A number of investigators have previously described the mechanism of induction of apoptosis by curcumin[1, 39]; however, the relationship between the early stages of induction of apoptosis and ROS formation by a wide variety of *o*-methoxyphenols is poorly understood. Figure 4 shows compound **4**-induced apoptosis in HL-60 cells, but not in HSG cells, whereas compounds **6** or **8** did not induce apoptosis as judged by externalization of annexin-V-targeted phosphatidylserine residues (Fig. 4). A loss of mitochondrial membrane potential (DertaPsim) for **4** was found in HL-60 cells (data not shown). Also, compounds **1** and **2** did not induce apoptosis in HL-60 cells at 10 μM. The ability of these compounds to induce apoptosis appears to vary not only with the cell type, but also with the potency of the compound. Quantitative structure-activity relationships can be used to characterize phenolic compounds with apoptotic activity with the aim of developing new agents for cancer therapy[29]. Log P and BDE are standard

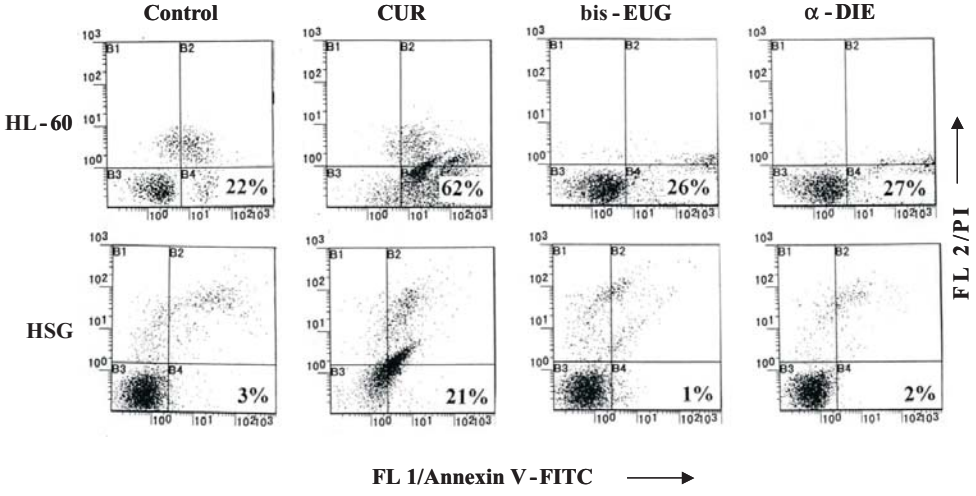

Figure 4. Effects of curcumin (**4**), bis-EUG (**6**) or α-DIE (**8**) on externalization of annexin-V-targeted phosphatidylserine residues in the early stage of apoptotic cell death, as assessed by Annexin V-FITC/PI staining of HL-60 and HSG cells. HSG cells were precultured for 2 days at 1×10^5/ml in medium containing 10% FBS. Then the cells were cultured for 5 h in serum-free medium with curcumin at a concentration of 10 μM. HL-60 cells were cultured in RPMI 1640 medium containing 1% FBS. The cells ($1\text{-}5\times10^5$) were stained with an Annexin V-FITC/PI kit. Stained cells were analyzed with a flow cytometer (Beckman ALTRA). At least 5,000 cells were analyzed per sample. Four distinct phenotypes become distinguishable: viable (lower left quadrant, B3); early apoptotic (lower right quadrant, B4); secondary necrotic (upper right quadrant, B2) and damaged cells (upper left quadrant, B1). The number inserted in B4 represents the early apoptotic cell number (%).

terms in such relationships. Log P for **1**, **2**, **4**, **6** and **8** is 2.55, 2.55, 2.48, 4.75 and 4.40, respectively, as calculated by the PM 3 method. Compounds **8** (log P=4.4) and **4** (log P=2.5) have similar 50% cytotoxic concentrations (CC_{50}) in HL-60 cells (about 2 μM), whereas CC_{50} values for **1**, **2** and **6** are approximately 178, 10, and 200 μM, respectively. Log P and BDE for **4** and **8** are identical to each other, but they show a great difference in biological activity. In contrast, the cytotoxicity of phenolic compounds in HSG or HGF cells was one order of magnitude less than that in HL-60 cells. The high cytotoxicity of *o*-methoxyphenols in HL-60 cells may be associated with the high oxidative activity of endogenous enzymes, such as myeloperoxidase, characteristic of these cells. The intracellular ROS generated by these compounds at a concentration of 10 μM was also determined with a CDFH-DA probe, indicating that intracellular ROS (FI/cell, about 0.2) for **4** was 10 times greater than that for **6**, **8** or controls. Such generation of ROS by compound **4** may permit efficient execution of the apoptotic signal. Even though compound **8** does not induce ROS, the cytotoxicity of compounds **4** and **8** is equipotent, suggesting that compound **8** causes toxicity by a mechanism that does not involve ROS. ROS are involved in the induction of apoptosis by the polyphenols EGCG (epigallocatechin-3-gallate)[58] and EGC (epigallocatechin)[18]. EGC can also induce apoptosis by mechanisms not involving ROS, for example in Jurkat T, HL-60, or K562 cells it inhibits S phase progression because of suppression of DNA copies[61]. Furthermore, the induction of apoptosis is dependent on the activity of transcription factors. Binding of TNF-α to the TNF receptor can initiate apoptosis, but also activates the transcription factor NF-κBi, which suppresses apoptosis by an unknown mechanism, although activation of NF-κB is known to block the activation of caspase-8. This suggests that suppression of activation of NF-κB could induce apoptosis[73]. Suppression of NF-κB and expression of c-jun, c-fos, and iNOS is associated with the apoptosis-inducing effects of curcumin[52]. Compound **4**, but not compounds **l** or **2**, can inhibit fos-jun dimerization[52]. NF-κB is stimulated by intracellular generation of ROS[3]. Hypoxia enhances intracellular ROS generation and leads to I-κBα phosphorylation and NF-κB activation through ROS[77]. Together, these findings suggest that low levels of intracellular ROS may enhance the sensitivity of cancer cells to phenol-induced cell death.

NO AND NF-κB

As found recently, not only ROS, but also •NO plays important roles in human physiology and pathology. •NO, nitrites, and nitrates are produced *in vitro* by macrophages incubated in the presence of LPS and interferon α. They originate from the semi-essential amino acid L-arginine by the activity of NO synthase. At higher concentrations, •NO has antimicrobial, antitumor, and cytotoxic effects. At low concentrations it stimulates guanylate cyclase, resulting in effects on blood flow and blood pressure regulation, neuronal signal transmission, and neuroendocrine activity[3]. Phenolic carboxylic acids, such as ferulic acid (**3**), caffeic acid, and *p*-coumaric acid, and *o*-methoxyphenols, such as eugenol (**1**), inhibit NO production by LPS-activated mouse macrophage-like Raw 264.7 cells[50]. Also, under cell-free conditions, •NO radicals generated by NOC-7 (a NO donor) are scavenged by phenolic carboxylic acids and eugenol, as measured by ESR spectroscopy[50]. The activity of •NO is controlled by cytokines, the effector and signaling factors of NO connecting the immune system with the cardiovascular, nervous, and endocrine systems. NF-κB is an important transcriptional factor, which is activated by phosphorylation-dependent proteolysis of inhibitor of NF-κB (I-κB), which regulates inflammatory responses and the expression of various inflammatory cytokine genes[14, 30, 38, 43, 74]. Phenolic antioxidants possess potent anti-inflammatory and antimutagenic activities. Recent studies have shown that Cox-2 is regulated by the eukaryotic transcription factor NF-κB. Cox-2 has been recognized as a molecular target for many of the chemopreventive effects of phenolic antioxidants[64]. Antioxidant and cyclooxygenase-inhibitory phenolic compounds from *Ocimum sanctum* Linn. have been investigated[33], showing that eugenol (**1**) possesses 97% Cox-1 inhibitory activity at a slightly higher level of Cox-2 inhibitory activity at 1000 μM. The activity of eugenol is less than that of other extracts. Compound **1** suppresses Cox-2 gene expression in LPS-stimulated mouse macrophages[35]. Also, curcumin (**4**)[51] and ECG[2] inhibit Cox-2 gene expression and the activation of NF-κB. We have previously investigated whether *o*-methoxyphenols and their dimers could inhibit LPS-stimulated NF-κB activation in RAW 264.7 cells. *bis*-Eugenol (**6**), but not eugenol (**1**), potently inhibited LPS-stimulated NF-κB activation, resulting in suppression of inflammatory cytokine expression in macrophages[45]. Compounds **6**, **7** and **9**, but not compound **8,** inhibited LPS-induced Cox-2 expression, whereas the corresponding monomers, compounds **2** and **3**, did not (data not shown). Also, *bis*-BHA inhibits NF-κB activation stimulated by a bacterial component, fimbriae of *Porphyromonas gingivalis* (an oral anaerobe), although the mechanism is unknown (unpublished data). These findings suggest that *o*-methoxyphenolic dimers may act as efficient inhibitors of NF-κB activation and Cox-2 expression. Dimerized *o*-me-

thoxyphenols may be useful in the design of potent chemopreventive and anticancer agents.

CONCLUSION

Dimers of the *o*-methoxyphenol compounds **1**, **2**, and **3** were synthesized. Their radical-scavenging activity under cell-free conditions, their cytotoxicity, ROS formation, and induction of apoptosis in HL-60, HSG, or HGF cells, and their effects on the expression of NF--κB and Cox-2 in RAW 264.7 cells were compared with those of the original monophenols, compound **4**, catechin, or other phenols. Kinetic polymerization studies of *o*-methoxyphenols by the induction period method in the absence or presence of the thiol MMI showed that the radical-scavenging behavior of **4** was similar to that of catechin, accompanied by oxygen uptake and ROS formation, whereas compound **1** led to quinone methide (**5**)/MMI conjugates without oxygen uptake. Compound **4** induced ROS formation and apoptosis in HL-60 cells. Compounds **6** and **7** inhibited LPS-stimulated NF-κB activation in RAW 264.7 cells. Furthermore, compounds **6**, **7** and **9** inhibited LPS-induced Cox-2 expression in RAW 264.7 cells in a dose-dependent manner, whereas the phenolic monomers **1**, **2** and **3** were ineffective.

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