Antioxidative Effects of Curcumin and its Analogues against the Free-radical-induced Peroxidation of Linoleic Acid in Micelles

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Curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, 1) is a yellow ingredient isolated from turmeric (curcumin longa). Many health benefits have been claimed for curcumin, and these have generally been ascribed to its radical-trapping antioxidant properties. In order to find more active antioxidants with 1 as the lead compound, we synthesized curcumin analogues, i.e., 1,7-bis(3,4-dihydroxyphenyl)-1,6-heptadiene-3,5-dione (2), 1-(3,4-dihydroxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione (3), 1-(4-hydroxy-3-methoxyphenyl)-7-(4-hydroxyphenyl)-1,6-heptadiene-3,5-dione (4), 1,7-bis(4-hydroxyphenyl)-1,6-heptadiene-3,5-dione (5), 1,7-bis(3,4-dimethoxyphenyl)-1,6-heptadiene-3,5-dione (6), 1,7-bis(4-methoxyphenyl)-1,6-heptadiene-3,5-dione (7), and 1,7-diphenyl-1,6-heptadiene-3,5-dione (8). Antioxidative effects of curcumin and these analogues against the peroxidation of linoleic acid were studied in sodium dodecyl sulfate (SDS) and cetyltrimethylammonium bromide (CTAB) micelles. The peroxidation was initiated thermally by a water-soluble initiator 2,2′-azobis(2-amidinopropane hydrochloride) (AAPH), and reaction kinetics were monitored by the formation of linoleic acid hydroperoxides. Kinetic analysis of the antioxidation process demonstrates that these compounds, except 6, 7 and 8, are effective antioxidants in micelles by H-atom abstraction from the phenolic groups. Compounds 2 and 3, which bear ortho-diphenoxyl functionality, possess significantly higher antioxidant activity than curcumin and other analogues, and the 4-hydroxy-3-methoxyphenyl group also plays an important role in the antioxidative activity. In addition, the synergistic antioxidant effect of these compounds with α-tocopherol (vitamin E) in micelles was also studied by following the formation of linoleic acid hydroperoxides and the consumption of α-tocopherol. It was found that these compounds could not synergistically interact with α-tocopherol in micelles. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: curcumin; antioxidants; reaction mechanism; lipid peroxidation; structure/activity relationship.

INTRODUCTION

Oxidative damage of cell membranes, DNA and proteins induced by free radicals has been implicated in the etiology of aging and several degenerative diseases, such as cancer and atherosclerosis (Finkel and Holbrook, 2000; Cooke et al., 2003; Perwez Hussain et al., 2003; Barnham et al., 2004). Therefore, inhibition of oxidative damage by supplementation of antioxidants becomes an attractive therapeutic strategy to reduce the risk of these diseases (Rice-Evans and Diplock, 1993; Brash and Harve, 2002; Surh, 2003).

Curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, 1) is a naturally occurring phenolic compound isolated as a yellow pigment from turmeric (dry rhizomes of curcumin longa) which is commonly used as a spice and food colorant (Buescher and Yang, 2000). This compound has attracted considerable attention due to its various biological and pharmacological activities, including antioxidant (Venkatesan and Rao, 2000; Naidu and Thippeswamy, 2002; Wright, 2002; Patro et al., 2002; Priyadarssini et al., 2003; Rukkumani et al., 2003; Daniel et al., 2004) and anticancer activities (Cheng et al., 2001; Lin et al., 2001; Shukla et al., 2002; Aggarwal et al., 2003). Much effort has also been devoted to investigating the radical-scavenging mechanisms of curcumin (Jovanovic et al., 1999; Barclay et al., 2000; Jovanovic et al., 2001; Sun et al., 2002; Priyadarssini et al., 2003; Shen et al., 2005). During our ongoing research project on kinetics and mechanisms of natural antioxidants (Hou et al., 2004a; Hou et al., 2004b; Zhou et al., 2004, 2005a, 2005b) we found recently that simple structural modification of resveratrol, which is an antioxidative component in red wine, could significantly enhance its antioxidative activity (Fang et al., 2002; Cai et al., 2003) and cytotoxicity against cancer cells (Cai et al., 2004). This motivated us to use curcumin as a lead compound to design more active potential antioxidants and chemopreventive agents against cancer. Reported herein is the synthesis of a set of curcumin analogues and a quantitative kinetic study on their antioxidant effects on free-radical-induced peroxidation of linoleic acid in micelles. The peroxidation was initiated thermally at physiological temperature by a water-soluble azo initiator 2,2′-azobis(2-amidinopropane hydrochloride) (AAPH) and conducted in sodium dodecyl sulfate (SDS) and cetyltrimethylammonium bromide (CTAB) micelles to mimic the micro-environment of biomembranes. The compounds studied...
were curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, 1), 1,7-bis(3,4-dihydroxyphenyl)-1,6-heptadiene-3,5-dione (2), 1-(3,4-dihydroxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione (3), 1-(4-hydroxy-3-methoxyphenyl)-7-(4-hydroxyphenyl)-1,6-heptadiene-3,5-dione (4), 1,7-bis(4-hydroxyphenyl)-1,6-heptadiene-3,5-dione (5), 1,7-bis(3,4-dimethoxyphenyl)-1,6-heptadiene-3,5-dione (6), 1,7-bis(3,4-methoxyphenyl)-1,6-heptadiene-3,5-dione (7), and 1,7-diphenyl-1,6-heptadiene-3,5-dione (8). The interaction of these compounds with α-tocopherol (TOH, vitamin E) was also investigated.

MATERIALS AND METHODS

Materials. Curcumin and its analogues 1–8 were synthesized by condensation of 2,4-pentanedione with two equivalents of substituted benzaldehyde based on the available methods (Roughley and Whiting, 1973; Mazumder et al., 1997). Generally, 2,4-pentanedione (1.0 g, 0.01 mol) and boron oxide (0.49 g, 0.007 mol) were dissolved in EtOAc (10 mL) and stirred for 0.5 h at 40 °C followed by addition of the corresponding benzaldehyde (0.02 mol) and tributyl borate (4.6 g, 0.02 mol) and stirred for an additional 0.5 h. Then n-butylamine (1 mL) in EtOAc (10 mL) was added dropwise during 30 min. After further stirring for 4 h at 40 °C the mixture was allowed to stand overnight to complete the reaction. The mixture was hydrolyzed by HCl (0.4 N, 15 mL) and the aqueous layer was extracted three times with EtOAc. The combined organic layers were washed with water and dried over Na₂SO₄. After removal of the solvent under reduced pressure, the residual paste was purified by column chromatography (silica gel, cyclohexane–EtOAc) and recrystallized from EtOH to give pure 1 and 4–8. Compounds 2 and 3 were obtained by demethylation of 1 with AlCl₃-pyridine as described in the literature (Mazumder et al., 1997). Their structures were fully identified with ¹H NMR and EI-MS or FAB-MS and the data are consistent with those reported in the literature (Mazumder et al., 1997).

1,7-Bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione (curcumin, 1): 48% yield from vanillin (3 g, 0.02 mol), m. p. 184–185 °C (lit. (Mazumder et al., 1997) m. p. 182–183 °C). ¹H NMR (400 MHz, (CD₃)₂CO): δ 3.91 (s, 6H), 5.97 (s, 1H), 6.68 (d, J = 15.8 Hz, 2H), 6.88 (d, J = 8.2 Hz, 2H), 7.17 (d, J = 8.2 Hz, 2H), 7.32 (s, 2H), 7.59 (d, J = 15.8 Hz, 2H); EIMS (m/z, %): 368 (M⁺, 45).

1,7-Bis(3,4-dihydroxyphenyl)-1,6-heptadiene-3,5-dione (2): 25% yield from 1 (5.5 g, 0.015 mol), m. p. 304–306 °C decom. [lit. (Mazumder et al., 1997) m. p. 306–308 °C decom]. ¹H NMR (200 MHz, (CD₃)₂CO): δ 5.99 (s, 1H), 6.50 (d, J = 15.8 Hz, 2H), 6.88 (d, J = 8.6 Hz, 2H), 7.07 (d, J = 8.6 Hz, 2H), 7.19 (s, 2H), 7.53 (d, J = 15.8 Hz, 2H); FAB/MS (m/z): 341 (M⁺ + 1)⁺.

1-(3,4-dihydroxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione (3): 40% yield from 1 (5.5 g, 0.015 mol), m. p. 166–168 °C (lit. (Mazumder et al., 1997) m. p. 165–167 °C). ¹H NMR (300 MHz, (CD₃)₂CO): δ 7.15 (s, 2H), 8.31 (t, J = 16.4 Hz, 1H), 7.73 (d, J = 2.8 Hz, 2H), 7.19 (d, J = 2.8 Hz, 2H), 6.86 (d, J = 15.8 Hz, 1H), 6.59 (d, J = 15.8 Hz, 1H), 6.69 (d, J = 15.8 Hz, 1H), 6.86 (d, J = 8 Hz, 2H), 7.02 (d, J = 8.1 Hz, 1H), 7.15 (d, J = 8.1 Hz, 1H), 7.16 (s, 1H), 7.31 (s, 1H), 7.51 (d, J = 16.5 Hz, 1H), 7.62 (d, J = 16.8 Hz, 1H); EIMS (m/z, %): 354 (M⁺, 2).

Figure 1. Molecular structures of curcumin and its analogues.

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General procedure for the following experiments. The linoleic acid micelles were prepared by mixing appropriate amounts of linoleic acid, α-tocopherol and curcumin and its analogues, when required, with an aqueous solution 0.1 M SDS or 15 mM CTAB followed by vigorous mixing with a vortex mixer for 2 min. AAPH was directly dissolved in phosphate buffered saline (pH = 7.4) and injected into the micelles to initiate the peroxidation. The rate of oxidation was measured in an open vessel of ca. 2 mL in volume, thermostated at 37 °C and provided with magnetic stirrer, using a Gilson model 702 high performance liquid chromatography (HPLC).

Determination of linoleic acid hydroperoxides. Aliquots of the reaction mixture were taken out of an open vessel at appropriate time intervals and subjected to HPLC analysis on a Gilson liquid chromatography with a ZORBAX ODS reversed-phase column (6 × 250 mm, Du Pont Instruments), then eluted with methanol/water (5:1, v/v). The flow rate was set at 1.0 mL min⁻¹. A Gilson 116 UV detector was used to monitor the total linoleic acid hydroperoxides at 235 nm. Every determination was repeated three times, and the experimental deviations were within ±10%.

Determination of α-tocopherol. The procedure was the same as described above for the determination of linoleic acid hydroperoxides, except that a Gilson 142 electrochemical detector set at +700 mV was used for monitoring TOH. The column was eluted with methanol/propan-2-ol/formic acid (80:20:1, v/v/v) containing sodium perchlorate (50 mmol L⁻¹) as supporting electrolyte.

RESULTS

Peroxidation of linoleic acid or its esters gives different hydroperoxides depending on the reaction conditions (Porter, 1986; Porter et al., 1995; Brash, 2000; Tallman et al., 2001; Pratt et al., 2003). The hydroperoxide substitution at the C-9 or C-13 positions produces either trans, trans- or cis, trans-conjugated dienes which are the major products in the absence of antioxidants or in the presence of a small amount of antioxidants, e.g., millimolar concentrations of α-tocopherol (Porter, 1986; Porter et al., 1995). It was found recently that these conjugated dienes were formed from the rapid β-scission of the primarily formed bis-allylic 11-peroxyl radical (Brash, 2000; Tallman et al., 2001), and the kinetically controlled product, the nonconjugated 11-substituted hydroperoxide, might become the major product in the presence of high concentrations of antioxidant, e.g., molar concentrations of α-tocopherol (Tallman et al., 2001). These experimental observations have been rationalized recently by theoretical calculations (Pratt et al., 2003). The present experiment used very small amount of antioxidants (micromolar α-tocopherol and/or curcumin and its analogues), hence the production of the nonconjugated 11-hydroperoxide should be negligible and the conjugated hydroperoxides were the predominant products. The latter showed characteristic ultra-violet absorption at 235 nm (Ohkawa et al., 1978) that was used to monitor the formation of the total hydroperoxides formed during the peroxidation after separation of the reaction mixture by HPLC.

A set of representative kinetic curves of the total hydroperoxide formation during the peroxidation of linoleic acid in SDS and CTAB micelles is shown in Figs 2 and 3, respectively. It can be seen from the figures that, upon AAPH initiation, the concentration of linoleic acid hydroperoxides increased quickly and linearly with time in the absence of antioxidants (line a in Figs 2 and 3), demonstrating the fast peroxidation of the substrate. The slope of this line corresponds to the rate of propagation, Rₚ, of the peroxidation. The formation of the hydroperoxides was remarkably inhibited by the addition of α-tocopherol (TOH) during the so-called ‘inhibition period’ or ‘induction period’ (line b in Figs 4 and 5). During the inhibition period, the concentration of the hydroperoxides also increased approximately linearly with time, and the slope of this line was designated as Rₚ, which reflects the antioxidative potential of the antioxidant. After the inhibition period, the rate
of the hydroperoxide formation increased to close to the original rate of the propagation, demonstrating the exhaustion of the antioxidant. The turning point from the inhibition period to the restoration of the fast peroxidation refers to the inhibition time, $t_{inh}$, which is also an indication of the efficacy of the antioxidant. Addition of curcumin (1) and its analogues (COHs) 2, 3, 4 or 5 to the micelles significantly decreased the rate of hydroperoxide formation from $R_p$ to $R_{inh}$, but no inhibition period was observed (lines b–f in Figs 2 and 3). Compounds 6, 7 and 8, showed no appreciable effect (lines not shown for clarity). The antioxidant activity can be expressed by the percentage inhibition of the peroxidation, $P_{inh} = (R_p - R_{inh})/R_p \times 100\%$, that follows the same sequence of 2 ~ 3 > 4 > 5 > 6 ~ 7 ~ 8 in both SDS and CTAB micelles (Table 1).

$\alpha$-tocopherol (TOH), the most abundant and active form of vitamin E, is well known and the principal lipid-soluble chain-breaking antioxidant in plasma and erythrocytes (Burton and Ingold, 1986). Its synergistic antioxidative effect with other antioxidants, such as L-ascorbic acid (vitamin C), (Liu et al., 1988) green tea polyphenols (Zhou et al., 2005b) and resveratrol and its analogues, (Fang et al., 2002) has been well documented. Therefore, it is interesting to see if TOH can also interact synergistically with curcumin and its analogues.

Table 1. Inhibition of AAPH-initiated peroxidation of linoleic acid by curcumin and its analogues (COHs) in micelles$^*$

<table>
<thead>
<tr>
<th>Micelle</th>
<th>COHs</th>
<th>$R_p/10^4$ M s$^{-1}$</th>
<th>$R_{inh}/10^4$ M s$^{-1}$</th>
<th>kcl, kclon</th>
<th>$P_{inh}$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDS</td>
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<td>7.4</td>
<td>22.4</td>
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<td></td>
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<tr>
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<td>1</td>
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<td>4.5</td>
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<tr>
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<td>78</td>
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<tr>
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<td>7.9</td>
<td>65</td>
<td></td>
</tr>
<tr>
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<td>4.3</td>
<td>13.0</td>
<td>42</td>
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<tr>
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<td>7.3</td>
<td>22.1</td>
<td>~ 0</td>
<td></td>
</tr>
<tr>
<td>SDS</td>
<td>7</td>
<td>7.6</td>
<td>23.0</td>
<td>~ 0</td>
<td></td>
</tr>
<tr>
<td>SDS</td>
<td>8</td>
<td>7.1</td>
<td>21.5</td>
<td>~ 0</td>
<td></td>
</tr>
<tr>
<td>CTAB</td>
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<td>17.8</td>
<td>20.5</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>2.3</td>
<td>89</td>
<td></td>
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<tr>
<td>CTAB</td>
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<td>1.0</td>
<td>1.1</td>
<td>94</td>
<td></td>
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<tr>
<td>CTAB</td>
<td>3</td>
<td>1.1</td>
<td>1.3</td>
<td>94</td>
<td></td>
</tr>
<tr>
<td>CTAB</td>
<td>4</td>
<td>3.0</td>
<td>3.4</td>
<td>83</td>
<td></td>
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<tr>
<td>CTAB</td>
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<td>9.3</td>
<td>10.7</td>
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<tr>
<td>CTAB</td>
<td>6</td>
<td>17.8</td>
<td>20.5</td>
<td>~ 0</td>
<td></td>
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<tr>
<td>CTAB</td>
<td>7</td>
<td>17.7</td>
<td>20.3</td>
<td>~ 0</td>
<td></td>
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<tr>
<td>CTAB</td>
<td>8</td>
<td>17.8</td>
<td>20.5</td>
<td>~ 0</td>
<td></td>
</tr>
</tbody>
</table>

$^*$ The reaction conditions and the initial concentration of substrates are the same as described in the legends of Figs 2 and 3 for reactions conducted in SDS and CTAB micelles, respectively. The calculations were based on the total reaction volume. Data are the average of three determinations which were reproducible with a deviation of less than ±10%.

$^*$ Taking $R_i$ as 3.3 and 8.7 nM s$^{-1}$ in SDS and CTAB micelles respectively, see text.
exhibited a clear turning point in the decay curves of resveratrol and its analogues (Fang et al., 2002; Zhou et al., 2004b). Addition of curcumin or its analogues together with TOH did not prolong the inhibition period of the latter, but decreased appreciably the rate of inhibition, \( R_p \), and decreased remarkably the rate of propagation after the inhibition time (Figs 4 and 5). The rate of propagation, \( R_p \), when curcumin or its analogues and TOH were used in combination was approximately equal to the rate of inhibition, \( R_p \), when curcumin or its analogues was used individually (compare data in Tables 1 and 2).

The decay kinetics of TOH during the antioxidation reaction has been used to determine the rate of initiation, \( R_i \) (Bowry and Stocker, 1993), and to study the antioxidant synergism of TOH with other antioxidants (Fang et al., 2002; Zhou et al., 2005a, 2005b). In the present work, the decay kinetics of TOH were studied by HPLC separation of the reaction mixture followed by electrochemical determination of TOH. It was found that in the two micelles TOH decayed approximately linearly with time in the initial two half-lives (line a in Figs 6 and 7), in accordance with the kinetic demand for antioxidant reactions (eqn 4, vide infra). The decay rate was 1.5 and 4.0 nM s\(^{-1}\) in SDS and CTAB micelles, respectively. Addition of 1, 2 or 3 did not appreciably change the decay rate of TOH, and no apparent turning point was observed (lines b–d in Figs 6 and 7). Similar decay curves of TOH were obtained for other curcumin analogues (data not shown). This is in contrast to the case of vitamin C (Niki et al., 1984), green tea polyphenols (Zhou et al., 2005b), and resveratrol and its analogues (Fang et al., 2002) which decreased significantly the decay rate of TOH and exhibited a clear turning point in the decay curves of TOH, demonstrating the antioxidant synergism with TOH. Therefore, it is obvious that TOH and curcumin or its analogues worked independently as antioxidants in micelles and they do not interact each other, in accordance with the experimental results mentioned in the previous section.

**DISCUSSION**

It has been proven that the reaction kinetics of lipid peroxidation in micelles and biomembranes follow the

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**Table 2. Inhibition of AAPH-initiated peroxidation of linoleic acid by curcumin and its analogues (COHs) and \( \alpha \)-tocopherol (TOH) in micelles**

<table>
<thead>
<tr>
<th>Micelle</th>
<th>Antioxidant</th>
<th>( R_p/10^8 ) M s(^{-1})</th>
<th>( R_{\text{prop}}/10^8 ) M s(^{-1})</th>
<th>( t_{\text{inh}}/10^2 ) s</th>
<th>( k_{\text{prop}}/10^4 ) M(^{-1}) s(^{-1})</th>
<th>( n' )</th>
<th>( k_i/R_i )</th>
<th>( k_i/R_i )</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDS</td>
<td>TOH</td>
<td>7.7( ^{a} )</td>
<td>1.1</td>
<td>3.0</td>
<td>3.5</td>
<td>2.0</td>
<td>23.3</td>
<td>3.3</td>
</tr>
<tr>
<td>SDS</td>
<td>1 + TOH</td>
<td>1.9( ^{c} )</td>
<td>0.8</td>
<td>3.0</td>
<td>4.8</td>
<td>0.66</td>
<td>5.8</td>
<td>2.4</td>
</tr>
<tr>
<td>SDS</td>
<td>2 + TOH</td>
<td>1.4( ^{c} )</td>
<td>0.7</td>
<td>3.0</td>
<td>5.5</td>
<td>0.66</td>
<td>4.2</td>
<td>2.1</td>
</tr>
<tr>
<td>SDS</td>
<td>3 + TOH</td>
<td>1.6( ^{c} )</td>
<td>0.7</td>
<td>3.0</td>
<td>5.5</td>
<td>0.66</td>
<td>4.8</td>
<td>2.1</td>
</tr>
<tr>
<td>SDS</td>
<td>4 + TOH</td>
<td>2.7( ^{c} )</td>
<td>0.9</td>
<td>3.0</td>
<td>4.3</td>
<td>0.66</td>
<td>8.2</td>
<td>2.7</td>
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<tr>
<td>SDS</td>
<td>5 + TOH</td>
<td>3.8( ^{c} )</td>
<td>0.8</td>
<td>3.0</td>
<td>4.8</td>
<td>0.66</td>
<td>11.5</td>
<td>2.4</td>
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<td>SDS</td>
<td>6 + TOH</td>
<td>7.8( ^{c} )</td>
<td>1.2</td>
<td>3.0</td>
<td>3.2</td>
<td>0.66</td>
<td>23.9</td>
<td>3.8</td>
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<tr>
<td>SDS</td>
<td>7 + TOH</td>
<td>7.8( ^{c} )</td>
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<td>3.0</td>
<td>3.9</td>
<td>0.66</td>
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<td>3.5</td>
<td>0.66</td>
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<td>3.3</td>
</tr>
<tr>
<td>CTAB</td>
<td>TOH</td>
<td>20.0( ^{c} )</td>
<td>1.5</td>
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<td>2.3</td>
<td>2.0</td>
<td>23.0</td>
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<td>1.7( ^{c} )</td>
<td>0.8</td>
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<td>4.3</td>
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<td>2.0</td>
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<tr>
<td>CTAB</td>
<td>2 + TOH</td>
<td>1.0( ^{c} )</td>
<td>0.6</td>
<td>2.3</td>
<td>5.8</td>
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<td>CTAB</td>
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<td>1.1( ^{c} )</td>
<td>0.6</td>
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<td>2.9( ^{c} )</td>
<td>0.7</td>
<td>2.3</td>
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<td>3.3</td>
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<td>CTAB</td>
<td>5 + TOH</td>
<td>10.0( ^{c} )</td>
<td>1.2</td>
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<td>1.0</td>
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<td>1.0</td>
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<td>1.6</td>
</tr>
<tr>
<td>CTAB</td>
<td>7 + TOH</td>
<td>18.2( ^{c} )</td>
<td>1.2</td>
<td>2.3</td>
<td>2.9</td>
<td>1.0</td>
<td>20.9</td>
<td>1.4</td>
</tr>
<tr>
<td>CTAB</td>
<td>8 + TOH</td>
<td>18.4( ^{c} )</td>
<td>1.2</td>
<td>2.3</td>
<td>2.9</td>
<td>1.0</td>
<td>21.1</td>
<td>1.4</td>
</tr>
</tbody>
</table>

\( ^{a} \text{The reaction conditions and the initial concentration of substrates are the same as described in the legends of Figs 4 and 5 for reactions conducted in SDS and CTAB micelles, respectively. The calculations were based on the total reaction volume. Data are the average of three determinations which were reproducible with a deviation of less than ±10%}.\n
\( ^{b} \text{Taking } R_i \text{ as 3.3 and 8.7 nM s}^{-1} \text{ in SDS and CTAB micelles respectively, see text.} \)

\( ^{c} \text{Rate of propagation after exhaustion of the antioxidants.} \)

\( ^{d} \text{Calculated from Eq. (2) by taking } k_p = 37 \text{ M}^{-1} \text{s}^{-1} \text{ (Pryor et al., 1988).} \)

\( ^{e} \text{n'} = R_i t_{\text{inh}}/([COH]_0 + [TOH]_0). \)

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same rate law as that in homogenous solutions (Barclay and Ingold, 1981; Barclay, 1993). The kinetics of linoleic acid (LH) peroxidation initiated by azo-compounds and its inhibition by chain-breaking antioxidant (AH) have been discussed in detail in our previous papers (Zhou et al., 2000). The rate of propagation ($R_p$) and the rate of peroxide formation in the inhibition period ($R_{inh}$) are given by Equations (1) and (2), respectively.

$$\frac{d[LOO^\cdot]}{dt} = R_p = \frac{k_p}{(2k_t)^{1/2}} R_i^{1/2} [LH]$$  \hspace{1cm} (1)

$$R_{inh} = k_{pR} [LH]/(n k_{inh}[AH])$$  \hspace{1cm} (2)

Here $k_p$, $k_R$, and $k_{inh}$ are rate constants for the chain propagation, chain termination and chain inhibition by antioxidants, respectively, and $R_i$ is the apparent rate of chain initiation, which can be obtained by measuring the inhibition period or decay rate of the antioxidant (AH), [Eqs (3) and (4), respectively] (Zhou et al., 2000).

$$R_i = n[AH]/t_{inh}$$  \hspace{1cm} (3)

$$R_i = -n[d[AH]/dt]$$  \hspace{1cm} (4)

Here $n$ is the stoichiometric factor that designates the number of peroxyl radicals trapped by each antioxidant molecule. Since the $n$ value of TOH is generally taken as 2, (Barclay and Ingold, 1981; Barclay, 1993) the $R_i$ value can be determined from the inhibition period or the decay rate of TOH (Barclay and Ingold, 1981; Barclay, 1993; Zhou et al., 2000).

The kinetic chain length ($kcl$) defines the number of chain propagations induced by each initiating radical and is given by Equations (5) and (6) for uninhibited and inhibited peroxidation respectively. The kinetic parameters deduced from Figs 2–7 are listed in Tables 1 and 2.

$$kcl_p = R_p/R_i$$  \hspace{1cm} (5)

$$kcl_{inh} = R_{inh}/R_i$$  \hspace{1cm} (6)

It can be seen from Figs 2–7 and Tables 1 and 2 that the reaction medium exerts significant effects on the rate of initiation, and the antioxidant activity of TOH and curcumin and its analogues (COHs). The $R_i$ values calculated from the inhibition time [Eqn (3)] are $3.3 \times 10^{-9}$ and $8.7 \times 10^{-9}$ nM s$^{-1}$ in SDS and CTAB micelles respectively, which are in good agreement with the values of $3.0 \times 10^{-9}$ and $8.0 \times 10^{-9}$ nM s$^{-1}$ respectively, obtained from the decay of TOH [Eqn (4)]. Taking the concentration of AAPH as 6.3 mM, the $R_i$ value in CTAB micelles corresponds to $1.4 \times 10^{-9}$ [AAPH] s$^{-1}$, in good agreement with that reported previously in liposomal dispersions (Bowry and Stocker, 1993). However, the $R_i$ value of AAPH in SDS micelles is appreciably smaller than that in CTAB micelles. This can be understood because AAPH is positively charged, hence it is prone to being adsorbed onto the surface of SDS micelles; this in turn reduces the effective initiation due to the cage effect. On the other hand, the inhibition rate constant, $k_{inh}$, of TOH is larger in SDS than in CTAB micelles. This is due to the fact that lipid peroxyl radicals are polar (dipole moment of ca. 2.6 Debye) and electrophilic (Barclay and Ingold, 1981). Thus they should move to the surface of micelles and subject to intra- and inter-micellar diffusion (Castle and Perkins, 1986) more quickly in SDS than in CTAB micelles, so as to react with the antioxidant whose reactive phenoxyl functional group shall reside on the surface of the micelles (Castle and Perkins, 1986). In addition, the microviscosity in the interior of CTAB micelle is 2.6 times larger than that of SDS micelle (Witte and Engberts, 1988), making the intra-micellar diffusion slower in CTAB micelle. It has been proved previously that intra- and inter-micellar diffusions are rate-limiting steps for the antioxidation reaction conducted in micelles (Castle and Perkins, 1986; Liu et al., 1992).

It can be seen from Figs 2 and 3 that addition of COHs in micelles only decrease the rate of hydroperoxide formation, but no inhibition period is observed. The similar antioxidant behavior was also found in β-carotene and tea polyphenols against AAPH-induced linoleic acid peroxidation in micelles (Liu et al., 1996) and solution (Jia et al., 1998), respectively. This indicates that, in micelles, COHs can only trap the initiating radicals (ROO$^\cdot$) derived from the thermal decomposition of AAPH but they are unable to trap the propagating peroxy radicals (LOO$^\cdot$). The antioxidative efficacy can be accessed by the percentage inhibition ($P_{inh}$) or the kinetic chain length in the inhibition period ($kcl_{inh}$) that follows the same sequence of $2 \sim 3 > 1 > 4 > 5 > 6 > 7 > 8$ in SDS and CTAB micelles.

The antioxidant mechanisms of curcumin have recently been a focus of interest of free radical chemists and biologists (Jovanovic et al., 1999; Barclay et al., 2000; Jovanovic et al., 2001; Sun et al., 2002; Priyadarshini et al., 2003; Shen et al., 2005). The central argument is whether the phenolic hydrogen or the central methylene hydrogen in the heptadienone moiety is responsible for its antioxidant activity. Jovanovic et al. (1999) studied the reaction of curcumin with methyl radical and tert-butoxy radical by laser flash photolysis and pulse radiolysis, and concluded that in acidic and neutral aqueous and acetonitrile solutions, curcumin is a superb H-atom donor by donating the H-atom from the central methylene group rather than from the phenolic group. On the other hand, Barclay et al. (2000) compared the antioxidant activity of curcumin (1) and dimethylcurcumin (6) against free radical initiated peroxidation of styrene in chlorobenzene solution and concluded that curcumin is a classical phenolic chain-breaking antioxidant, donating H-atoms from the

Figure 7. Decay of α-tocopherol (TOH) during the inhibition of linoleic acid peroxidation in 15 mM CTAB micelles at 37°C, initiated with AAPH and inhibited with TOH and COHs. (a) Decay of TOH in the absence of COHs; (b) Decay of TOH in the presence of 1; (c) Decay of TOH in the presence of 3; (e) Decay of TOH in the presence of 2. [LH]$_0$ = 15.2 mM; [AAPH]$_0$ = 6.3 mM; [TOH]$_0$ = 10 μM; [COHs]$_0$ = 10 μM.

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phenolic groups. Recently Priyadarsini et al. (2003) also compared the antioxidant activity of 1 and 7 against radiation-induced lipid peroxidation of rat liver microsomes and the free radical scavenging activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH), and concluded that the phenolic group plays a major role in the activity of curcumin. Theoretical calculations by density functional theory (DFT) demonstrated that the enol form of curcumin is significantly more stable than the diketo form and that the bond dissociation enthalpy (BDE) of the phenolic O-H bond is significantly lower than the BDE of the central C-H bond, suggesting that the H-atom abstraction shall take place in the phenolic group (Sun et al., 2002; Wright, 2002; Priyadarsini et al., 2003). It was also pointed out that the relative contribution of the phenolic group and the central methylene group on the antioxidant activity depends on the activity of the attacking radical and the reaction medium (Wright, 2002; Shen et al., 2005). Litwinienko and Ingold (2004) recently compared the rate constants of the reaction of DPPH with curcumin, dehydrozingerone and isoeugenol in ionizing solvents and nonionizing solvents, and resolved the curcumin antioxidant controversy by the mechanism of sequential proton loss electron transfer (SPLET). That is, in solvents that support ionization, curcumin reacts with electrophilic radicals initially at ionized keto-enol moiety and the resulting neutral radical loses a phenolic proton, thus yielding the same phenoxyl radical, as would have been formed by hydrogen atom transfer (HAT) from the phenolic hydroxyl group of the curcumin anion to the radical (Litwinienko and Ingold, 2004). However, in nonionizing solvent, the SPLET mechanism cannot occur and the reaction involves only HAT from a phenolic hydroxyl group of the neutral curcumin to the radical (Litwinienko and Ingold, 2004).

In the present work, compounds 6, 7 and 8 which bear no phenolic group are totally inactive on inhibition of AAPH-induced linoleic acid peroxidation in micelles, indicating unambiguously that in the present case the phenolic group is responsible for the antioxidant activities of curcumin and its analogues. Compounds 2 and 3 which bear an ortho-diphenolic functionality are the most active ones. Obviously, this is due to, not only the number of phenolic groups, but also to the high reactivity of ortho-methoxyphenolic and ortho-hydroxyphenolic functionalities. It has been proven that the ortho-methoxyl group can form intramolecular hydrogen bond with the phenolic hydrogen, making the H-atom abstraction from the ortho-methoxyphenols remarkably easy (de Heer et al., 2000). It was also known that the ortho-hydroxyl substitution on phenol would make the oxidation intermediate, ortho-hydroxyphenoxyl radical, more stable due to the intramolecular hydrogen bonding interaction as reported recently from both experiment (Foti and Ruberto, 2001) and theoretical calculations (Wright et al., 2001). The theoretical calculation showed that the intramolecular H-bond in ortho-OH phenoxyl radical is ca. 4 kcal/mol stronger than that in the parent molecule catechol and the BDE of catechol is 9.1 kcal/mol lower than that of phenol (Wright et al., 2001). We have also found the significantly higher antioxidant activity of molecules bearing ortho-diphenoxyl functionality in flavonols (Hou et al., 2004a; Hou et al., 2004b; Zhou et al., 2005a) and resveratrol analogues (Fang et al., 2002; Cai et al., 2003).

The synergistic antioxidative effect of TOH with other antioxidants, such as L-ascorbic acid (vitamin C) (Niki et al., 1984; Liu et al., 1988), green tea polyphenols (Zhou et al., 2000, 2005b), and resveratrol (Fang et al., 2002) has been well documented and proved to be due to the reduction of \( \alpha \)-tocopheroxyl radical (TO\( \cdot \)) by the co-existent antioxidant (ArOH) to regenerate TOH [Eqn (7)].

\[
\text{TO}^\cdot + \text{ArOH} \rightarrow \text{TOH} + \text{ArO}^- \tag{7}
\]

It can be seen from Figs 4–7 and Tables 1 and 2 that the overall inhibition time when COH and TOH were used in combination is approximately equal to that TOH when it was used individually, and the decay of TOH is almost not changed in the presence of COHs. However, the \( R_p \) of TOH is remarkably decreased after the inhibition time in the presence of COH. This \( R_p \) is approximately equal to the \( R_{oh} \) of COH when it is used individually. These facts suggest that TOH and the curcumin analogues shall act independently, i.e., no synergistic antioxidant interaction takes place between the curcumin analogues and TO (Eqn 7). This is understandable because the oxidation potential of curcumin (0.77 V at pH 7) is considerably higher than that of TOH (0.48 V at pH 7) (Jovanovic et al., 1999), rendering the \( \alpha \)-tocopherol regeneration reaction [Eqn (7)] by curcumin an unfavorable process. Therefore, TOH shall act firstly, and COH continuously works after the exhaustion of TOH.

In brief, curcumin and its analogues, except 6, 7 and 8, are effective antioxidants against AAPH-initiated peroxidation of linoleic acid in micelles, and the H-atom abstraction from the phenolic group is responsible for the activity. Of particular interest is the finding that the compounds bearing ortho-diphenoxyl and/or ortho-methoxyphenoxyl functionalities exhibit remarkably higher antioxidative activity than the ones bearing no such functionalities, which gives us useful information for antioxidant drug design. Synergistic antioxidant interaction between curcumin and its analogues and TOH is not observed in micelles.

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