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Structure–Activity Relationships for the Inhibition of Lipid Peroxidation and the Scavenging of Free Radicals by Synthetic Symmetrical Curcumin Analogues

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Abstract

A number of ring substituted analogues of curcumin were synthesized. Their antioxidant properties were studied using three models, inhibition of lipid peroxidation, scavenging of 1,1′-diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-azinobis(3-ethyl-benzthiazoline-6-sulphonate radical ($ABTS^{+\cdot}$).

In all the models, the phenolic analogues were more active than the non-phenolic analogues, some of which were inactive. The highest antioxidant activity was obtained when the phenolic group was sterically hindered by the introduction of two methyl groups at the *ortho* position. This and several other compounds were more active than the standard antioxidants α-tocopherol and trolox.

This study has demonstrated that the phenolic group is important for the antioxidant activity of curcumin and that the structural features that enhance the antioxidant properties of phenols are optimized in curcumin to a significant extent.

Curcumin is a yellow pigment present in the rhizomes of *Curcuma longa*, which has been shown to have significant antioxidant (Noguchi et al 1994; Sreejayan & Rao 1996) and therapeutic properties (Srimal 1997). Many of the biological activities of curcumin have been attributed to its antioxidant properties (Perchellet & Perchellet 1989; Mukundan et al 1993; Ruby et al 1998). Several studies have demonstrated that the presence of a phenolic group is important for these properties and that activity is enhanced by the presence of a methoxy group in the ring (Sharma 1976; Rao 1995; Ruby et al 1995; Sreejayan & Rao 1996). However, Tonnesen & Greenhill (1992) found that 5-hydroxy-1,7-diphenyl-1,4,6-heptatriene-3-one, a curcumin analogue lacking phenolic and methoxy groups, was as active as curcumin in scavenging hydroxyl radicals and in other redox properties. Conney et al (1990) used similar analogues and studied their inhibitory effect on 12-*O*-tetradecanoyl phorbol-13-acetate-induced tumour promotion in mouse skin. That author concluded that the phenolic group was not essential for antitumour activity. To understand the importance of the phenolic group and other substituents we have synthesized various analogues of curcumin and studied their antioxidant properties.

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Materials and Methods

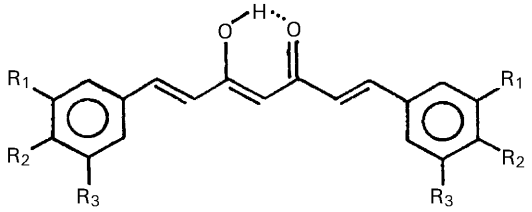
Materials

Soyphosphatidylcholine, 2-thiobarbituric acid, butylated hydroxytoluene (BHT), 1,1′-diphenyl-2-picryl hydrazyl (DPPH), 2,2′-azino bis (3-ethyl-benzthiazoline)-6-sulphonic acid (ABTS), potassium persulphate, trolox and α-tocopherol were purchased from Sigma Chemical Co. (St Louis, MO). Other chemicals were of analytical grade.

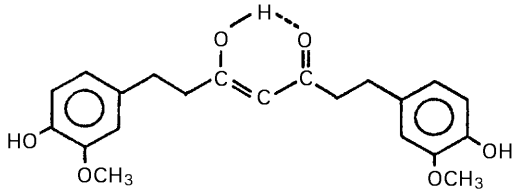
Synthesis

Curcumin and analogues were synthesized as described by Pabon (1964) (Table 1). Tetrahydrocurcumin was a gift sample from Sami Chemicals and Extracts (Bangalore, India). All the compounds except **III** are known in the literature. Structures were confirmed by melting point deter-

Table 1. Effect of curcumin analogues on lipid peroxidation and scavenging of DPPH and $ABTS^{+\cdot}$ radicals.



1,7-Bis-(R-phenyl-) 1,6-heptadiene-3,5-dione



XIII Tetrahydrocurcumin

Compound	R_1	R_2	R_3	Lipid peroxidation inhibition[a] IC50 (μM)[c]
I	H	OH	H	2·19 (2·17–2·21)
II curcumin	OCH_3	OH	H	1·30 (1·29–1·31)
III	OEt	OH	H	1·11 (1·09–1·13)
IV	CH_3	OH	CH_3	0·63 (0·56–0·66)
V	t-C_4H_9	OH	t-C_4H_9	6·48 (6·32–6·68)
VI	H	H	H	NA
VII	H	CH_3	H	NA
VIII	H	SCH_3	H	NA
IX	H	OCH_3	H	NA
X	OCH_3	OCH_3	H	NA
XI	OCH_3	OCH_3	OCH	15·32 (15·15–15·59)
XII	OCH_3	$OCOCH_3$	H	1·85 (1·84–1·86)
XIII tetrahydrocurcumin				1·83 (1·79–1·96)
α-Tocopherol				12·53 (12·35–12·71)
Trolox				44·68 (44·04–45·49)

Compound	DPPH scavenging[b]		$ABTS^{+\cdot}$ scavenging		
	IC50 (μM)[c]	$t_{\frac{1}{2}}$ (min)[d]	TEAC[e]		
			3 min	9 min	15 min
I	32·08 (30·99–36·27)	< 1	3·04 ± 0·09	4·31 ± 0·18	4·96 ± 0·20
II curcumin	20·02 (19·59–21·11)	< 1	2·61 ± 0·09	3·09 ± 0·15	3·37 ± 0·12
III	30·32 (29·52–34·01)	< 1	2·36 ± 0·14	3·07 ± 0·13	3·32 ± 0·13
IV	21·75 (20·86–25·56)	34·49	0·89 ± 0·06	1·13 ± 0·05	1·28 ± 0·05
V	23·72 (23·51–25·93)	51·28	0·81 ± 0·03	0·98 ± 0·04	1·07 ± 0·04
VI	> 250	5·79	1·57 ± 0·12	2·78 ± 0·08	3·36 ± 0·13
VII	> 250	10·45	0·67 ± 0·04	1·52 ± 0·06	1·95 ± 0·09
VIII	NA till 90	10·17	1·09 ± 0·07	–	–
IX	> 250	4·48	2·05 ± 0·06	2·04 ± 0·09	2·14 ± 0·10
X	> 250	4·03	1·86 ± 0·12	2·49 ± 0·07	2·67 ± 0·09
XI	NA	11·24	1·90 ± 0·09	2·98 ± 0·09	3·43 ± 0·12
XII	NA	10·81	1·33 ± 0·10	2·01 ± 0·08	2·33 ± 0·07
XIII tetrahydrocurcumin	18·22 (17·62–19·01)	1·88	2·08 ± 0·05	2·37 ± 0·07	2·52 ± 0·08
α-Tocopherol	91·50 (87·37–100·19)	f	0·95 ± 0·02	0·96 ± 0·01	0·96 ± 0·01
Trolox	58·03 (57·39–58·77)	f	RS	RS	RS

[a]Percent inhibition of formation of thiobarbituric acid reactive substances (TBARS) by test compounds was measured. The amount of TBARS formation in the control (containing Fe^{3+}/ascorbate but without test compound) was 14·03 ± 0·5 nmol mL^{-1} (mean ± s.d.), which was calculated using a molar extinction coefficient value of $1{\cdot}56 \times 10^5$ M^{-1} cm^{-1}. [b]Reduction of DPPH (100 μM) by curcumin analogues was estimated by measuring the change in absorbance at 517 nm. [c]IC50 value is the concentration required for inhibition of 50% of lipid peroxidation or scavenging of DPPH radical and was determined from the dose–response curve; values given in parentheses are 95% confidence intervals. [d]$t_{\frac{1}{2}}$ measures the time taken by the test compound at 5 μM to bleach the $ABTS^{+\cdot}$ solution (initial absorbance ca. 0·70–0·73) to 50%. [e]TEAC is the trolox equivalent antioxidant capacity, which is defined as the millimolar concentration of a trolox solution having the antioxidant capacity equivalent to a 1·0 mM solution of the substance under investigation. IC50 for the test compounds and trolox were measured from the dose–response curve made from the activity vs concentration (1–7·5 μM) plot. The ratio of IC50 of trolox to the test compound gives TEAC. [f]Activity of both trolox and tocopherol in scavenging the $ABTS^{+\cdot}$ radical remains the same at 3 and 15 min, hence $t_{\frac{1}{2}}$ value was not calculated. NA, not active. RS, relative standard. Results above are derived from three different sets of experiments (n = 3).

mination, and UV, IR and NMR spectra. The purity of the compounds was tested by TLC and elemental analysis (C, H) of the compounds showed less than 0·5% variation.

Inhibition of lipid peroxidation

Inhibition of Fe^{3+}/ascorbate-induced lipid peroxidation in liposomes by curcumin analogues was measured as described by Rajakumar & Rao (1995). Soyphosphatidylcholine liposome dispersion was prepared by the "film" method.

Soyphosphatidylcholine (25 mg) was dissolved in chloroform (2 mL) and heated in a round-bottomed flask in a rotary evaporator until a thin homogeneous film was formed, which was then hydrated with normal saline (5 mL). The mixture was sonicated for 10 min to obtain a homogeneous suspension and then further diluted 10 times with normal saline. The incubation mixture contained 0·5 mL liposomes in normal saline (final concentration 250 μg), 0·1 mL potassium chloride (2·31 M) and 0·7 mL methanolic solution of test compound. Peroxidation was initiated by adding 0·1 mL ferric chloride (8 mM) and 0·1 mL ascorbic acid (1 mM) into the liposomal system. After 20 min incubation, the reaction was stopped by the addition of 0·1 mL BHT (0·5%) under ice-cold conditions. 2-Thiobarbituric acid (0·75%; 0·4 mL) and 0·5 mL sodium acetate–acetic acid buffer (pH 3·5) were then added and heated over a boiling-water bath for 15 min. After cooling, the red chromogen was extracted with 2·5 mL butanol–pyridine (15 : 1) and measured at 532 nm. Control experiments without the test compound were conducted in an identical manner.

A correction was made in both test and control for spontaneous peroxidation by conducting experiments in the absence of inducing agents. The IC50 (inhibitory concentration for 50%) value was calculated from the linear regression curve of percent inhibition of formation of thiobarbituric acid reactive substances (TBARS) plotted against concentration of test compounds. Nodal points for drawing the graph ranged from 5 to 7.

Free radical scavenging activity by DPPH method

Test compound was dissolved in methanol or dimethylsulphoxide (DMSO) and added to a methanolic solution of DPPH (final concentration 100 μM) and incubated at ambient temperature. The organic solvent present was 50% in the final reaction mixture and the remaining was made up with phosphate-buffered saline, pH 7·4 (5 mM). After 20 min, the blue violet chromogen was measured at 517 nm. Most of the phenolic derivatives were dissolved in methanol but for those compounds poorly soluble in methanol DMSO was used. A similar treatment was carried out on curcumin (**II**) for comparison. A suitable control containing vehicle only (without test compound) was kept and the values, if any, were subtracted from the test values.

Free radical scavenging activity by $ABTS^{+\cdot}$ method

Scavenging a radical cation $ABTS^{+\cdot}$ by the test compounds was measured as described by Re et al (1999). $ABTS^{+\cdot}$ radical cation was prepared by adding potassium persulphate solution (200 μL, 70 mM solution) to the ABTS solution (50 mL, 2 mM solution). Oxidation of ABTS started immediately, but the absorbance was not maximal and stable until approximately 6-h later. Despite the radical being stable for two days at room temperature in the dark, for all experiments $ABTS^{+\cdot}$ was prepared freshly and used after 12 h. To study the antioxidant activity, the final reaction mixture contained test compound in methanol (ca. 0·3–0·7 mL), phosphate-buffered saline (5 mM, pH 7·4; a quantity sufficient to obtain a final volume of 3·5 mL after adding all the components) and 0·3 mL $ABTS^{+\cdot}$ solution. After 3 min the absorbance was measured at 734 nm. The final concentration of methanol in the incubation mixture ranged from 8 to 20%. Suitable controls containing solvent (without the test compound) and $ABTS^{+\cdot}$ were kept and the values were subtracted from the respective test readings. The absorbance of the incubation mixture containing $ABTS^{+\cdot}$ only ranged from 0·70 to 0·73. For the relative standard trolox, the concentration required for 50% inhibition (IC50) of $ABTS^{+\cdot}$ radical was calculated from the linear regression curve of percent inhibition against concentration. Similarly IC50 values for other test compounds were calculated. The ratio of IC50 value of trolox to test compound gives the trolox equivalent antioxidant activity (TEAC).

Statistical analysis

Confidence interval (95%) was calculated by regression analysis.

Results

Inhibition of lipid peroxidation

The phenolic analogues were more active than the non-phenolic analogues (Table 1). The highest activity (IC50 = 0·63 μM) was exhibited by compound **IV** where the phenolic group was hindered by the presence of two methyl groups at the *ortho* position to the phenolic group. Curcumin (**II**) was almost half as active (IC50 = 1·30 μM) as com-

pound **IV**. When the two methyl groups *ortho* to the phenolic group (compound **IV**) was replaced with more bulkier *tert*-butyl groups (compound **V**), the activity was reduced significantly (IC50 = 6·48 μM). The importance of the methoxy group in curcumin was demonstrated by the fact that its deletion as in compound **I** reduced the activity to a considerable extent (IC50 = 2·19 μM). Similarly when the methoxy group was replaced by the ethoxy group (compound **III**) the activity improved to some extent (IC50 = 1·11 μM). All the non-phenolic analogues were either less active or inactive. However, the diacetyl derivative of curcumin (compound **XII**) showed appreciable activity (IC50 = 1·85 μM). It is possible that the acetyl group may be hydrolysing to some extent during the test to release the free phenolic group. It is interesting to note that tetrahydrocurcumin (**XIII**) showed significant activity comparable with curcumin, suggesting that the presence of unsaturation in the side chain of curcumin may not be important for the activity. All the phenolic compounds showed higher activity than the standard antioxidants α-tocopherol and trolox.

Free radical scavenging activity by DPPH method
Antioxidants react with the nitrogen centred radical DPPH and converts it to 1,1′-diphenyl-2-picryl hydrazine. The change in the absorbance produced in this reaction has been used as a measure of radical scavenging potential (Blois 1958; Hadjipavlou-Litina & Geronikaki 1996; Mathiesen et al 1997). Table 1 shows the scavenging of DPPH radical by curcumin analogues. In this model, phenolic analogues showed good activity. Non-phenolic analogues were either less active or inactive. Tetrahydrocurcumin showed highest activity (IC50 = 18·22 μM) followed by curcumin (IC50 = 20·02 μM). Sterically hindered phenolic analogues such as compounds **IV** and **V** were slightly less active than curcumin. The importance of the methoxy group adjacent to the phenolic group as in curcumin is evident from the fact that deletion of the methoxy group, as in compound **I**, resulted in a significant reduction in the activity. In this model the standard antioxidants α-tocopherol and trolox were less active than the phenolic analogues.

Free radical scavenging activity by $ABTS^{+\cdot}$ method
Antioxidants reduce the coloured $ABTS^{+\cdot}$ radical cation to colourless ABTS. The extent of decolorization is related to the antioxidant activity. The $ABTS^{+\cdot}$ method has been found to be useful in evaluating various antioxidants (Salah et al 1995; Plumb et al 1996). Table 1 gives the time and concentration dependent scavenging of $ABTS^{+\cdot}$ by curcumin analogues. The time taken to scavenge 50% of $ABTS^{+\cdot}$ at 5 μM was measured ($t_{\frac{1}{2}}$). Curcumin, bisdesmethoxy curcumin (**I**) and ethoxy analogue (**III**) showed very high activity ($t_{\frac{1}{2}} < 1$ min). Tetrahydrocurcumin also showed significant activity. Sterically hindered phenolic analogues (compounds **IV** and **V**) showed poor activity. In this model many non-phenolic analogues showed significant activity. The ring unsubstituted analogue (**VI**), the methoxy analogue (**IX**) and the dimethoxy analogue (**X**) showed significant activity although these are non-phenolic analogues. TEAC is a useful parameter for comparing antioxidant activities and has been applied in studying a number of antioxidants (Romay et al 1996; Miller et al 1997). TEAC is defined as the millimolar concentration of a trolox solution having the antioxidant capacity equivalent to a 1·0 mM solution of substance under investigation. Higher TEAC values indicate higher antioxidant activity. Table 1 gives the TEAC calculated based on 3, 9 and 15 min. Curcumin and its ethoxy analogue (**III**) were less active. The phenolic analogues were more active compared with non-phenolics. Most of the compounds showed higher activity than the standard antioxidants α-tocopherol and trolox.

Discussion

The phenolic group is important for the antioxidant activity of curcumin. In all of the three models studied the phenolic analogues were more active than the non-phenolic analogues, which were sometimes inactive. The introduction of a methoxy group at the *ortho* position to phenol increased the activity to a significant extent in the lipid peroxidation model and in the DPPH model. Studies have shown that the *ortho* substitution with an electron donor such as methoxy, methyl etc., increases the antioxidant activity of phenols by enhancing the stability of phenoxyl radical by inductive effect (Burton et al 1985; Cuvelier et al 1992). It has been suggested that the steric crowding at *ortho* positions frees the phenolic group from hydrogen bonding to the media and enhances the facile transfer of the hydrogen atom (Barclay et al 1993). Steric crowding also increases the stability of the phenoxy radical (Burton et al 1985; Cuvelier et al 1992). The good antioxidant activity of α-tocopherol is attributed to the steric crowding effect of two methyl groups at the *ortho* position to the phenolic group. A similar mechanism may be

responsible for the high activity of compound **IV** where two methyl groups were present *ortho* to phenol. Compound **IV** was more active than curcumin in the lipid peroxidation model. Nurfina et al (1997) reported that compound **IV** was more active than curcumin as an anti-inflammatory agent. However, when the *ortho* position was substituted with a bulkier group such as *tert*-butyl group, as in compound **V**, the activity was reduced to a significant extent in the lipid peroxidation model. Thus it appears that there is an optimum requirement for the steric crowding. Phenols with *para*-substitution stabilize the phenoxyl radical either directly or vinylogously for better activity (Andersson et al 1996). The presence of a double bond in conjugation with the phenolic ring in curcumin helps in increasing the stability of the phenoxyl radical by electron delocalization. When the double bond is saturated, as in tetrahydrocurcumin, the electron delocalization is reduced. However, the antioxidant activity of tetrahydrocurcumin was reduced to a slight extent only in the lipid peroxidatioin model and $ABTS^{+\cdot}$ model. In the DPPH model it was slightly more active than curcumin.

This study has demonstrated that the phenolic group is important for the antioxidant activity of curcumin and that the structural features that enhance the antioxidant properties of phenols are optimized in curcumin to a significant extent. Compounds with more activity could be designed by investigating steric and electronic factors in a systematic manner.

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