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Synthesis and evaluation of curcumin analogues as potential thioredoxin reductase inhibitors

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ABSTRACT

Series of curcumin derivatives were synthesized; the inhibitory activities on thioredoxin reductase (TrxR) of all analogues were evaluated by DTNB assay in vitro. It is found that most of the analogues can inhibit TrxR in the low micromolar range; Structure-activity relationship analysis reveals that analogues with furan moiety have excellent inhibitory effect on TrxR in an irreversible manner, indicating that the furan moiety may serve as a possible pharmacophore during the interaction of curcumin analogues with TrxR. The effect of selected curcuminoids on growth of different TrxR overexpressed cancer cell lines was also investigated and discussed.

1. Introduction

Thioredoxin reductase (TrxR EC 1.8.1.9) belongs to a well-known class of homodimeric pyridine nucleotide disulfide oxidoreductases.[1] As part of a major thiol regulating system, TrxR catalyzes NADPH-dependent reduction of the redox-active disulfide in thioredoxin (Trx), which serves a wide range of functions in cell proliferation and intracellular redox control.[2,3] Many tumor cells have shown elevated TrxR level.[4–7] Moreover, it is believed that TrxR plays an important role in tumor proliferation and drug resistance of tumor cells.[8–10] Inhibition of TrxR and its related redox reactions may thus contribute to a successful cancer therapy.

Several effective natural and synthetic TrxR inhibitors are now available, possessing antitumor potential ranging from induction of oxidative stress to cell cycle arrest and apoptosis.[11–13] Curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione), a yellow spice and pigment isolated from the rhizome of *Curcuma longa*, has been found useful in various bioactivities,[14–17] such as antioxidant, anti-inflammatory and anti-HIV protease activities. Furthermore, curcumin was found to be a chemopreventive agent of tumor initiation and proliferation.[18–20] Many explanations on the cancer preventative activity of curcumin have been proposed.[21,22] Arne Holmgren et al. first reported that the rat TrxR activity could be inhibited by curcumin.[23] It was proposed that the modification of TrxR by curcumin should be the mechanistic explanation for the cancer preventive activity of curcumin. Although some reports on biological evaluation of curcumin analogues have been published in recent years, studies on the curcumin analogues as TrxR inhibitors remain scarce up to now. To search for useful drug candidates in the development of chemotherapeutic agents, we thought it would be interesting to evaluate some symmetrical and unsymmetrical curcumin analogues with different substituents on aromatic or heterocyclic rings for their TrxR inhibitory effect and cancer cell growth inhibitory activity.

2. Results and discussion

2.1. Chemistry

Various classes of unsymmetrical analogues ***1a–1e***, ***2a–2g***, ***3a–3b*** were designed by altering the substituted group, aryl ring and ketene moiety respectively (Scheme 1).

All of the unsymmetrical curcumin derivatives were synthesized according to a solid phase synthetic route we developed in our previous letter.[24]

To extend the structure diversity of curcumin analogues for further structure–activity relationship (SAR) information, several symmetrical curcumin derivatives ***4a–4h*** also designed and synthesized by treating the methene protected acetyl acetone with aromatic aldehydes in the presence of tributyl borate and *n*-butylamine (Scheme 2).

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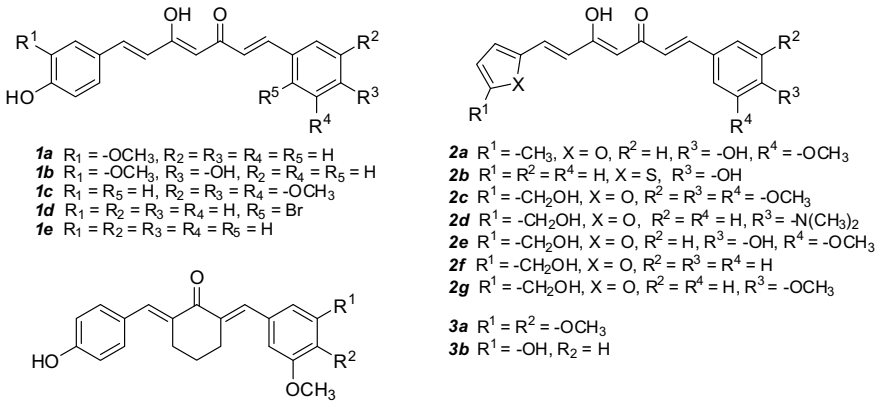
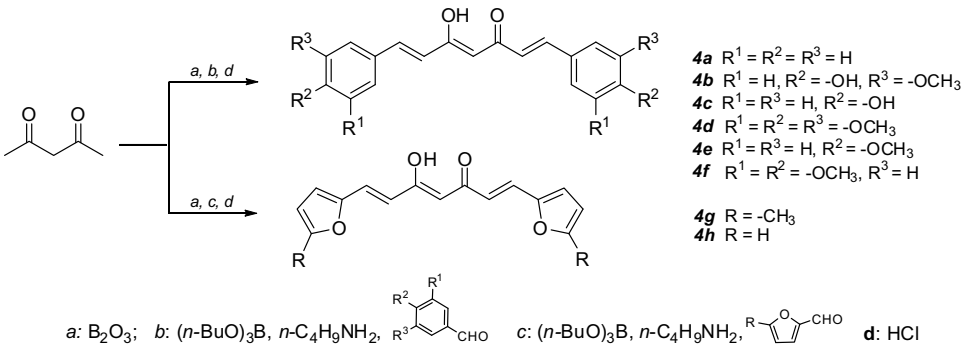


Scheme 1. Structure of unsymmetrical curcumin analogues.



Scheme 2. Synthesis of symmetrical curcumin derivatives ***4a***–***4h***.

2.2. Evaluation of the TrxR inhibitory activities of curcumin analogues

The TrxR inhibitory activities of all curcumin analogues were determined with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) assay.[25,26] The IC_{50} (half maximal inhibitory concentration) values are summarized in Table 1. The results showed that most of unsymmetrical curcumin analogues exhibited excellent inhibitory effects on TrxR, while all the tested traditional symmetrical curcumin analogues (***4a***, ***4c***–***4f***) except the natural curcumin (***4b***) showed poor activities. Compounds ***4a***, ***4d***–***4f*** do not possess hydroxylated moiety, compared with the TrxR inhibition activity of other hydroxylated compounds, this indicates that the hydroxylated moiety contribute the inhibitory activity. On the other hand, the low activity of dihydroxylated compound ***4c*** may result from its relatively higher polarity.

It should be noted that the IC_{50} value for the curcumin was determined to be 38.3 μM, which was approximately 10-fold larger than what was reported earlier.[23] We reasoned that this result was due to the different incubation time of curcumin with TrxR during the inhibition assay; 4 min assay was performed in current studies and 2 h assay was performed in the earlier report. We noted a similar result that the natural curcumin could inhibit TrxR in a time-dependent manner (data not shown), and with an IC_{50} value of 4.2 μM after a 2-h incubation time.

As a whole, the unsymmetrical analogues with benzene rings showed moderate inhibitory effect on TrxR. As compared to ***1e***, the introduction of a halide group Br at the R^5 (namely ***1d***) decreased the inhibitory activity. In addition, the introduction of one terminal hydroxyl or/and methoxyl to ***1e*** did not contribute to the inhibitory activity (***1a***, ***1b***); on the contrary, it led to a decrease of inhibitory activity for ***1a***.

Noticeably, all unsymmetrical compounds with a furan moiety (***2a***, ***2c***,***2e***–***2g***) except ***2d*** exerted a much higher inhibitory activity than that of the natural curcumin. Symmetrical analogues ***4g*** and ***4h***, which bear furan moieties on both terminals of the molecule, were designed and synthesized therefore based on this primary result. Expectedly, both of ***4g*** and ***4h***, especially ***4g***, showed excellent inhibitory activities against TrxR, indicating that the furan moiety may serve as a possible pharmacophore. It may be also due to the interaction of furan moiety with TrxR by hydrogen bonding, which induced the conformational changes of TrxR, making the active sites of TrxR easier to access. Compared to ***2f***, compound with thiophene moiety (***2b***) did not increase the activity. Interestingly, the introduction of dimethylamino group at the benzene moiety (namely ***2d***) decreased the activity significantly compared with ***2f*** in spite of the furan moiety. It might be explained that the high polarity of protonized ***2d*** led to the negative inhibitory effect on TrxR.

Analogues with dimethylenecyclohexanone moiety (***3a***, ***3b***) displayed activities against TrxR; however, no evidence supported that dimethylenecyclohexanone moiety would be a positive or negative factor to the inhibitory activity, since the activity of ***1b***/***3b*** and ***1c***/***3a*** showed ruleless and had no apparent coherence.

2.3. Irreversible inhibition of TrxR by curcumin analogue *4g*

It has been well known that mammalian TrxR contains a C-terminal selenocysteine active site, which is essential for catalysis and can be irreversibly modified by many electrophilic agents.[27] To investigate the nature of the TrxR inhibition by curcumin analogues, we determined the reversibility of TrxR inactivation by curcumin analogue ***4g***. As illustrated in Figure 1, when ***4g*** was incubated with TrxR for 2 h and then subjected to ultrafiltration, the TrxR activity was found unrecoverable. This suggested that

Table 1
Inhibition of TrxR by curcumin analogues

Entry	R^1	R^2	R^3	R^4	R^5	IC_{50} (μM)
1a	OCH_3	H	H	H	H	57.0
1b	OCH_3	H	OH	H	H	20.0
1c	H	OCH_3	OCH_3	OCH_3	H	13.8
1d	H	H	H	H	Br	50.0
1e	H	H	H	H	H	23.3

Entry	R^1	R^2	R^3	R^4	X	IC_{50} (μM)
2a	CH_3	H	OH	OCH_3	O	0.5
2b	H	H	OH	H	S	20.0
2c	CH_2OH	OCH_3	OCH_3	OCH_3	O	2.0
2d	CH_2OH	H	$N(CH_3)_2$	H	O	62.2
2e	CH_2OH	H	OH	OCH_3	O	1.0
2f	CH_2OH	H	H	H	O	1.3
2g	CH_2OH	H	OCH_3	H	O	0.8

Entry	R^1	R^2	IC_{50} (μM)
3a	OCH_3	OCH_3	24.9
3b	OH	H	5.1

Entry	R^1	R^2	R^3	IC_{50} (μM)
4a	H	H	H	>100
4b (Curcumin)	OCH_3	OH	H	38.3
4c	H	OH	H	>100
4d	OCH_3	OCH_3	OCH_3	>100
4e	H	OCH_3	H	>100
4f	OCH_3	OCH_3	H	>100

Entry	R	IC_{50} (μM)
4g	CH_3	0.3
4h	H	1.6

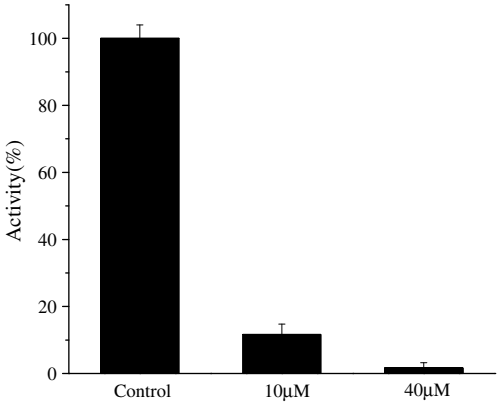


Figure 1. TrxR could be irreversibly inhibited by compound ***4g***. NADPH-reduced TrxR was incubated with indicated concentrations of IG3 at room temperature. The enzyme solutions were subjected to ultrafiltration after 2 h. TrxR activity was expressed as the percentage of the control in the absence of ***4g***. Data are presented as means ± SD of triplicate experiments. $^{*}p < 0.001$ compared with the control.

TrxR was irreversibly inhibited by curcumin analogue ***4g***. It might be due to the covalent modification of the nucleophilic C-terminal active site in TrxR by α,β-unsaturated ketone moieties in curcumin analogues, as Arne Holmgren et al. proposed.[23]

2.4. Effects of curcumin analogue on TrxR activity in cancer cells

To further investigate the effect of curcumin analogues in cancer cells, TrxR activities in human HeLa cervical carcinoma cells and human MCF-7 breast adenocarcinoma cells were examined using insulin reduction assay after the treatment with compound ***4g***. As shown in Fig. 2A, TrxR activity in cell lysates declined by approximately 30% after the exposure of HeLa cells to 50 μM of ***4g***. Similar findings were observed in ***4g*** treated MCF-7 cells (Fig. 2B). These results demonstrated that curcumin analogue could reduce TrxR activities in both HeLa and MCF-7 cancer cells at relatively high concentrations.

2.5. Cytotoxicity evaluation of selected curcumin analogues

Our present study shows that the curcuminoids, especially analogues bearing furan moiety, can irreversibly inhibit mammalian TrxR under physiologic conditions. We further investigated the effect of selected curcuminoids on the growth of TrxR overexpressed cancer cell lines including human A549 lung adenocarcinoma epithelial cell line, cis-diamminedichloroplatinum (II) (CDDP) resistant A549/R cell line, doxorubicin resistant MCF-7/R cell line, and human HepG2 hepatocellular liver carcinoma cell line using MTT method (Fig. 3 and Table 2). The results showed that analogues ***2a***, ***2e***, ***2g***, and ***4g***, which turned out to be potent inhibitors of TrxR, exhibited stronger toxicity to A549/R cells than that of the natural curcumin (Fig. 3B), suggesting that the cell death of A549/R induced by curcuminoids may result from the inhibition of TrxR system; unfortunately, the most potent TrxR inhibitor ***4g*** only showed moderate inhibitory activities on the growth of MCF-7/R, A549/R, and A549. Compounds ***2e*** and ***2g*** could also inhibit the growth of the MCF-7/R at relatively lower micromolar concentrations; however, the cytotoxicity of ***2e*** and ***2g*** did not increase at higher concentrations, suggesting that some other induction systems

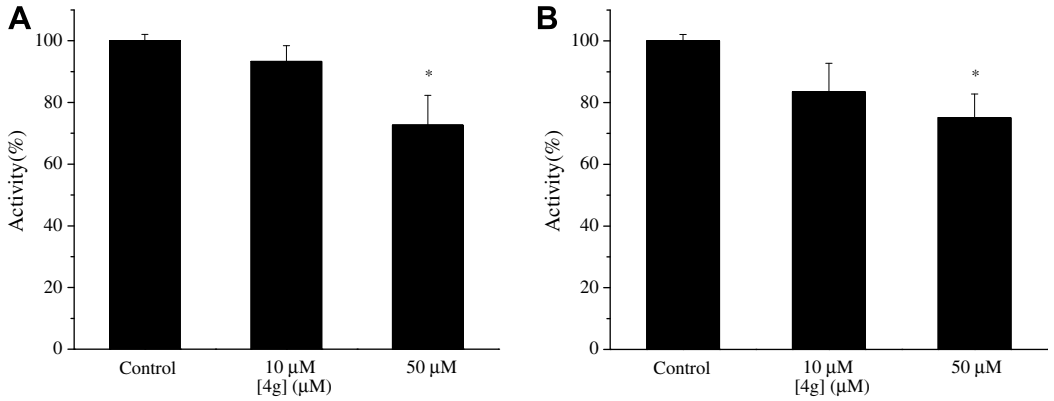


Figure 2. Effects of curcumin analogue ***4g*** on TrxR in HeLa and MCF-7 cells. HeLa cells (A) and MCF-7 cells (B) were cultured in the absence or presence of various concentrations of ***4g*** for 12 h. TrxR activity in cell lysates was measured by insulin reduction assay as described in Section 4. Activities are given as the percentage of the control. Data are means ± SD of at least three determinations. $^{*}p < 0.05$ compared with the control.

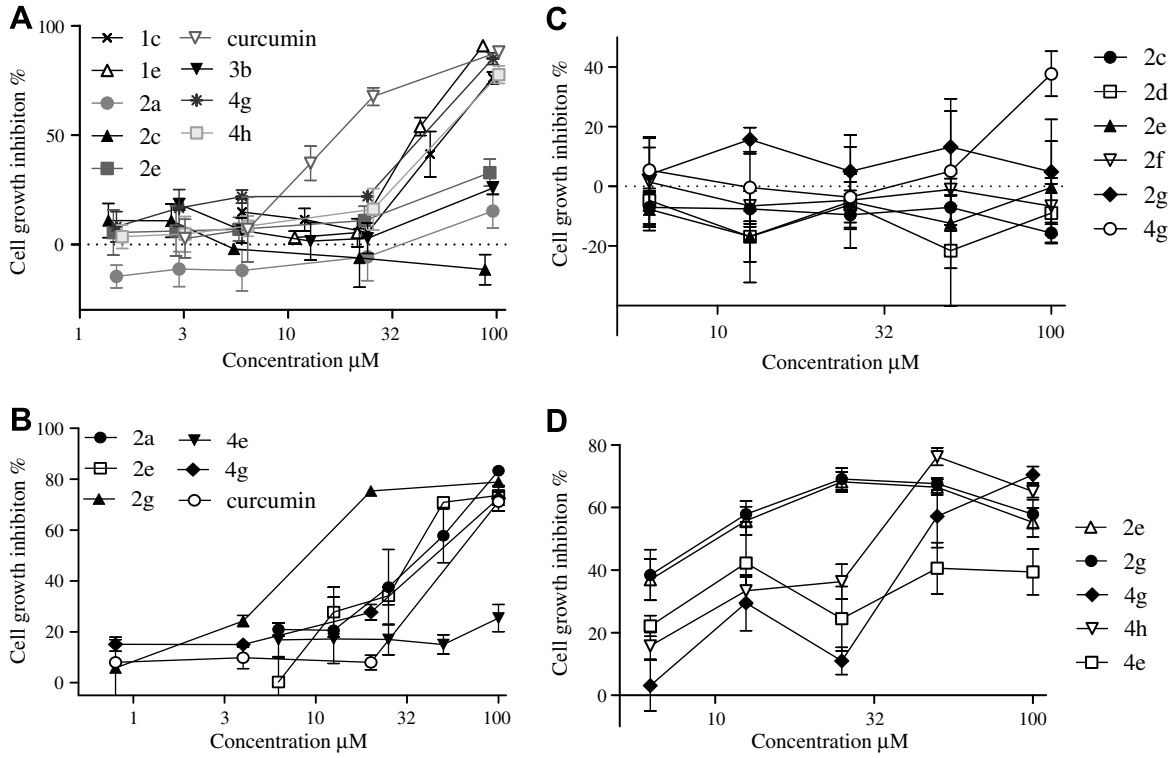


Figure 3. Effect of different curcuminoids on growth of TrxR overexpressed cancer cell lines A549 (A), cis-diamminedichloroplatinum (II) (CDDP) resistant A549/R (B), HepG2 (C), and doxorubicin resistant MCF-7/R (D). Activities are given as the percentage of the control. Data are means ± SD of at least three determinations.

against ***2g*** and ***2e*** might be functional in MCF-7/R cells. Interestingly, HepG2 seemed to be more resistant to curcuminoids as compared to the other cell lines (Fig. 3C) since all of the curcumin analogues could not induce effectively cell death even at concentration up to 100 μM.

4g and ***4h*** have moderate inhibitory activities on the growth of both MCF-7/R and A549 (Fig. 3A). Noticeably, while compounds with relatively higher IC_{50} on TrxR (curcumin, ***1e*** and ***1c***) can induce the A549 cell death effectively, most of the analogues with low IC_{50} on TrxR (***2a***, ***2e***, ***2g***, and ***4h***) have lower toxicities to A549 and/or HepG2 than to drug resistant cell lines A549/R and MCF-7/R (Table 2); the reason is not clear; it probably owns to the multi-mechanisms of cell death induced by curcuminoids. Furthermore, it was reported[28,29] that the CDDP or doxorubicin resistant cell lines have higher TrxR level than sensitive cells; it might render the cell lines to be more dependent on the TrxR activities. Nevertheless, further investigation should be done to unclose this phenomenon.

In summary, we synthesized series of curcumin derivatives; the inhibitory activities on TrxR of all analogues were evaluated by DTNB assay. Most of the curcumin analogues were found to show inhibitory activity against TrxR in vitro. Especially, the analogues with furan moiety have excellent activities against TrxR in an irreversible manner, indicating that the furan moiety may act as a possible pharmacophore during the interaction of analogues with TrxR. The effects of selected curcuminoids on the growth of TrxR overexpressed cancer cell lines were investigated and discussed. The detailed mechanism is under active exploration.

Table 2
Effect of different curcuminoids on growth of TrxR overexpressed cancer cell lines

Compounds	TrxR inhibition (IC_{50}, μM)	Cytotoxicity (GC_{50}[a], μM)			
		A549	A549/R	MCF-7/R	HepG2
1c	13.8	60.1	ND	ND	ND
1e	23.3	41.3	ND	ND	ND
2a	0.5	>100	39.4	ND	ND
2c	2.0	>100	ND	ND	>100
2d	62.2	ND	ND	ND	>100
2e	1.0	>100	35.8	10.6	>100
2f	1.3	ND	ND	ND	>100
2g	0.8	ND	12.1	10.0	>100
3b	5.1	>100	ND	ND	ND
4e	>100	ND	>100	>100	ND
4g	0.3	52.9	59.6	48.5	>100
4h	1.6	66.4	ND	33.6	ND
Curcumin	38.3	18.2	73.0	ND	ND

ND, not determined.
[a] The GC_{50} represents the compound concentration required for the reduction of the mean cell viability to 50%.

3. Experiment

3.1. General

All reagents are available commercially. Solvents were purified using standard techniques. Curcumin and its analogues used in this work were all synthesized and characterized in our lab. TrxR was purified from porcine brains as described by Cheung et al.[30] The enzyme purity was judged by SDS–PAGE and IEF. A Varian^{UNITY} INOVA 500 MHz spectrometer was used to record the ^{1}H NMR spectra in which TMS was the internal standard. The MS analysis was performed on a Finnigan LCQ Deca XP ion trap mass spectrometer.

3.2. Synthesis of curcumin analogues

3.2.1. Synthesis of unsymmetrical curcumin analogues *1a–e*, *2a–g*, *3a–b*

All of the unsymmetrical curcumin derivatives ***1a–e***, ***2a–g***, ***3a–b*** were synthesized according our previous work, and the ^{1}H NMR, HRMS or MS and EA have been described in our early report.[24]

3.2.2. Synthesis of curcumin analogues *4a–4h*

3.2.2.1. 5-Hydroxy-1,7-diphenylhepta-1,4,6-trien-3-one (*4a*). Compound ***4a*** was prepared according to Pedersen method[31] with slight modification. Boric anhydride (0.35 g, 5 mmol) was suspended in 10 mL EtOAc in the presence of acetylacetone (1.00 g, 10 mmol), and the mixture was stirred for 3 h at 70 °C. After removing the solvent, the resultant white solid was washed with hexane and then, 20 ml EtOAc, benzaldehyde (2.12 g, 20 mmol), and tributyl borate (4.60 g, 20 mmol) were added, and the mixture was stirred for a further 30 min. Butylamine (73 mg, 1 mmol) dissolved in EtOAc was added dropwise for 15 min. The mixture reacted at 70 °C for 24 h. Then 1 N HCl was added to adjust the pH to 5, and the solution was heated for 30 min at 60 °C. EtOAc was used to extract the product from the water layer. The ***4a*** was purified by recrystalization from EtOAc to yield yellow crystals: 2.21 g (80% yield); ^{1}H NMR(500 MHz, DMSO-d_6, ppm) δ:6.19 (s, 1H, enol), 6.86 (d, 2H, J = 16 Hz), 7.41 (m, 6H), 7.61(d, 2H, J = 16 Hz), 7.66 (dd, 4H, J = 8, 2 Hz); MS(ESI) m/z: 277.1 $(M+H)^+$.

3.2.2.2. 5-Hydroxy-1,7-bis(4-hydroxy-3-methoxyphenyl)hepta-1,4,6-trien-3-one (*4b*). Compound ***4b*** was prepared from 4-hydroxy-3-methoxybenzaldehyde by using the similar procedure described above for ***4a***. 3.04 g (20 mmol) of 4-hydroxy-3-methoxybenzaldehyde was used and 2.88 g of product was obtained as yellow powder, yield: 78%; ^{1}H NMR(500 MHz, $CDCl_3$) δ: 3.94 (s, 3 H), 5.79 (s, 1H, enol), 6.46 (d, 2H, J = 16 Hz), 6.92 (d, 2H, J = 8 Hz), 7.04 (d, 2H, J = 1.5 Hz), 7.11(dd, 2H, J = 8, 1.5 Hz), 7.58 (d, 2H, J = 16 Hz); MS (ESI) m/z: 369.1 $(M+H)^+$.

3.2.2.3. 5-Hydroxy-1,7-bis(4-hydroxyphenyl)hepta-1,4,6-trien-3-one (*4c*). ***4c*** was prepared from 4-hydroxybenzaldehyde by using the similar procedure described above for ***4a***. 2.44 g (20 mmol) of 4-hydroxybenzaldehyde was used and 2.24 g of product was obtained as yellow powder, yield: 73%; ^{1}H NMR(500 MHz, DMSO-d_6, ppm) δ:5.97 (s, 1H, enol), 6.57 (d, 2H, J = 16 Hz), 6.77 (d, 4H, J = 9 Hz), 7.45 (d, 4H, J = 9 Hz), 7.46 (d, 2H, J = 16 Hz); MS(ESI) m/z: 309.3 $(M+H)^+$.

3.2.2.4. 5-Hydroxy-1,7-bis(3,4,5-trimethoxyphenyl)hepta-1,4,6-trien-3-one (*4d*). Compound ***4d*** was prepared from 3,4,5-trimethoxybenzaldehyde by using the similar procedure described above for ***4a***. 3.92 g (20 mmol) of 3,4,5-trimethoxybenzaldehyde was used and 3.82 g of product was obtained as orange powder, yield: 84%; ^{1}H NMR(500 MHz, $CDCl_3$) δ:3.71 (s, 6H), 3.84 (s, 12H), 6.17(s, 1H, enol), 6.91(d, 2H, J = 16 Hz), 7.07 (s, 4H), 7.59(d, 2H, J = 16 Hz); MS(ESI) m/z: 457.1$(M+H)^+$.

3.2.2.5. 5-Hydroxy-1,7-bis(4-methoxyphenyl)hepta-1,4,6-trien-3-one (*4e*). Compound ***4e*** was prepared from 4-methoxybenzaldehyde by using the similar procedure described above for ***4a***. 2.72 g (20 mmol) of 4-methoxybenzaldehyde was used and 2.94 g of product was obtained as yellow powder, yield: 87%; ^{1}H NMR(400 MHz, $CDCl_3$) δ: 7.62 (d, 2H, J = 15.6 Hz), 7.51 (dd, 4H, J = 7.8,2 Hz), 6.92(dd, 4H, J = 7.8, 2 Hz), 6.50 (d, 2H, J = 15.6 Hz), 5.78(s, 1H), 3.85(s, 6H); MS(APCI) m/z: 337.1$(M+H)^+$.

3.2.2.6. 1,7-Bis(3,4-dimethoxyphenyl)-5-hydroxyhepta-1,4,6-trien-3-one (*4f*). Compound ***4f*** was prepared from 3,4-dimethoxybenzaldehyde by using the similar procedure described above for ***4a***. 3.32 g (20 mmol) of 3,4-dimethoxybenzaldehyde was used and 3.55 g of product was obtained as orange powder, yield: 90%; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.61(d, 2H, J = 16 Hz), 7.14(dd, 2H, J = 2, 8.4 Hz), 7.07(d, 2H, J = 2 Hz), 6.88(d, 2H, J = 8.4 Hz), 6.50(d, 2H, J = 16 Hz), 5.82(s, 1H), 3.94(s, 3H), 3.92(s, 3H); MS(APCI) m/z: 397.2$(M+H)^+$.

3.2.2.7. 5-Hydroxy-1,7-bis(5-methylfuran-2-yl)hepta-1,4,6-trien-3-one (*4g*). Compound ***4g*** was identified as a new compound and prepared by using the similar procedure described above for ***4a***. Boric anhydride (0.35 g, 5 mmol) and acetylacetone (1.00 g, 10 mmol) were suspended in 10 mL EtOAc, and the mixture was stirred for 3 h at 70 °C. After removing the solvent, the resultant solid was washed with hexane and then, 20 ml of EtOAc, 5-methylfuran-2-carbaldehyde (2.20 g, 20 mmol), and tributyl borate (4.60 g, 20 mmol) were added, and the mixture was stirred for a further 30 min. Butylamine (73 mg, 1 mmol) dissolved in EtOAc was added dropwise in 15 min. The mixture reacted at 70 °C for 24 h. Then 1 N HCl was added to adjust the pH to 5, and the solution was heated for 30 min at 60 °C. EtOAc was used to extract the product from the water layer. The ***4g*** was purified by recrystalization from EtOAc to yield 2.55 g red crystal, yield 90%; ^{1}H NMR(400 MHz, CDCl3) δ: 2.36 (s, 6H), 5.70 (s, 1H), 6.08 (dd, 2H, J = 3.2 Hz), 6.42 (d, 2H, J = 15.6 Hz), 6.50 (d, 2H, J = 3.2 Hz), 7.33(d, 2H, J = 15.6 Hz); ^{13}C NMR(100 MHz, $CDCl_3$) δ: 183.0, 155.6, 150.6, 126.7, 120.3, 116.5, 109.1, 101.9, 13.9; MS(EI) m/z: 285.1$(M+H)^+$; HRMS-EI m/z: calcd for $C_{17}H_{16}O_4$, 284.1043; found 284.1044.

3.2.2.8. 1,7-Di(furan-2-yl)-5-hydroxyhepta-1,4,6-trien-3-one (*4h*). Compound ***4h*** was prepared from furan-2-carbaldehyde by using the similar procedure described above for ***4a***, 1.92 g (20 mmol) furan-2-carbaldehyde was used and the final product was purified by column chromatography to yield 0.76 g yellow powder, yield: 30%; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.48(d, 2H, J = 1.6 Hz), 7.40(d, 2H, J = 15.6 Hz), 6.60 (d, 2H, J = 3.2 Hz), 6.51 (d, 2H, J = 15.6 Hz), 6.47 (dd, 2H, J = 1.6, 3.2 Hz), 5.74 (s, 1H); MS(APCI) m/z: 257.1(M+H)$^+$.

3.3. The inhibition assay of TrxR by curcumin analogues

For determining the TrxR inhibitory activity of curcumin analogues, the DTNB reduction assay was employed. All assays were conducted at 37 °C in a total volume of 600 μl. In each measurement, 18 μl of TrxR was added to an assay mixture containing 100 mM potassium phosphate, 2 mM EDTA, pH 7.4, and 46 μl of NADPH and 5 μl of inhibitor at various concentrations. After 4 min pre-incubation, the reaction was initiated with the addition of 18 μl of DTNB. The control was incubated with the same amount of DMSO. The increase in absorbance at 412 nm ($\Delta\varepsilon_{412}$ = 13.6 mM^{-1} cm^{-1}) was monitored in the initial 80 s. The IC_{50} values were calculated to represent the TrxR inhibitory effect of compounds.

3.4. Reversibility of inhibition of TrxR by curcumin analogue *4g*

Different concentrations of ***4g*** (10 μM and 40 μM, respectively) were incubated with NADPH-reduced TrxR (0.8 μM) at room temperature for 2 h. The control was treated with the same amounts of DMSO. Compound ***4g*** was then removed by filtering through a Millipore Centricon-30 centrifugal filter with 30,000 nominal molecular weight limits. The enzyme in the retentate was washed with 70 μl of PE buffer and then resuspended in 60 μl of PE buffer; 50 μl of the mixture was taken out to determine the enzyme activity as described in Table 1. The activity was given as the percentage of the control.

3.5. Cell culture

Four different human cancer cell lines (human A549 lung adenocarcinoma epithelial cell line (CDDP resistant A549/R) human MCF-7 breast carcinoma cells (doxorubicin resistant MCF-7/R), and human HEPG2 hepatocellular liver carcinoma cell line, and human HeLa cervical cancer cells) were cultured in RPMI 1640 medium (Gibco-BRL Grand Island, NY) supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine at 37 °C in incubator with humidified atmosphere of 5.0% CO_2 and 95% air.

3.6. Cell growth inhibition assay

The growth inhibitory effects of curcumin analogues on different cancer cell lines were evaluated by means of the MTT (3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyl-2H-tetrazolium bromide) assay as described by Carmichael[32] and Mossman[33] with modifications. The cells were plated at a density of 1×10^4 per well in 96-well microplates and allowed to incubate overnight. Curcumin analogues were added to the wells at increasing concentrations (0–100 μM). After 48 h, each well was treated with 20 μl of 5 mg/ml MTT solution, and the cells were further incubated at 37 °C for 4 h. At the end of incubation, the untransformed MTT was removed and 150 μl of DMSO was added. The microplates were shaken well to dissolve the formazan dye, and the absorbance at 570 nm was measured using a microplate-reader (Bio-Rad). Cytoviability of the control cells was taken as 100%. Tests were conducted in quadruplicates. For each dose, the mean cell viability was expressed as a percentage of the control. The GC_{50} represents the compound concentration required for the reduction of the mean cell viability to 50%.

3.7. TrxR activity in cell lysates

Cells (1.5×10^6) were seeded in 6-well plates in 5 ml of RPMI 1640 medium. Different concentrations of curcumin analogues were incubated with the cells for 12 h, and the control cells were treated with less than 1% DMSO. In the harvesting, cells were trypsinized and lysed in cell lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM NaF, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) in the presence of protease inhibitors (Roche). After centrifugation at 16,000g for 10 min, protein concentrations of supernatants were determined using the Bio-Rad assay kit. TrxR activity was determined by end point insulin reduction assay as described by Arner et al.[25] with slight modifications. Briefly, 38 μg of extract were incubated with 2.5 mg/ml bovine insulin, 2 mM EDTA, 400 μM NADPH, 0.8 μM Trx in 60 μl of 100 mM HEPES (pH 7.4) at 37 °C for 30 min. The reaction was terminated by the addition of 200 μl of 6 M guanidine hydrochloride/1 mM DTNB. The reaction mixtures with the omission of Trx were used as the control. The absorbance was measured at 412 nm. The percentage of TrxR activity in comparison with the control was determined.

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