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Novel curcumin analogs targeting TNF-induced NF-κB activation and proliferation in human leukemic KBM-5 cells

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Abstract—Novel curcumin analogs were synthesized using Knoevenagel condensation to convert enolic diketones of curcumin into non-enolizable ones and Schiff bases were prepared using a bioactive thiosemicarbazide pharmacophore. Copper(II) conjugates of all synthesized ligands were prepared and structurally characterized as well as evaluated for their potential of inhibiting TNF-induced NF-κB activation and proliferation in human leukemic KBM-5 cells wherein compound **13** was found to be more potent than curcumin. Compounds were further examined on other tumor cell lines such as Jurkat, H1299, and MM1, respectively.

1. Introduction

Curcumin (diferuloylmethane), which is a naturally occurring yellow pigment obtainable from the rhizomes of perennial herb *Curcuma longa* Linn., has been shown to act upon several important molecular targets in malignancy and inflammatory cascades and hence is used to treat various disorders including arthritis, Crohn's disease, cardiovascular disorders, psoriasis, cancers, and other pathologies.[1,2] It is found to be effective in treating almost all types of cancers at all stages of the disease.[3–6] Curcumin's biological effects include modulation of several cellular receptors (EGFR and HER2), signal transcription factors (NF-κB, AP-1, Egr-1, β-catenin, and PPAR-γ), various oxygenases [cycloxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX)], inducible nitric oxide synthase (iNOS), cell cycle proteins (cyclin D_1p[21]), cytokines (TNF, IL-1, IL-6, chemokines), as well as cell surface adhesion molecules.[7] Among the transcription factors affected by curcumin, NF-κB is the most important one as it plays a pivotal role in various inflammatory responses leading to host defense and activation of many gene expressions.[8] Although NF-κB is ubiquitous transcription factor, it performs a critical role in cells of the immune system, where it controls the expression of various cytokines and histocompatibility of complex genes. Inappropriate regulation of NF-κB has been shown to give rise to various pathological disorders including inflammation, viral replication, atherosclerosis, and growth of almost all types of tumors.[8,9] Hence, it is considered as an important therapeutic target for cancer drug development.

Curcumin has been shown to inhibit tumor initiation induced by benz(α)pyrene and 7,12-dimethylbenz(α)-anthracene as well as tumor promotion induced by phorbol esters which are known to trigger NF-κB activation.[10] It is found to inhibit gene expression induced by long terminal repeat (HIV-LTR) of type-1 human immunodeficiency virus and virus replication stimulated by TNF and phorbol esters.[10,11] Amongst the potent NF-κB blocking agents, COX-2-specific inhibitors have been marginalized[12] due to associated cardiotoxicities leaving perhaps curcumin as the only safe drug which does not show any adverse effects even upto doses as high as 8 g per day.[13] In addition, there are no reports on development of resistance against curcumin.[13] However, poor water solubility and unsatisfactory pharmacokinetics of curcumin necessitate search for new curcumin analogs.

Keywords: Curcumin; NF-κB; Thiosemicarbazone; Copper; Knoevenagel condensation.
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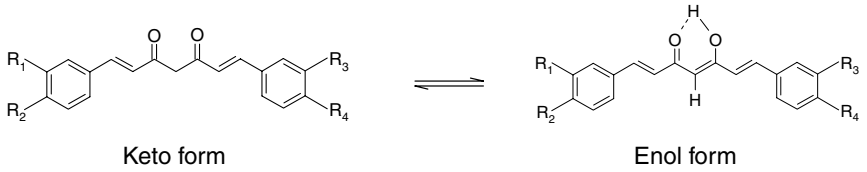
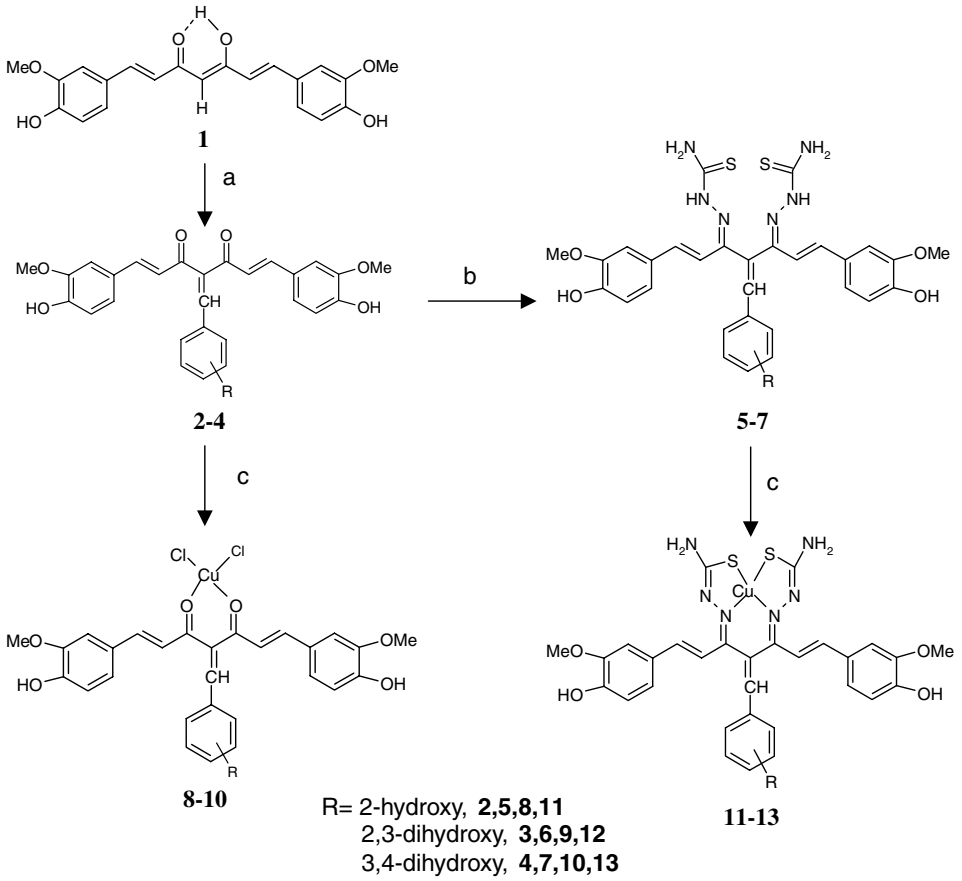


R_1= R_3= -OCH_3, R_2= R_4= -OH, Curcumin (**1**) (77%)
R_1= -H, R_3= -OCH_3, R_2= R_4= -OH, Demethoxycurcumin (17%)
R_1= R_3= -H, R_2= R_4= -OH, Bisdemethoxycurcumin (3%)

Scheme 1.



Scheme 2. Reagents and conditions: (a) piperidine, 48 h, methanol; (b) $H_2NCSNHNH_2$·HCl, 24 h, piperidine, methanol, rt (1:2); (c) $CuCl_2$·$2H_2O$, methanol, piperidine (1:1).

It is well established that herbal extract containing curcumin is often accompanied by the demethoxy and bisdemethoxy curcumin components, which together are referred to as the curcuminoids (Scheme 1). In our work, we have used only the fractionated sample of curcumin (**1**) which is appended with pharmacophoric side chains. Such modifications attempted earlier on the diketone functionalities in **1** remain suspected since the β-diketone system is found to be stable in the enolic form due to intramolecular hydrogen bonding.[14,15] It has been established that the diketo species can be functionalized only when they are made non-enolizable through derivatization of the active methylene group in **1**.[16] In the present work, we describe preparation and characterization of three curcumin Knoevenagel condensates (**2**–**4**), their Schiff bases (**5**–**7**), and corresponding copper conjugates (**8**–**13**) as described in Scheme 2. The choice of copper for metal conjugation was based upon our recent work showing it has synergistic effects on the antiproliferative activities against breast, prostate, and pancreatic cancer cells.[17,18] The Knoevenagel condensates and their Schiff bases both form 1:1 copper complexes. All synthesized compounds are evaluated for their potential of inhibiting TNF-induced NF-κB activation and proliferation in human leukemic KBM-5 cells.

2. Results and discussion

All synthetic manipulations were performed on **1** fractionated from commercial samples of curcumin by column chromatography using silica gel with chloroform/

methanol (9:1) as eluting solvents. IR spectra of the Knoevenagel condensates (**2**–**4**) showed a carbonyl stretching frequency in the range 1655–1633 cm^{-1} compared to 1620 cm^{-1} for **1**. Incorporation of thiosemicarbazide side chain (**5**–**7**) is characterized by the disappearance of the carbonyl frequency and appearance of additional bands around 1595 and 870 cm^{-1} due to azomethine and thiocarbonyl functionalities. Upon metal complexation the thiocarbonyl stretch shifts to lower frequency indicating its involvement in metal conjugation, which is further confirmed by the appearance of strong absorptions in the region 450–300 cm^{-1} indicative of Cu–N and Cu–S stretching vibrations. On the other hand, copper conjugates of the Knoevenagel condensates (**8**–**10**) without thiosemicarbazide side chains exhibit the carbonyl stretching frequency at lower wave number around 1585 cm^{-1} with Cu–O and Cu–Cl stretching vibrations located around 550 and 330 cm^{-1}, respectively.

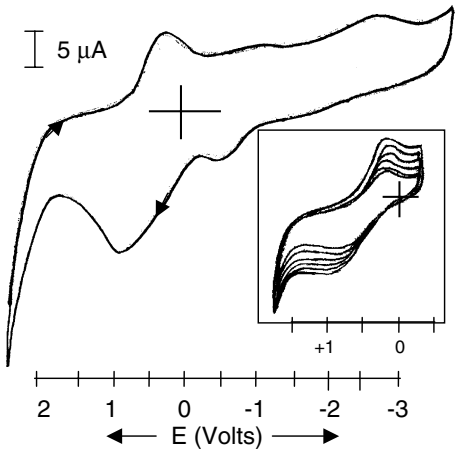


Figure 1. Cyclic voltammogram of **8** (v = 100 mV/s) in DMSO where inset shows scan rate dependence of the copper redox couple.

In the electronic spectra of all ligands absorptions between 325 and 415 nm are attributed to intraligand transitions, while intense and prominent charge transfer bands can be observed for their metal conjugates around 450 nm. The broad band observed in the range 500–600 nm is the metal-based d–d transition. In copper conjugates having the side chains (**11**–**13**), these bands were shifted towards lower energy and were assigned to the combination of $^2B_{1g} \rightarrow {}^2E_g$ and $^2B_{1g} \rightarrow {}^2B_{2g}$ transitions indicative of distorted planar geometry.[19,20] Room temperature magnetic moments (μ_{eff}) of the copper(II) conjugates were found to be in the range of 1.74–1.82 BM suggestive of monomeric species having spin only magnetic moments (Table 1).

The electrochemical profile of **1** (not shown) shows a quasi-reversible peak centered at −0.84 V ascribed to the reduction of its carbonyl functions.[14] This peak is shifted to more negative potentials in the Knoevenagel condensates (**2**–**4**) indicative of resistance to reduction. An additional peak observed at −0.6 V probably arises out of the abstraction of one of the methylenic protons. Copper conjugates (**8**–**10**) of the Knoevenagel condensates exhibit reversible Cu^{2+}/Cu^{+} redox couple ($E_{1/2}$) at +0.33 V, while it is observed at 0.40 V in their counterparts with side chains indicative of facile copper reduction. In Figure 1, we have shown a representative cyclic voltammogram of **8** with inset showing scan rate dependence of the copper redox couple.

The X-band EPR spectra of all copper conjugates with side chains were recorded in DMSO at liquid nitrogen temperature available as supplementary material. The spin Hamiltonian parameters calculated for these are included in Table 1. All compounds exhibit a typical four-line copper hyperfine pattern and follow the relationship $g_{\parallel} > g_{\perp} > 2.0$ characteristic of monomeric copper complexes. Kivelson and Niemen have pointed out that compounds having $g_{\parallel} \geqslant 2.3$ are ionic while those with $g_{\parallel} < 2.3$ are covalent in character.[21] The $g_{\parallel}$ values for the present series of complexes reveal appreciable covalency with $d_{x^2-y^2}$ as the ground state. The degree of distortion $F(g_{\parallel}/A_{\parallel})$ is regarded as an index of deviation from the idealized geometry. The values of 110–135 cm^{-1} are typical for square planar complexes, while the range of 150–250 cm^{-1} is characteristic of moderate distortion.[22] The distortion factor (F), calculated for the present series of complexes **8**–**10** (117–132 cm^{-1}) and **11**–**13** (145–156 cm^{-1}) indicates square planar and moderate distortion towards tetrahedral geometry, respectively.

All compounds were evaluated for their ability to inhibit the redox active transcription factor NF-κB in human leukemic KBM-5 cells as they expressed TNF receptors and were responsive to TNF activation. The KBM-5 cells were pre-incubated for 48 h with different concentrations of curcumin analogs and their metal conjugates, and were treated with TNF (0.1 nM) for 30 min at 37 °C. The nuclear extracts were prepared and assayed for the NF-κB activation by Electrophoretic Mobility Shift Assay (EMSA).

Curcumin inhibited TNF-mediated NF-κB activation in a dose dependent manner with maximum inhibition

Table 1. Magnetic, ESR, and electrochemical data on copper conjugates

Compound	μ_{eff} (BM)	Ep_c	Ep_a	$E_{1/2}$ (Cu^{2+}/Cu^{+})	$g_{\parallel}$	$g_{\perp}$	$A_{\parallel}$ (G)	F (cm^{-1})
8	1.76	0.200	0.475	0.3375	2.212	2.069	188	117
9	1.79	0.125	0.550	0.3375	2.232	2.073	175	127
10	1.82	0.125	0.600	0.3625	2.260	2.077	170	132
11	1.80	0.250	0.475	0.3625	2.280	2.066	157	145
12	1.77	0.075	0.725	0.4000	2.291	2.070	156	146
13	1.80	0.250	0.550	0.4000	2.294	2.072	147	156

taking place at 50 μM (Fig. 2). None of the curcumin analogs and their copper conjugates including that of **1** by themselves inhibited NF-κB activation. TNF-induced NF-κB activation, however, was inhibited by most of the synthesized curcumin analogs and their metal conjugates in a dose dependent manner. In another experiment, the cells were pre-incubated with curcumin for different times before the addition of TNF and then were treated with TNF for 30 min. The maximum inhibition of NF-κB activation was observed (Fig. 2) only when the cells were pretreated for 4 h with curcumin (50 μM) and hence this period was considered as optimal for evaluation of other curcumin analogs and their metal conjugates.

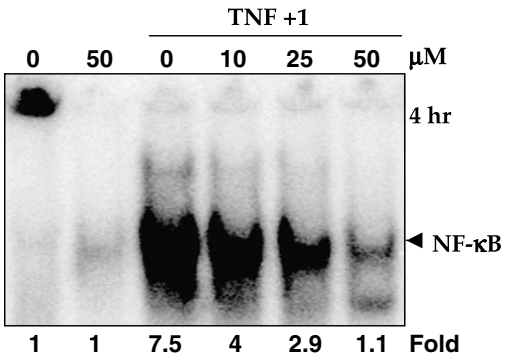


Figure 2. Concentration-dependent effect of curcumin (**1**) on TNF-induced NF-κB activation.

The most active Knoevenagel condensate was found to be compound **4** (Fig. 3A) suggesting an aryl hydroxy pharmacophore at the methylene center (**3** and **4**) contributing to the enhanced antioxidant potential is an important determinant of the requirement of NF-κB inhibition. Complexing with copper does not seem to help endowing them with inhibitory activity (**8** and **10**). The Schiff base derivatives of these condensates themselves (**5**–**7**) are not active. However, their copper complexes (**11**–**13**) show very distinct inhibitory activities (Fig. 4).

All copper conjugates exhibited good to moderate activity and comparable NF-κB deactivation as that of curcumin and may enhance their potency at higher concentrations with prolonged incubation period. The

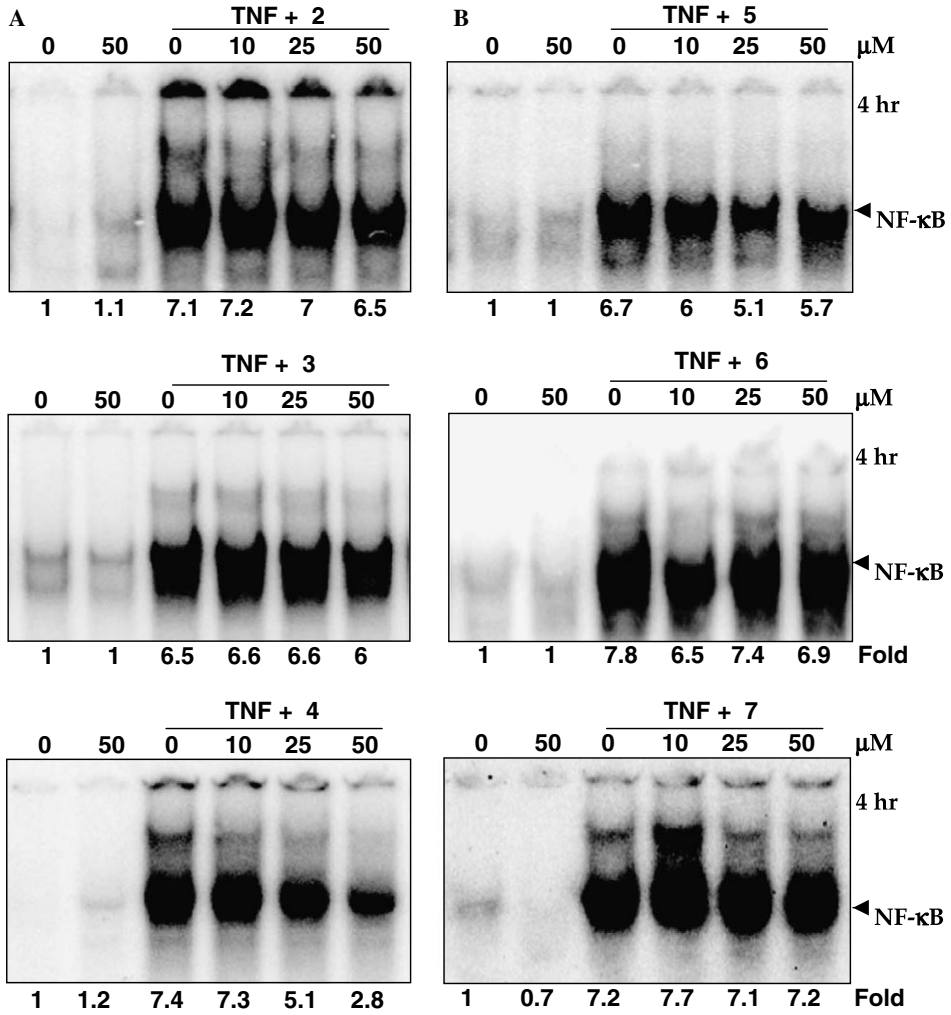


Figure 3. Suppression of TNF-induced NF-κB activation by (A) **2**, **3**, **4** and (B) **5**, **6**, **7**.

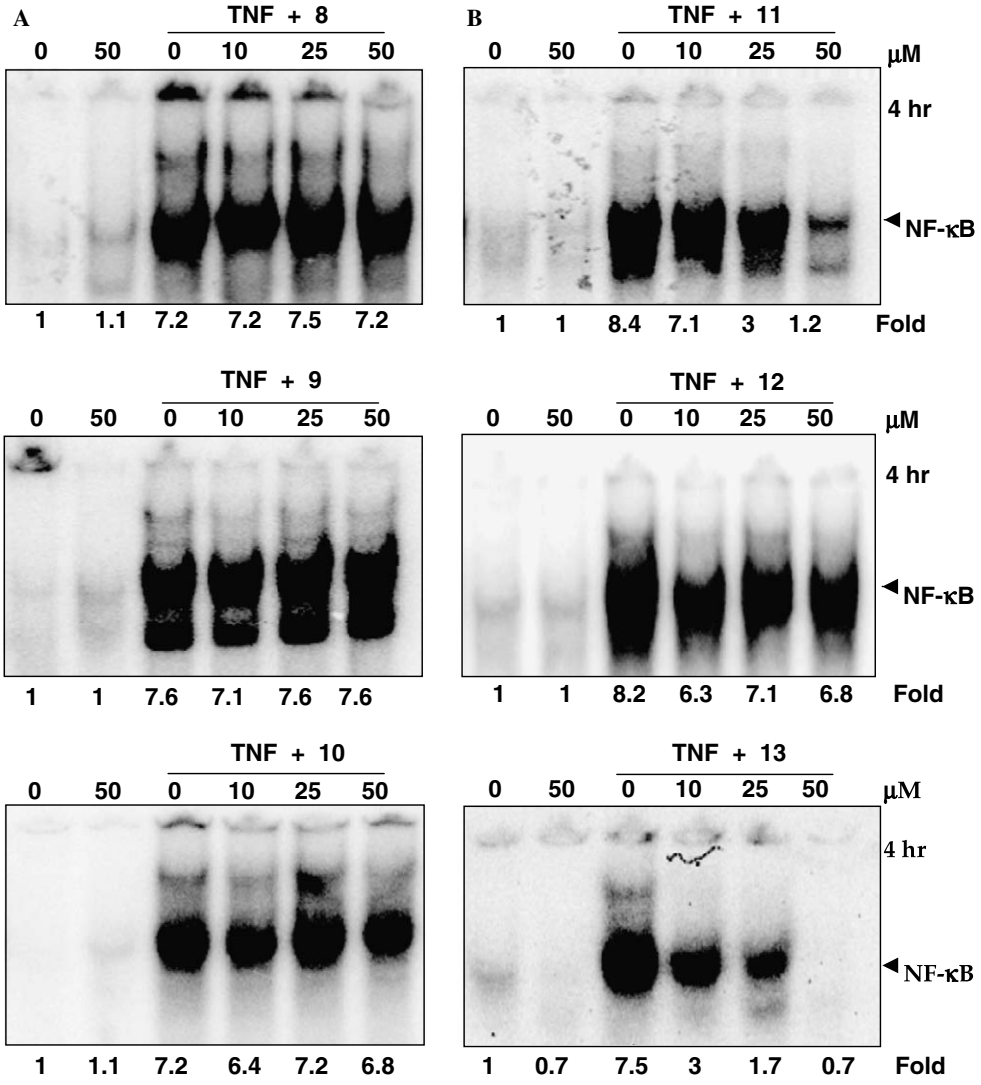


Figure 4. Suppression of TNF-induced NF-κB activation by (A) **8**, **9**, **10** and (B) **11**, **12**, **13**.

inhibition in case of **13** was more than that of curcumin. This is interesting because, we believe this probably arises out of its favored electrostatic interactions through H-bonding and π–π interactions with residues at the active site of protein. It is also possible that scavenging of excessive reactive oxygen species (ROS) by divalent copper conjugates may also contribute to this process.

Because NF-κB activation is known to regulate proliferation of tumor cells,[23] we also examined the effect of curcumin analogs on proliferation of various tumor cells. The effect of curcumin and its analogs was compared at equimolar concentrations. The antiproliferative effect (cytotoxicities) against different cell lines is shown in Table 2. In KBM-5 cells, almost all Knoevenagel curcumin condensates (**2**–**4**) showed nearly equal cytotoxicity at 50 μM concentration as that of curcumin (**1**). However, at lesser concentrations they were less active. Amongst Schiff base derivatives (**5**–**7**) of these condensates, compound **5** exhibits comparable activity even at lower concentrations.

In general, copper complexes of Knoevenagel condensates of curcumin (**8**–**10**) are less inhibitory than curcumin. However, compounds **9** and **10** are found to be more cytotoxic than curcumin even at 10 μM concentration in H1299 cells. In KBM-5 cells, compounds **11** and **13** displayed maximum cytotoxicity greater than curcumin at 10 μM concentration. A similar trend is also observed in H1299 cells. At higher concentrations copper complexes exhibit cytotoxicity comparable with curcumin. It should be noted that Knoevenagel condensates having phenolic group are especially effective as antiproliferative agents through NF-κB inhibition.

3. Conclusions

Present work shows that Knoevenagel condensates of curcumin restricting enolization can serve as starting blocks for developing effective curcumin analogs capable of inhibiting NF-κB activation. Copper conjugation appears to be beneficial especially in case

Table 2. Effect of curcumin and its analogs on proliferation of various tumor cell types

Compound	(μM)	KBM-5		Jurkat		H1299		MM1	
		% cytotoxicity	SD	% cytotoxicity	SD	% cytotoxicity	SD	% cytotoxicity	SD
Curcumin (**1**)	10	76	3.09	69	1.60	27	1.95	28	4.82
	50	83	0.06	85	0.40	77	2.43	67	3.74
2	10	29	10.22	11	12.5	3	1.65	18	5.67
	50	83	0.56	77	1.45	36	1.10	29	2.36
3	10	25	1.66	6	1.60	7	2.08	9	16.74
	50	76	0.15	72	0.74	37	1.03	29	0.30
4	10	42	3.80	0	0.17	18	2.38	12	4.90
	50	82	0.29	84	2.79	66	0.38	49	1.49
5	10	72	4.85	52	2.60	40	2.56	40	1.25
	50	84	0.12	66	3.90	36	3.30	34	4.47
6	10	15	0.84	24	3.50	7	1.85	0	2.53
	50	40	3.16	40	3.75	11	4.23	11	0.34
7	10	9	2.22	0	0.68	10	0.17	0	2.92
	50	54	3.74	38	4.01	15	1.65	16	1.91
8	10	25	6.73	2	1.00	6	0.35	7	3.32
	50	77	2.99	80	0.70	38	2.12	27	3.46
9	10	42	14.10	0	0.92	44	2.14	26	1.86
	50	79	1.23	78	0.55	77	1.56	69	1.56
10	10	17	2.17	0	3.37	34	1.82	6	3.72
	50	34	6.87	26	1.59	48	1.17	19	1.25
11	10	81	0.43	52	1.23	52	0.48	45	4.72
	50	87	0.12	80	1.96	73	1.55	67	3.45
12	10	46	6.29	30	2.98	45	3.09	21	1.86
	50	80	0.59	83	0.74	79	1.17	77	0.46
13	10	15	5.84	0	0.43	56	0.70	2	2.33
	50	82	0.16	85	5.68	82	1.18	79	0.35

Human myeloid (KBM-5), human T-cell leukemia (Jurkat), human lung adenocarcinoma (H1299), and human multiple myeloma (MM1) cells (2000 cells/0.1 ml) were incubated with indicated concentrations of curcumin and its analogs for 72 h and then examined for cell viability by the MTT method.[27] Percent cytotoxicity was calculated by dividing the absorbance of treated group with that of untreated group and multiplied with 100. The resulting value was subtracted from 100 to obtain the percent cytotoxicity. All samples were run in triplicate. The results are means of triplicate determinants.

of ligands appended with thiosemicarbazone functionality. This suggests a probable role of S and N in providing a more favorable environment for the metal center in regard to offer better inhibition of TNF-induced activation of NF-κB as well as antiproliferative activity in various human cancer cells tested. Further investigations are obviously needed to evolve the optimized structure.

4. Experimental

4.1. General

All reagents and solvents were of analytical reagent grade or were purified by standard methods prior to use.[24] Curcumin was obtained from Sigma (St. Louis, MO) and curcuminoids were separated by column chromatography on silica gel 60 (Merck 60–120 mesh). The reaction progress was monitored through thin-layer chromatography (TLC) on precoated silica gel on aluminum plates (Merck). Characterization of all synthesized ligands and their metal conjugates was performed as reported previously.[25]

4.2. General procedure for preparation of various Knoevenagel condensates (2–4)

Fractionated curcumin (368 mg, 1 mmol) purified by column chromatography was dissolved in minimum amount of chloroform/methanol mixture in a round-bottomed flask (RBF). Methanolic solution of aromatic aldehyde (1 mmol) was added dropwise to the above solution with continuous stirring along with catalytic amount of piperidine (0.05 cm^3). The reaction mixture was further stirred for 48 h at room temperature and set aside when the product separated out which was washed with excess of petroleum ether/hexane mixture to remove any unreacted reagents. The washing out process was repeated two to three times and the compound was recrystallized from chloroform/hexane mixture to give a pure dark brown Knoevenagel condensate.

4.2.1. 4-Salicylidene-1,7-bis(4-hydroxy, 3-methoxyphenyl)-1,6-heptadiene-3,5-dione (2). Yield: 76%, mp: 96–98 °C, ^{1}H NMR ($CDCl_3$): δ 3.73 (s, 6H, OCH_3), 6.66 (s, 2H, ArH), 6.71 (dd, 4H, J = 7.8, 7.8 Hz, ArH), 7.1 (d, 2H, J = 15.6 Hz, =C–H), 7.6 (d, 2H, J = 15.6 Hz, H–C=), 7.49 (m, 4H, ArH), 7.98 (s, 1H, =CH–Ar); IR

(KBr, cm^{-1}): ~3400 ($\nu_{O\text{-}H\ br}$), ~3012, 2945 ($\nu_{=C-H,\ C-H}$), ~1633 ($\nu_{C=O}$), ~1508 ($\nu_{C=C}$), ~1388, 1029 (ν_{C-H}), ~975 ($\nu_{H-C=C-H(trans)}$), ~821 ($\nu_{C-H(arom)}$); UV–vis: λ_{max} (nm, DMSO): 327; MS (+ES-MS): m/z = 472.48 (475) (M+2); Anal. Calcd for $C_{28}H_{24}O_7$: C, 71.18; H, 5.12. Found: C, 71.07; H, 5.20.

4.2.2. 4-(2,3-Dihydroxy benzyl)-1,7-bis(4-hydroxy, 3-methoxyphenyl)-1,6-heptadiene-3,5-dione (3). Yield: 63%, mp: 79–81 °C, ^1H NMR ($CDCl_3$): δ 3.82 (s, 6H, OCH_3), 6.97 (m, 2H, ArH), 6.80 (m, 6H, J = 8.62 Hz, ArH), 6.42 (d, 2H, J = 15.6 Hz, =C–H), 7.54 (d, 2H, J = 15.4 Hz, H–C=), 7.34 (d, 1H, J = 8.62 Hz, ArH), 7.98 (s, 1H, =CH–Ar); IR (KBr, cm^{-1}): ~3417 ($\nu_{O-H\ br}$), ~3050, 2947 ($\nu_{=C-H,C-H}$), ~1655 ($\nu_{C=O}$), ~1514 ($\nu_{C=C}$), ~1382, 1029 (ν_{C-H}), ~977 ($\nu_{H-C=C-H(trans)}$), ~819 ($\nu_{C-H(arom)}$); UV–vis: λ_{max} (nm, DMSO): 329, 428; MS (+ES-MS): m/z = 488.48 (490) (M+1); Anal. Calcd for $C_{28}H_{24}O_8$: C, 68.85; H, 4.95. Found: C, 68.78; H, 4.82.

4.2.3. 4-(3,4-Dihydroxy benzyl)-1,7-bis(4-hydroxy, 3-methoxyphenyl)-1,6-heptadiene-3,5-dione (4). Yield: 80%, mp: 104–106 °C, ^1H NMR ($CDCl_3$): δ 3.83 (s, 6H, OCH_3), 6.80 (m, 6H, ArH), 7.38 (m, 3H, ArH), 7.98 (s, 1H, =CH–Ar), 6.43 (d, 2H, J = 14.85 Hz, =C–H), 7.54 (d, 2H, J = 15.68 Hz, H–C=); IR (KBr, cm^{-1}): ~3500 ($\nu_{O-H\ br}$), ~2929, 2856 ($\nu_{=C-H,C-H}$), ~1633 ($\nu_{C=O}$), ~1521 ($\nu_{C=C}$), ~1382, 1028 (ν_{C-H}), ~954 ($\nu_{H-C=C-H(trans)}$), ~823 ($\nu_{C-H(arom)}$); UV–vis: λ_{max} (nm, DMSO): 341, 420; MS (+ES-MS): m/z = 488.48 (512) $(M+Na)^+$; Anal. Calcd for $C_{28}H_{24}O_8$: C, 68.85; H, 4.95. Found: C, 68.88; H, 4.91.

4.3. General procedure for preparation of various hydroxy substituted 1,7-bis(4-hydroxy, 3-methoxyphenyl)-1,6-heptadiene-3,5-dithiosemicarbazones (5, 6, and 7)

The methanolic solutions of Knoevenagel condensates (**2**, **3**, and **4**) (1 mmol) were reacted with thiosemicarbazide (2 mmol) with continuous stirring and catalytic amount of piperidine (0.05 cm^3) was added slowly to the reaction mixture for more than 5–10 min. The reaction mixture was further stirred at room temperature for 24 h and set aside when a dark brown solid precipitated out. It was washed with excess of petroleum ether/hexane mixture thrice to remove any unreacted reagents. The compound was purified by recrystallization from chloroform/methanol to yield pure dark brown thiosemicarbazide Schiff base of Knoevenagel condensate.

4.3.1. 4-Salicylidene-1,7-bis(4-hydroxy, 3-methoxyphenyl)-1,6-heptadiene-3,5-dithiosemicarbazone (5). Yield: 82%, mp: 108–110 °C, ^1H NMR ($CDCl_3$): δ 2.63 (s, 4H, NH_2 br), 3.78 (s, 6H, OCH_3), 6.6 (s, 2H, NH br), 6.84 (m, 14H, ArH), 8.00 (s, 1H, =CH–Ar); IR (KBr, cm^{-1}): ~3500 ($\nu_{O-H\ br}$), ~3349 (ν_{N-H}), ~3244, 3156 (ν_{C-NH2}), ~3035, 2949 ($\nu_{=C-H,C-H}$), ~1610 ($\nu_{C=N}$), ~1502 ($\nu_{C=C}$), ~1367, 1028 (ν_{C-H}), ~943 ($\nu_{H-C=C-H(trans)}$), ~823.5 ($\nu_{C-H(arom)}$), ~870 ($\nu_{C=S}$); UV–vis: λ_{max} (nm, DMSO): 332, 410; MS (+ES-MS): m/z = 618.72 (620) (M+1); Anal. Calcd $C_{30}H_{30}N_6O_5S_2$: C, 58.24 ; H, 4.89; N, 13.58. Found: C, 58.29; H, 4.84; N, 13.52.

4.3.2. 4-(2,3-Dihydroxy benzyl)-1,7-bis(4-hydroxy, 3-methoxyphenyl)-1,6-heptadiene-3,5-dithiosemicarbazone (6). Yield: 68%, mp: 127–129 °C, ^1H NMR ($CDCl_3$): δ 3.14 (s, 4H, NH_2 br), 3.85 (s, 6H, OCH_3), 6.8 (s, 2H, ArH), 7.1 (s, 2H, NH br), 7.4 (m, 7H, ArH), 7.99 (s, 1H, =CH–Ar), 6.33 (d, 2H, J = 15.61 Hz, =C–H), 7.6 (2H, d, J = 15.5 Hz, H–C=); IR (KBr, cm^{-1}): ~3751 (ν_{N-H}), ~3553 ($\nu_{O-H\ br}$), ~3289, 3164 (ν_{C-NH2}), ~3009, 2943 ($\nu_{=C-H,C-H}$), ~1596 ($\nu_{C=N}$), ~1514 ($\nu_{C=C}$), ~1379, 1028 (ν_{C-H}), ~945 ($\nu_{H-C=C-H(trans)}$), ~817 ($\nu_{C-H(arom)}$), ~874 ($\nu_{C=S}$); UV–vis: λ_{max} (nm, DMSO): 322, 403; MS (+ES-MS): m/z = 634.72 (636) (M+1); Anal. Calcd for $C_{30}H_{30}N_6O_6S_2$: C, 56.77; H, 4.76; N, 13.24. Found: C, 56.81; H, 4.73; N, 13.30.

4.3.3. 4-(3,4-Dihydroxy benzyl)-1,7-bis(4-hydroxy, 3-methoxyphenyl)-1,6-heptadiene-3,5-dithiosemicarbazone (7). Yield: 79%, mp: 132–134 °C, ^1H NMR ($CDCl_3$): δ 2.96 (s, 4H, NH_2 br), 3.87 (s, 6H, OCH_3), 6.56 (s, 2H, NH br), 6.88 (dd, 4H, J = 8.2 Hz, ArH, J = 16.01 Hz, H–C=), 7.04 (s, 2H, ArH), 7.49 (m, 5H, ArH), 6.2 (d, 2H, J = 15.67 Hz, =C–H), 8.0 (s, 1H, =CH–Ar); IR (KBr, cm^{-1}): ~3683 (ν_{N-H}), ~3617 ($\nu_{O-H\ br}$), ~3262, 3155 (ν_{C-NH2}), ~3012, 2949 ($\nu_{=C-H,C-H}$), ~1610 ($\nu_{C=N}$), ~1517 ($\nu_{C=C}$), ~1380, 1026 (ν_{C-H}), ~945 ($\nu_{H-C=C-H(trans)}$), ~844 ($\nu_{C-H(arom)}$), ~869 ($\nu_{C=S}$); UV–vis: λ_{max} (nm, DMSO): 326, 404; MS (+ES-MS): m/z = 634.72 (635.5) (M+1); Anal. Calcd for $C_{30}H_{30}N_6O_6S_2$; C, 56.77; H, 4.76; N, 13.24. Found: C, 56.73; H, 4.72; N, 13.28.

4.4. General procedure for preparation of copper(II) conjugates of hydroxy substituted 1,7-bis(4-hydroxy, 3-methoxyphenyl)-1,6-heptadiene-3,5-diones (8, 9, and 10)

The copper(II) conjugates were prepared in situ by dissolving the Knoevenagel condensates (**2**, **3**, and **4**) in methanol. To the resulting solution, piperidine (0.05 cm^3) was added dropwise with continuous stirring followed by addition of methanolic solution of $CuCl_2{\cdot}2H_2O$ (1 mmol). The reaction mixture was stirred for the period of 2–5 h. The light brown precipitate separated out at the end was isolated by vacuum filtration, washed with cold methanol, and dried overnight in vacuo at room temperature.

4.4.1. [4-Salicylidene-1,7-bis(4-hydroxy, 3-methoxyphenyl)-1,6-heptadiene-3,5-dione] copper(II) (8). Yield: 82%, IR (KBr, cm^{-1}): ~3453 ($\nu_{O-H\ br}$), ~3110, 2949 ($\nu_{=C-H,C-H}$), ~1614 ($\nu_{C=O}$), ~1589 ($\nu_{C=C}$), ~1390, 1031 (ν_{C-H}), ~829 $\nu_{C-H(arom)}$), ~551 (ν_{Cu-O}), ~332 (ν_{Cu-Cl}); UV–vis: λ_{max} (nm, DMSO): 353, 465; Anal. Calcd for $C_{28}H_{24}Cl_2CuO_7$: C, 55.41; H, 3.99; Cu, 10.47. Found: C, 55.62; H, 4.12; Cu, 10.38.

4.4.2. [4-(2,3-Dihydroxy benzyl)-1,7-bis(4-hydroxy, 3-methoxyphenyl)-1,6-heptadiene-3,5-dione] copper(II) (9). Yield: 87%, IR (KBr, cm^{-1}): ~3479 ($\nu_{O-H\ br}$), ~3018, 2945 ($\nu_{=C-H,C-H}$), ~1591 ($\nu_{C=O}$), ~1506 ($\nu_{C=C}$), ~1406, 1028 (ν_{C-H}), ~827 ($\nu_{C-H(arom)}$), ~559 (ν_{Cu-O}), ~336 (ν_{Cu-Cl}); UV–vis: λ_{max} (nm, DMSO): 335, 476, 506 ($dd_{trans.}$); Anal. Calcd for $C_{28}H_{24}Cl_2CuO_8$: C,

53.99; H, 3.88; Cu, 10.20. Found: C, 54.12; H, 3.96; Cu, 10.34.

4.4.3. [4-(3,4-Dihydroxy benzyl)-1,7-bis(4-hydroxy, 3-methoxyphenyl)-1,6-heptadiene-3,5-dione] copper(II) (10). Yield: 81%, IR (KBr, cm^{-1}): ~3544 ($\nu_{O-H\ br.}$), ~3026, 2941 ($\nu_{=C-H,C-H}$), ~1587 ($\nu_{C=O}$), ~1525 ($\nu_{C=C}$), ~1357, 1026 (ν_{C-H}), ~823 ($\nu_{C-H(arom)}$), ~555 (ν_{Cu-O}), ~331 (ν_{Cu-Cl}); UV–vis: λ_{max} (nm, DMSO): 398, 509 (dd_{trans}); Anal. Calcd for $C_{28}H_{24}Cl_2CuO_8$: C, 53.99; H, 3.88; Cu, 10.20; Found: C, 54.21; H, 4.07; Cu, 10.11.

4.5. General procedure for preparation of copper(II) conjugates of hydroxy substituted 1,7-bis-(4-hydroxy, 3-methoxyphenyl)-1,6-heptadiene-3,5-dithiosemicarbazones (11, 12, and 13)

The copper(II) conjugates were prepared by interaction of the methanolic solutions of Schiff base of Knoevenagel condensates (**8**, **9**, and **10**) (1 mmol) with $CuCl_2{\cdot}2H_2O$ (1 mmol) in presence of catalytic amount of piperidine (0.05 cm^3). The light brown precipitate separated was isolated after 2–5 h of continuous stirring, washed with cold methanol, and dried overnight in vacuo at room temperature.

4.5.1. [4-Salicylidene-1,7-bis(4-hydroxy, 3-methoxyphenyl)-1,6-heptadiene-3,5-dithiosemicarbazone] copper(II) (11). Yield: 78%, IR (KBr, cm^{-1}): ~3575($\nu_{O-H\ br}$), ~3215, 3126 (ν_{C-NH2}), ~3012, 2941 ($\nu_{=C-H,C-H}$), ~1593 ($\nu_{C=N}$), ~1508 ($\nu_{C=C}$), ~1360, 1028 (ν_{C-H}), ~815 ($\nu_{C-H(arom)}$), ~856 ($\nu_{C=S}$), ~435 (ν_{Cu-N}), ~350 (ν_{Cu-S}); UV–vis: λ_{max} (nm, DMSO): 306, 459; Anal. Calcd for $C_{30}H_{30}CuN_6O_5S_2$: C, 52.81; H, 4.43; N, 12.32; Cu, 9.31. Found: C, 53.13; H, 4.27; N, 12.48, Cu, 9.54.

4.5.2. [4-(2,3-Dihydroxy benzyl)-1,7-bis(4-hydroxy, 3-methoxyphenyl)-1,6-heptadiene-3,5-dithiosemicarbazone] copper(II) (12). Yield: 83%, IR (KBr, cm^{-1}): ~3607 ($\nu_{O-H\ br}$), ~3264, 3154 (ν_{C-NH2}), ~3030, 2947 ($\nu_{=C-H,C-H}$), ~1575 ($\nu_{C=N}$), ~1514 ($\nu_{C=C}$), ~1369, 1028 (ν_{C-H}), ~817 ($\nu_{C-H(arom)}$), ~867 ($\nu_{C=S}$), ~441 (ν_{Cu-N}), ~345 (ν_{Cu-S}); UV–vis: λ_{max} (nm, DMSO): 329, 395, 515 (dd_{trans}); Anal. Calcd for $C_{30}H_{30}CuN_6O_6S_2$; C, 51.60; H, 4.33; N, 12.04; Cu, 9.10. Found: C, 51.09; H, 4.78; N, 12.18, Cu, 9.22.

4.5.3. [4-(3,4-Dihydroxy benzyl)-1,7-bis(4-hydroxy, 3-methoxyphenyl)-1,6-heptadiene-3,5-dithiosemicarbazone] copper(II) (13). Yield: 79%, IR (KBr, cm^{-1}): ~3692 ($\nu_{O-H\ br.}$), ~3249, 3077 (ν_{C-NH2}), ~3019, 2943 ($\nu_{=C-H,C-H}$), ~1600 ($\nu_{C=N}$), ~1517 ($\nu_{C=C}$), ~1359, 1026 (ν_{C-H}), ~814($\nu_{C-H(arom)}$), ~856 ($\nu_{C=S}$), ~448 (ν_{Cu-N}), ~352 (ν_{Cu-S}); UV–vis: λ_{max}(nm, DMSO): 395, 473, 557 ($dd_{trans.}$); Anal. Calcd for $C_{30}H_{30}CuN_6O_6S_2$: C, 51.60; H, 4.33; N, 12.04; Cu, 9.10. Found: C, 51.78; H, 4.51; N, 12.26; Cu, 9.28.

4.6. Cell lines

Human myeloid KBM-5 cells, human T-cell leukemia Jurkat cells, human lung adenocarcinoma H1299 cells, and human multiple myeloma MM1 cells were obtained from American Type Culture Collection.KBM-5 cells were cultured in IMDM supplemented with 15% FBS. H1299 cells, MM1 cells, and Jurkat cells were cultured in RPMI 1640 medium supplemented with 10% FBS. All media were also supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin.

4.7. Electrophoretic mobility shift assay

To assess NF-B activation, we performed EMSA as previously described.[26]Briefly, nuclear extractsprepared from treated cells (2×10^6/ml) were incubated with ^{32}P end-labeled 45-mer double-stranded NF-B oligonucleotide(15 μg of protein with 16 fmol DNA) from the HIV longterminal repeat, 5′-TTGTTACAA **GGGACTTTC** CGCTG **GGGACTTTC** CAGGGAGGCGTGG-3′ (boldface indicates NF-B binding sites), for 30 min at 37 °C, and the DNA–protein complex formed was separated from free oligonucleotide on 6.6% native polyacrylamide gels. The dried gels were visualized with a Storm820 and radioactivebands were quantitated using Imagequant software (Amersham Biosciences).

4.8. Cytotoxicity assay

Penicillin, Streptomycin, RPMI 1640 medium, and fetal bovine serum were obtained from Life Technologies, Inc. (Grand Island, NY). Tris, Glycine, NaCl, and bovine serum aibumin were obtained from Sigma and phenyl arseine oxide from Aldrich. Bacteria-derived recombinant human TNF, purified to homogeneity with a specific activity of 5×10^7 U/mg, was provided by Genentech, Inc. (South San Francisco, CA).

The cytotoxicity of curcumin and its analogs in KBM-5, H1299, MM1, and Jurkat cell lines was determined by the MTT uptake method as previously described.[27] Briefly, 2000 cells were treated with curcumin and its analogs for 72 h in triplicate on 96-wellplates at 37 °C. Thereafter, 20 μl MTT solution (5 mg/ml) was added to each well. After 2 h of incubation at 37 °C, solubilization buffer (20% SDS and 50% dimethylformamide) was added, the cells were incubated overnight at 37 °C, and then the OD was measured at 570 nm using a 96-well multiscanner (MRX Revelation; Dynex Technologies).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2006.06.056.

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