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TPA-induced up-regulation of activator protein-1 can be inhibited or enhanced by analogs of the natural product curcumin

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AP-1, activator protein-1
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TRE, TPA-responsive element

ABSTRACT

The activator protein-1 (AP-1) family of transcription factors, including the most common member c-Jun-c-Fos, participates in regulation of expression of numerous genes involved in proliferation, apoptosis, and tumorigenesis in response to a wide array of stimuli including pro-inflammatory cytokines, growth factors, stress, and tumor promoters. A number of plant polyphenols including curcumin, a yellow compound in the spice turmeric, have been shown to inhibit the activation of AP-1. Curcumin is a polyphenolic dienone that is potentially reactive as a Michael acceptor and also is a strong anti-oxidant. Multiple activities reported for curcumin, including inhibition of the stress-induced activation of AP-1, have been suggested to involve the anti-oxidant properties of curcumin. In the present study, a library of analogs of curcumin was screened for activity against the TPA-induced activation of AP-1 using the Panomics AP-1 Reporter 293 stable cell line which is designed for screening potential inhibitors. Numerous analogs were identified that were more active than curcumin, including analogs that were not anti-oxidants and analogs that were not Michael acceptors. Clearly, anti-oxidant activity or reactivity as a Michael acceptor is not an essential feature of active compounds. In addition, a number of analogs were identified that enhanced the TPA-induced activation of AP-1. The results from screening were confirmed using BV-2 microglial cells where curcumin and analogs were shown to inhibit LPS-induced COX-2 expression; analogs identified as more potent than curcumin in the screening assay were also more potent than curcumin in preventing COX-2 expression.

1. Introduction

Activator protein-1 (AP-1) is a family of dimeric transcription factors containing homo- or heterodimers of members of the Jun, Fos, ATF and Maf families of proteins. AP-1-mediated gene transcription contributes to many cellular processes such as proliferation, cell cycle regulation, differentiation, and apoptosis [1–6]. The major AP-1 proteins in mammalian cells are Jun-Fos dimers, mainly c-Jun-c-Fos. Active AP-1 dimers can bind to TPA-responsive elements (TREs) in the promoters of AP-1 responsive genes. In addition to tumor promoters, AP-1 binding to TREs is rapidly induced by growth factors,

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cytokines, and oncoproteins. This has contributed to the general view that activation of AP-1 is oncogenic by contributing to proliferation, survival and transformation of cells. This view is supported by the observation that several AP-1 proteins, including c-Jun and c-Fos, can transform cells in culture [7-9]. Development of inhibitors of activation of AP-1 may be a promising approach to development of new anti-cancer therapeutics [10,11]. It is noteworthy, however, that certain AP-1 dimers can be anti-oncogenic [4,12,13]. Whether or not AP-1 is oncogenic depends upon cell type, genetic background, nature of the stimulus, and state of differentiation [4,5]. This makes it difficult to make generalized statements about the physiological roles of AP-1 in the absence of specific information concerning cell type and environmental conditions.

Compounds that have been shown to inhibit the activation of AP-1 include a number of phytochemicals such as curcumin, resveratrol, epigallocatechin gallate, and theaflavins [6,14–16]. Curcumin is especially of interest. This phytochemical, obtained from the rhizome of the herb *Curcuma longa*, is a yellow compound in the spice turmeric, which has been used for centuries in Asia as a herbal medicinal to treat a wide range of health problems. Numerous studies of the anti-inflammatory and anti-cancer properties of curcumin have been reported (reviewed in refs. [16–19]). Curcumin is considered a non-toxic phytochemical. Doses as high as 12 g/day have been used in recent clinical studies of curcumin for the treatment of a wide variety of malignancies [20–22]. Curcumin (Fig. 1) is also a strong anti-oxidant by virtue of its phenolic groups [23–25]. In the multiple studies of the activities ascribed to curcumin, it is often suggested that the anti-oxidant properties of curcumin contribute to the observed biological activities. However, experiments designed specifically to answer this question are rarely included in these studies. In spite of reports indicating that curcumin is an unstable compound [26] and is especially reactive as a Michael acceptor involving its α,β-unsaturated ketone functionalies [27], the intrinsic reactivity of curcumin is generally overlooked in many of the studies of its biological properties.

In the present study, a library of analogs of curcumin was examined for activity as inhibitors of the TPA-induced activation of AP-1 in HEK293 cells transfected with an AP-1-dependent luciferase construct. This study compared analogs that retained the 7-carbon dienone spacer between the aromatic rings, as in curcumin, but with various ring substituents including non-phenolic groups; this provided analogs that were no longer active anti-oxidants and could be used to address the question as to whether the anti-oxidant activity is required for biological activity. In addition, analogs were included that contained 5-carbon or 3-carbon enone spacers as well as analogs devoid of the enone functionality. We report here that analogs devoid of anti-oxidant activity as well as analogs that are not Michael acceptors can retain or even exhibit increased activity compared to curcumin as inhibitors of the TPA-induced activation of AP-1. Moreover, some analogs can function as enhancers of the TPA-induced activation of AP-1.

2. Materials and methods

2.1. Synthesis of analogs of curcumin

Curcumin is a relatively simple bis-phenolic compound that can be prepared in a single reaction. The procedures used to prepare curcumin analogs in the 7-carbon, 5-carbon, and 3-carbon series were described previously, and are summarized in Fig. 2 [28–38]. The following describes the synthesis of analogs of curcumin that were not included in our previous studies [28,29] but were included in this study. The bold, underlined numbers refer to the numbering of the analogs as they appear in Figs. 3–8.

1,5-Bis(2-thienyl)-1,4-pentadien-3-one (**59**). Yellow solid: mp 115–117 °C [literature [39] 115–117 °C]; ^{13}C NMR: δ 124.4, 128.2, 128.7, 131.7, 135.5, 140.2, 187.5.

4-Benzyl-1,7-bis(4-hydroxy-3-methoxyphenyl)-heptane-3,5-dione (**20**). The compound was prepared [31] by reaction of 4-benzyl-1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione (0.25 g, 0.5 mmol) with hydrogen in the presence of palladium on activated carbon (0.20 g, 10%) to give 0.12 g (48%) of compound **20** as a pale yellow oil; ^{1}H NMR δ enol form: 2.66 (m, 8H), 3.53 (s, 2H), 3.77 (s, 6H), 5.55 (s, 2H), 6.56 (m, 4H), 6.77 (d, 2H, J = 7.6 Hz), 7.06 (m, 6H), 7.20 (m, 2H); keto form: 2.66 (m, 8H), 3.08 (d, 2H, J = 7.3 Hz), 3.82 (s, 6H), 3.92 (t, 1H, J = 8.3 Hz), 5.55 (s, 2H), 6.56 (m, 4H), 6.77 (d, 2H, J = 7.6 Hz), 7.06 (m, 6H), 7.20 (m, 2H); ^{13}C NMR: δ 29.0, 31.1, 31.8, 34.3, 37.7, 44.6, 55.8, 69.2, 111.0, 114.2, 120.7, 120.8, 126.6, 127.4, 128.5, 128.6, 132.3, 132.6, 137.9, 143.8, 146.3, 193.4, 204.5. Exact mass calcd. for $C_{28}H_{30}O_6$: 462.2042, observed (M + H) 463.2073.

1,5-Bis(4-dimethylaminophenyl)-1,4-pentadien-3-one (**41**). Orange solid: mp 179–181 °C [literature [40] 174–176 °C]; ^{1}H NMR: δ 3.01 (s, 12H), 6.69 (d, 4H, J = 8.7 Hz), 6.87 (d, 2H, J = 15.7 Hz), 7.50 (d, 4H, J = 8.7 Hz), 7.67 (d, 2H, J = 15.7 Hz); ^{13}C NMR: δ 40.2, 98.9, 111.8, 121.2, 122.9, 129.9, 142.8, 151.6.

1,5-Bis(1-naphthyl)-1,4-pentadien-3-one (**39**). Yellow solid: mp 132–133 °C [literature [41] 128 °C]; ^{13}C NMR: δ 123.4, 125.1, 125.4, 126.2, 126.9, 128.1, 128.7, 130.7, 131.7, 132.2, 133.7, 140.3, 188.5.

2,6-Bis(4-methylphenyl)-1-methyl-4-piperidone (**83**). White solid: mp 120–121 °C [literature [42] 105–107 °C]; ^{1}H NMR δ 1.79 (s, 3H), 2.36 (s, 6H), 3.10 (d, 2H, J = 14.9 Hz), 2.79 (t, 2H, J = 13.1 Hz), 3.35 (dd, 2H, J = 11.9, 2.4 Hz), 7.15 (d, 4H, J = 8.0 Hz), 7.21 (d, 4H, J = 7.9 Hz); ^{13}C NMR: δ 21.2, 40.7, 50.9, 70.0, 126.9, 129.4, 137.2, 140.2, 207.1.

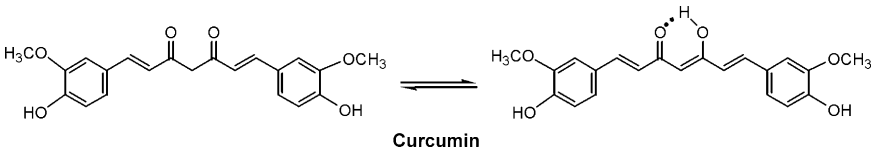


Fig. 1 – Curcumin exists as an equilibrium mixture of the keto and enol forms.

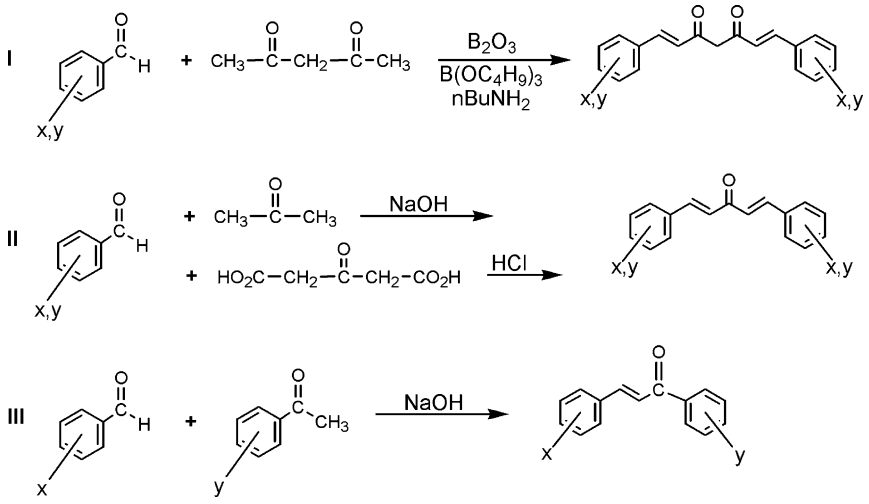


Fig. 2 – Synthesis of enone analogs of curcumin. Series I analogs, maintaining the 7-carbon dienone spacer between the aromatic rings as in curcumin, were synthesized from aromatic aldehydes by condensation with 2,4-pentanedione in an aldol type reaction [30–33]. This involves base-catalyzed condensation in the presence of a trialkylborate to complex with the carbonyl groups, which prevents enolization and guides the reaction. If a mixture of two different aldehydes is used, unsymmetrical analogues are formed. Series II analogs, containing a 5-carbon enone spacer, were synthesized either by base-catalyzed condensation of the appropriate aldehyde with acetone or by acid-catalyzed condensation with 3-oxo-glutaric acid [34,35]. Series III analogs, containing a 3-carbon enone spacer, were synthesized by base-catalyzed condensation of the appropriate aldehyde with a substituted acetophenone [36–38].

2,6-Bis(4-methoxyphenyl)-1-methyl-4-piperidone (**84**). White solid: mp 141–143 °C [literature [42] 129-130 °C]; ^{1}H NMR δ 1.77 (s, 3H), 2.45 (d, 2H, J = 14.5 Hz), 2.78 (t, 2H, J = 12.9 Hz), 3.33 (d, 2H, J = 11.9 Hz), 3.79 (s, 6H), 6.88 (d, 4H, J = 8.5 Hz), 7.32 (d, 4H, J = 8.5 Hz); ^{13}C NMR: δ 40.6, 50.9, 55.3, 69.5, 114.1, 128.0, 135.3, 158.9, 207.1.

2,6-Bis(2-methylphenyl)-1-methyl-4-piperidone (**85**). The compound was prepared [42] by the reaction of 1,5-bis(2-methylphenyl)-1,4-pentadien-3-one (0.50 g, 1.9 mmol) with methylamine (1.0 mL, 11.6 mmol) to give 0.29 g (52%) of compound **85** as a white solid: mp 155–157 °C; ^{1}H NMR δ 1.82 (s, 3H), 2.40 (s, 6H), 2.44 (d, 2H, J = 11.9 Hz), 2.77 (t, 2H, J = 13.3 Hz), 3.74 (d, 2H, J = 11.9 Hz), 7.16 (m, 6H), 7.67 (d, 2H, J = 7.0 Hz); ^{13}C NMR: δ 19.5, 39.9, 49.3, 65.8, 126.7, 126.9, 130.6, 134.8, 140.9, 207.2. Exact mass calcd. for $C_{20}H_{23}NO$: 293.1779, observed (M + H) 294.1856.

2,6-Bis(2-methoxyphenyl)-1-methyl-4-piperidone (**80**). The compound was prepared [42] by the reaction of 1,5-bis(2-methoxyphenyl)-1,4-pentadien-3-one (0.26 g, 0.9 mmol) with methylamine (0.40 mL, 4.6 mmol) to give 0.16 g (55%) of compound **80** as a white solid: mp 146–148 °C; ^{1}H NMR δ 1.89 (s, 3H), 2.50 (d, 2H, J = 13.7 Hz), 2.65 (t, 2H, J = 11.9 Hz), 3.82 (s, 6H), 4.11 (d, 2H, J = 11.5 Hz), 6.87 (d, 2H, J = 8.3 Hz), 7.03 (t, 2H, J = 7.2 Hz), 7.23 (t, 2H, J = 5.8 Hz), 7.72 (d, 2H, J = 7.6 Hz); ^{13}C NMR: δ 40.3, 49.2, 55.4, 61.2, 110.7, 121.0, 127.6, 127.8, 131.5, 156.3, 208.1. Exact mass calcd. for $C_{20}H_{23}NO_3$: 325.1678, observed (M + H) 326.1754.

2,6-Bis(2-naphthyl)-1-methyl-4-piperidone (**82**). The compound was prepared [42] by the reaction of 1,5-bis(2-naphthyl)-1,4-pentadien-3-one (0.82 g, 2.5 mmol) with methylamine (1.30 mL, 15.1 mmol) to give 0.20 g (22%) of compound **22** as a white solid: mp 209–212 °C; ^{1}H NMR δ 1.89 (s, 3H), 2.59 (d, 2H, J = 13.9 Hz), 2.97 (t, 2H, J = 11.3 Hz), 3.66 (d, 2H, J = 11.7 Hz), 7.49 (m, 4H), 7.81 (m, 10H); ^{13}C NMR: δ 41.1, 50.7, 70.3, 124.6, 125.9, 126.0, 126.2, 127.6, 127.7, 128.9, 133.0, 133.4, 140.4, 206.6. Exact mass calcd. for $C_{26}H_{23}NO$: 365.1780, observed (M + H) 366.1852.

2,6-Bis(2,5-dimethoxyphenyl)-1-methyl-4-piperidone (**86**). The compound was prepared [42] by the reaction of 1,5-bis(2,5-dimethoxyphenyl)-1,4-pentadien-3-one (0.85 g, 2.20 mmol) with methylamine (1.85 mL, 24 mmol) to give 0.68 g (68%) of compound **86** as white needles: mp 169–170 °C; ^{1}H NMR: δ 1.90 (s, 3H), 2.56 (m, 4H), 3.75 (s, 6H), 3.80 (s, 6H), 4.05 (dd, 2H, J = 11.7, 3.2 Hz), 6.76 (m, 4H), 7.28 (d, 2H, J = 2.8 Hz); ^{13}C NMR: δ 40.3, 49.1, 55.7, 56.1, 61.3, 112.0, 113.6, 132.7, 150.7, 154.1, 207.5. Exact mass calcd. for $C_{22}H_{27}NO_5$: 385.1889, observed (M + H) 386.1967.

2,6-Bis(2,4-dimethoxyphenyl)-1-methyl-4-piperidone (**87**). The compound was prepared [42] by the reaction of 1,5-bis(2,4-dimethoxyphenyl)-1,4-pentadien-3-one (0.85 g, 2.20 mmol) with methylamine (1.85 mL, 24 mmol) to give 0.61 g (66%) of compound **87** as white needles: mp 164–165 °C; ^{1}H NMR: δ 1.84 (s, 3H), 2.45 (d, 2H, J = 12.9), 2.63 (t, 2H, J = 12.1 Hz) 3.77 (s, 6H), 3.80 (s, 6H), 4.96 (dd, 2H, J = 11.3, 3.2 Hz,), 6.74 (d, 2H, J = 2.4 Hz), 6.54 (dd, 2H, J = 7.7, 2.2 Hz,), 7.54 (d, 2H, J = 8.6 Hz); ^{13}C NMR: δ 39.9, 49.4, 55.3, 55.5, 60.9, 98.2, 100.8, 105.2, 124.0, 128.2, 157.4, 159.5, 208.2. Exact mass calcd. for $C_{22}H_{27}NO_5$: 385.1889, observed (M + H) 386.1967.

4-Benzyl-1,7-diphenylheptane-3,5-dione (**17**). The compound was prepared [31] by reaction of 4-benzyl-1,7-diphenyl-1,6-heptadiene-3,5-dione (0.26 g, 0.7 mmol) with hydrogen in the presence of palladium on activated carbon (0.25 g, 10%) to give 0.18 g (69%) of compound **17** as white needles: mp 74–75 °C; ^{1}H NMR δ enol form: 2.61 (m, 10H), 7.14 (m, 15H); keto form: 2.61 (m, 8H), 3.07 (d, 2H, J = 7.2 Hz), 3.90 (t, 1H, J = 7.6 Hz), 7.14 (m, 15H); ^{13}C NMR: δ 29.3, 34.3, 44.3, 69.2, 126.1, 126.7, 128.3,

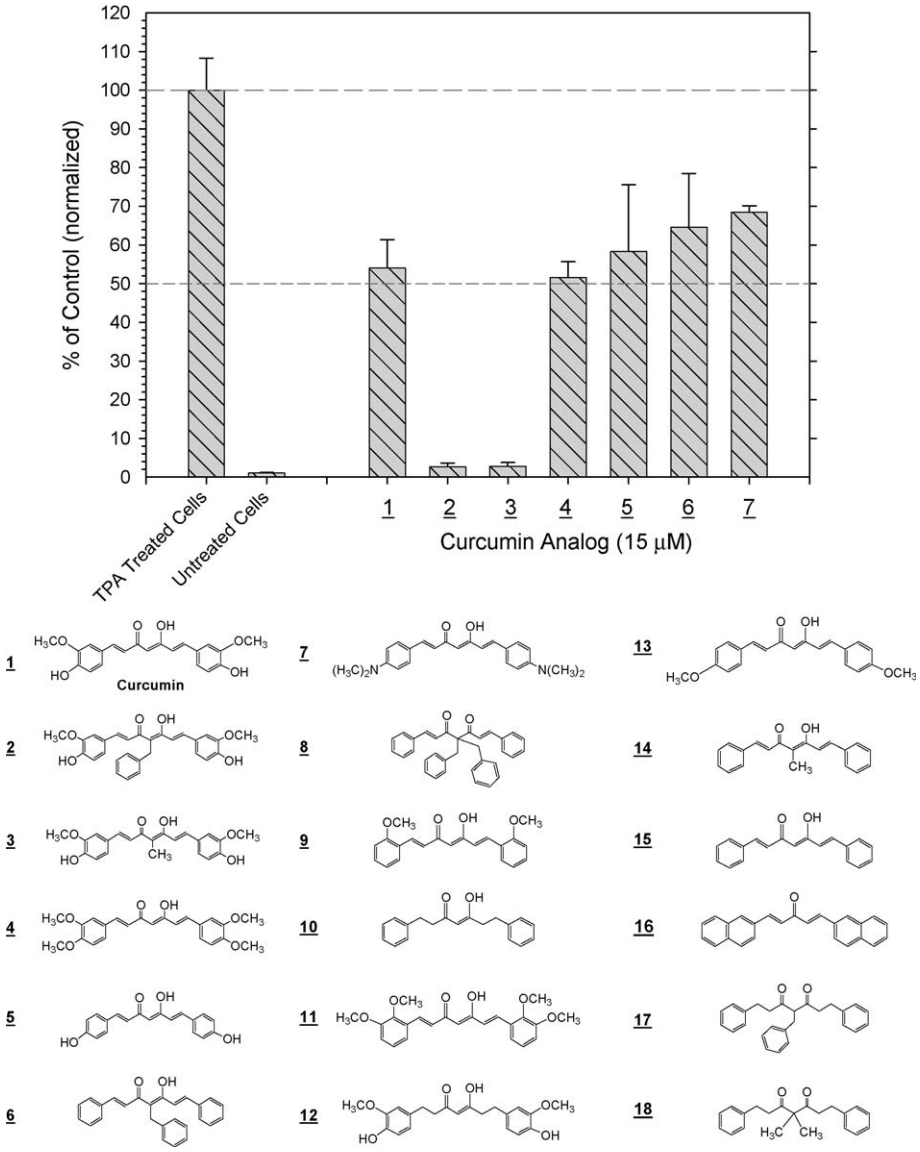


Fig. 3 – Inhibition of the TPA-induced activation of AP-1 by curcumin and analogs in the 7-carbon series. Curcumin and analogs were screened in triplicate at 15 μM concentrations. Analogs 2 and 3 were selected for further study (Table 1).

128.4, 128.6, 128.7, 137.9, 140.4, 204.3. Exact mass calcd. for $C_{26}H_{26}O_2$: 370.1933, observed (M + H) 371.2014.

4,4-Dimethyl-1,7-diphenylheptane-3,5-dione (**18**). The compound was prepared [31] by reaction of 4,4-dimethyl-1,7-diphenyl-1,6-heptadiene-3,5-dione (0.15 g, 0.5 mmol) with hydrogen in the presence of palladium on activated carbon (0.2 g, 10%) to give 0.12 g (80%) of compound **18** as a pale yellow oil; ^{1}H NMR δ 1.25 (s, 6H), 2.60 (t, 4H, J = 7.4 Hz), 2.80 (t, 4H, J = 7.0 Hz), 7.18 (m, 10H); ^{13}C NMR: δ 21.1, 29.8, 40.2, 62.4, 126.1, 128.3, 140.7, 208.4. Exact mass calcd. for $C_{21}H_{24}O_2$: 308.1776, observed (M + H) 309.1843.

2.2. Assay of the anti-oxidant activities of curcumin and analogs

The anti-oxidant activities of curcumin and analogs were determined using two standard assays [43]. The *total radical-trapping anti-oxidant parameter* assay (TRAP assay) measures the ability of an analog to react with the pre-formed radical monocation of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) [44]. ABTS was reacted with potassium persulfate in the dark, overnight, to generate the colored $ABTS^{\bullet+}$ radical cation, which has an absorption maximum at 734 nm. The activities of curcumin and analogs were determined by their abilities to quench the color of the radical cation. The *ferric reducing/anti-oxidant power* assay (FRAP assay) measures the ability of an analog to reduce a ferric tripyridyltriazine complex [45]. The ferric complex of 2,4,6-tripyridyl-*s*-triazine was prepared at acidic pH, and the anti-oxidant activities of curcumin and analogs were determined by their abilities to reduce the ferric complex to the ferrous complex, monitored by formation of the ferrous complex at 593 nm. In both colorimetric assays, the Vitamin E analog Trolox was used as a control.

2.3. Assay of the activities of curcumin and analogs as inhibitors of the TPA-induced activation of AP-1

An AP-1 reporter stable cell line derived from human 293T embryonic kidney cells transfected with a luciferase reporter construct containing three AP-1 binding sites in the promoter (293T/AP-1-luc, Panomics, Inc., Redwood City, CA) was grown in a humidified atmosphere at 37 °C in 5% CO_2/95% air. The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM—high glucose containing 4 mM glutamine) supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 100 units/mL penicillin, 100 μg/mL streptomycin and 100 μg/mL hygromycin (Gibco/Invitrogen, Carlsbad, CA) to maintain cell selection. One day prior to treatment, the 293T/AP-1-luc cells were plated into 24-well cell culture plates (Costar, Cambridge, MA) in the above media without hygromycin. The following day, the cells, which were at approximately 60% confluency, were fed fresh media with or without TPA, 10 ng/mL (Calbiochem) and immediately treated with curcumin or analog prepared in DMSO stock solutions. The cells were placed again in a humidified atmosphere at 37 °C in 5% CO_2/95% air for 24 h. Plate wells were gently washed with phosphate buffered saline, pH 7.4, and lysed with 1× passive lysis buffer (Promega, Madison, WI). The subsequent lysates were analyzed with the Luciferase Assay System (Promega) utilizing a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). The firefly luciferase relative light units were normalized to protein (mg/mL) with BCA™ Protein Assay Kit (Pierce, Rockford, IL) and standardized to percent of control (TPA control).

2.4. Inhibition of COX-2 expression by curcumin and analogs

Mouse microglial cells (BV-2) were kindly provided by Dr. Paul M. Stemmer (Institute of Environmental Health Sciences, Wayne State University, Detroit, MI). Cells were cultured in RPMI-1640 (Cellgro, Herndon, VA) supplemented with 10% FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 μg/mL streptomycin sulfate and 100 units/mL penicillin. Cells were grown on culture plates, pre-treated with 1% gelatin for 30 min, at 37 °C and passaged twice weekly.

BV-2 cells were incubated in the absence or presence of 0.2 μg/mL lipopolysaccharide (LPS) (Sigma, St. Louis, MO) to induce the inflammatory gene response. Those cells that were treated with LPS were incubated in parallel with curcumin or curcumin analogs **27** or **80** for 24 h at the indicated concentrations. Total RNA was purified using RNeasy (Qiagen, Valencia, CA) and converted to cDNA using TaqMan Reverse Transcriptase (Applied Biosystems, Branchburg, NJ). Cyclooxygenase-2 (COX-2) mRNA levels were measured using quantitative Real Time PCR analysis (qRT-PCR) of cDNA samples. Primers specific for COX-2 were designed to amplify a 132 base pair sequence flanking intron 7. Primer sequences for COX-2 were: upstream, TGGGGTGATGAGGCAACTATT; downstream, AAGGAGCTCTGGGTCAAACT. qRT-PCR was performed using ABsolute QPCR SYBR Green Mix (Fisher Scientific, Atlanta, GA) with the following cycling parameters: 1 cycle, 95 °C, 15 min; 40 cycles, 95 °C, 15 s, 60 °C, 1 min. β-Actin mRNA levels were quantitated using identical cycling conditions and used to normalize values obtained for COX-2 expression.

3. Results

3.1. Inhibition or enhancement of the TPA-induced activation of AP-1 by curcumin analogs in the 7-carbon series

Curcumin (**1**) and 23 analogs of curcumin in the 7-carbon series were compared for their effects on the TPA-induced activation of AP-1 in an initial screening at 15 μM concentration of the analogs. Analogs that inhibited the activation of AP-1 and showed activities comparable to or better than curcumin (analogs **2**–**7**) are shown in Fig. 3 along with the structures of analogs (**8**–**18**) that showed little or no activity (data not shown). The most active compounds, analogs **2** and **3**, retain the same ring substituents as curcumin but contain either an alkyl or arylalkyl group attached to the central carbon in the 7-carbon spacer. Both **2** and **3** were shown previously to possess anti-oxidant activities [29]. However, analogs **4** and **7**, which exhibit activities comparable to curcumin, do not show anti-oxidant activities in the FRAP or TRAP assays. We conclude, therefore, that anti-oxidant activity is not an essential property of analogs that inhibit the TPA-induced activation of AP-1.

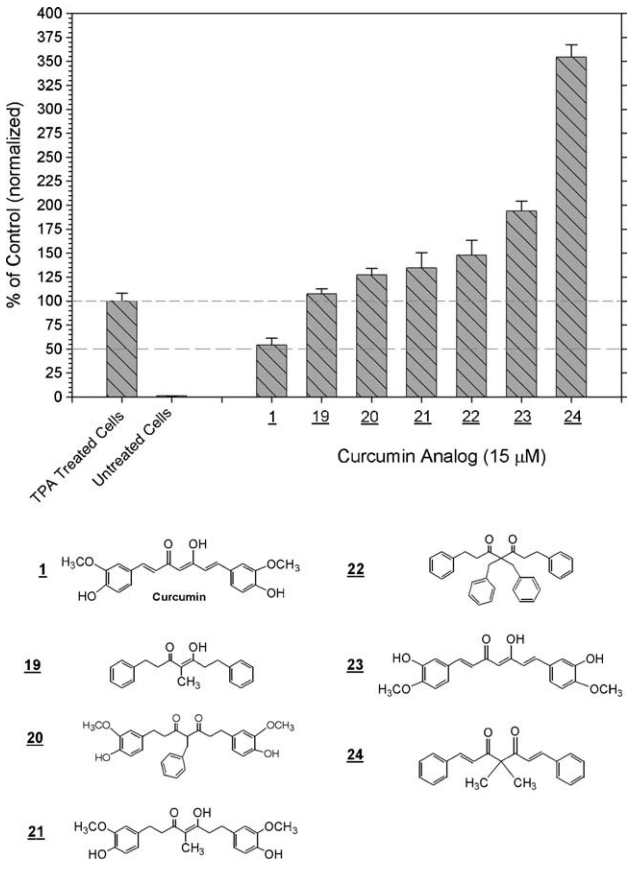


Fig. 4 – Enhancement of the TPA-induced activation of AP-1 by analogs in the 7-carbon series. Conditions were as described in Fig. 3. Analog 24 gave the strongest enhancement.

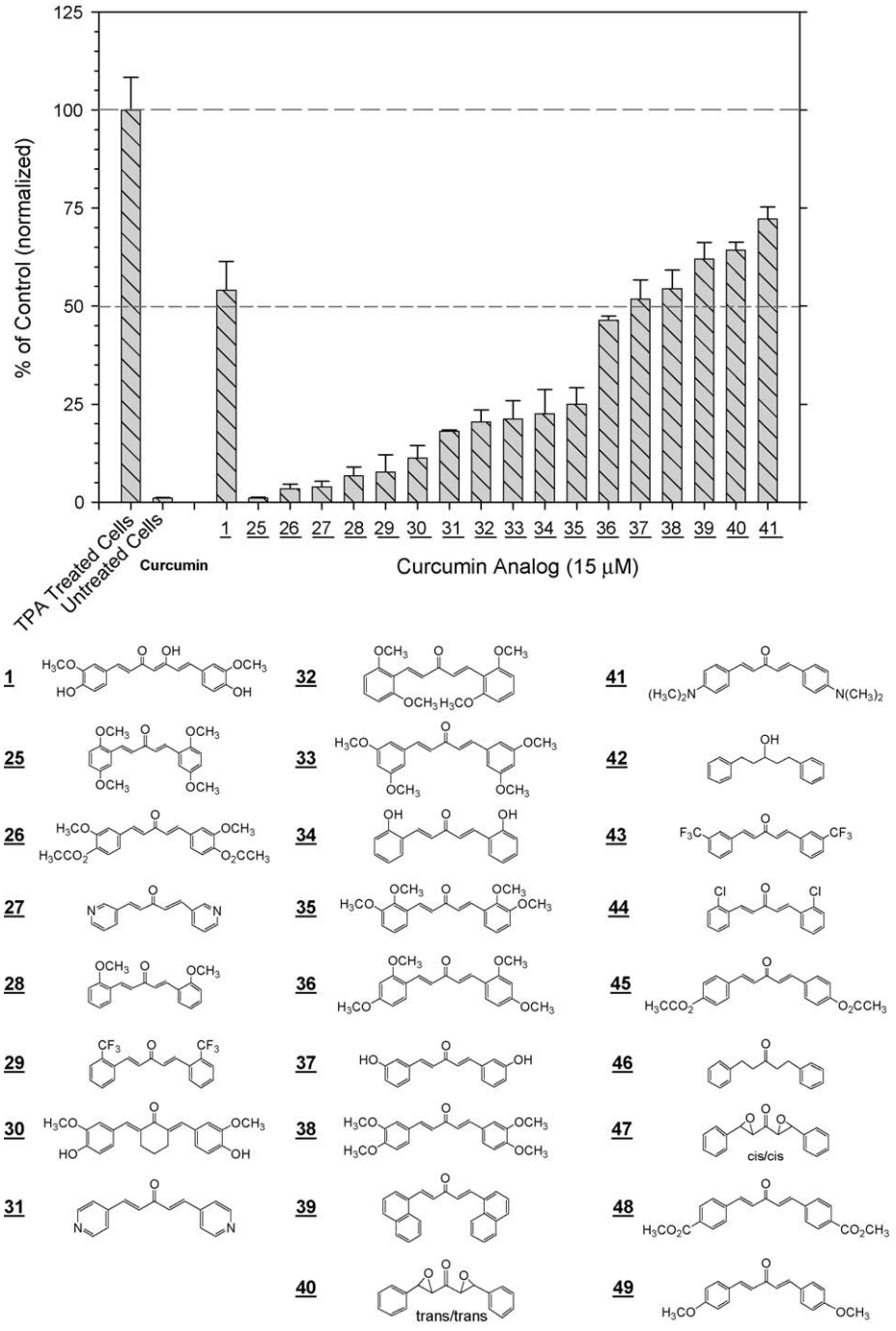


Fig. 5 – Inhibition of the TPA-induced activation of AP-1 by analogs in the 5-carbon series. Conditions were as described in Fig. 3. Analogs 25–35 were selected for further study (Table 1).

A number of analogs in the 7-carbon series (19–24, Fig. 4) enhance the activation of AP-1 in response to stimulation with TPA. For analog 24, which is devoid of anti-oxidant activity, the enhancement was almost four-fold. Analog 23, which gave a two-fold enhancement, is an isomer of curcumin, differing only in the locations of the ring substituents. Clearly, small structural changes can convert an inhibitor into an enhancer. The enhancer activities of the analogs 19–24 are only observed when TPA is present to stimulate the cells. These analogs have no effect on AP-1 expression in the absence of TPA.

3.2. *Inhibition or enhancement of the TPA-induced activation of AP-1 by curcumin analogs in the 5-carbon series*

Forty-three compounds in the 5-carbon series, analogs 25–67, were compared with curcumin as inhibitors or enhancers of TPA-induced activation of AP-1. Analogs 25–41 inhibited activation, with many showing greater activity than curcumin (Fig. 5). Most of these analogs were devoid of anti-oxidant activity, which further supports the conclusion that anti-oxidant activity is not an essential property of analogs that

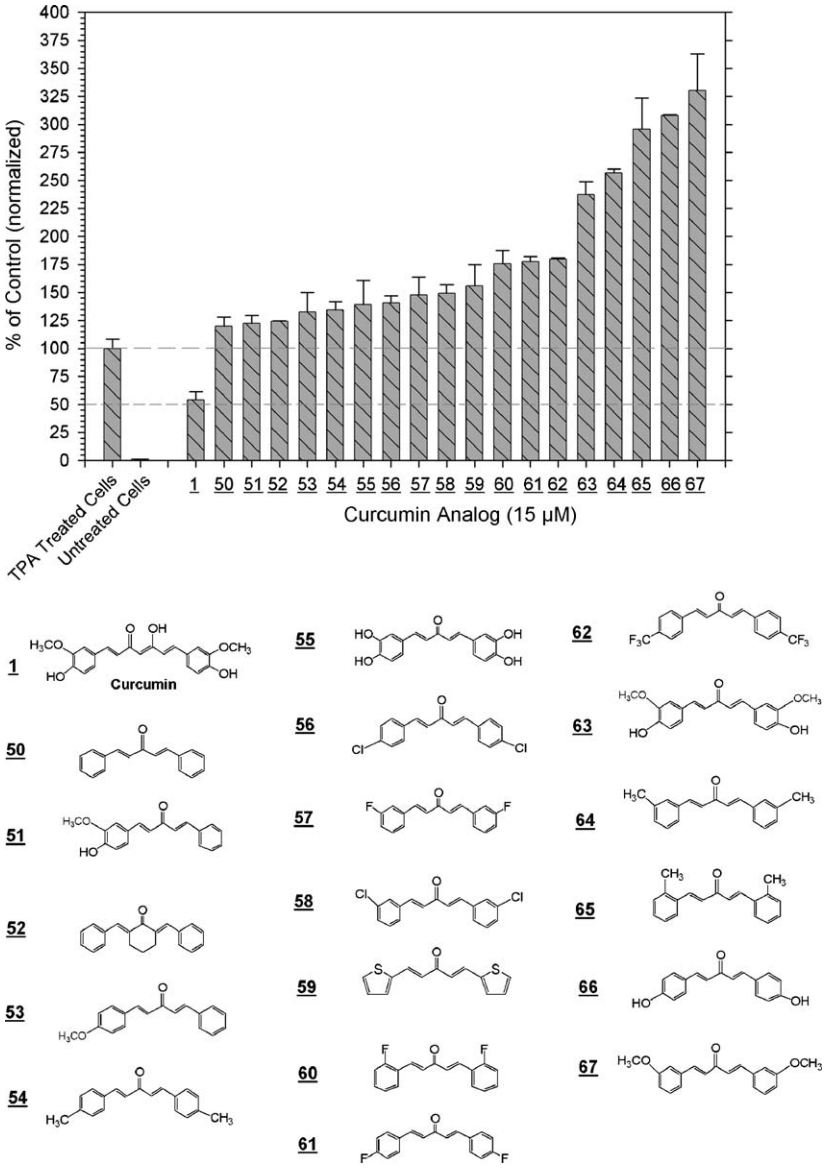


Fig. 6 – Enhancement of the TPA-induced activation of AP-1 by analogs in the 5-carbon series. Conditions were as described in Fig. 3. Analogs 64–67 gave the strongest enhancement.

effectively inhibit activation of AP-1. Analogs 42–49, whose structures are shown in Fig. 5, exhibited little or no activity (data not shown).

Analogs 50–67 of the 5-carbon series were enhancers of the TPA-induced activation of AP-1, with enhancement activities up to three-fold for analogs 65–67 (Fig. 6). As was observed with the 7-carbon series, anti-oxidant activity is not essential for enhancement activity. For example, analog 66 exhibits anti-oxidant activity whereas analog 67 does not.

3.3. *Inhibition or enhancement of the TPA-induced activation of AP-1 by curcumin analogs in the 3-carbon series*

Twelve analogs in the 3-carbon series were compared with curcumin, as shown in Fig. 7. Analog 68 showed activity comparable to curcumin as an inhibitor of the TPA-induced activation of AP-1, while analogs 69–76 exhibited some activity. Analogs 77–79 showed little or no activity (data not shown). Most of the analogs in the 3-carbon series were devoid of anti-oxidant activity.

3.4. *Michael adducts retain activity as inhibitors or enhancers of the TPA-induced activation of AP-1*

Curcumin and most of the analogs in Figs. 3–7 are α,β-unsaturated ketones that are potential Michael acceptors in reactions with nucleophiles such as sulfhydryl groups or amino groups. Therefore, the question was addressed as to whether activity, either as inhibitors or as enhancers of the TPA-induced activation of AP-1, requires retention of this enone functionality. A series of analogs in the 5-carbon series was reacted with methylamine to produce piperidone derivatives, which resulted from a double Michael addition. The activities of these analogs (80–87) are shown in Fig. 8. Analogs

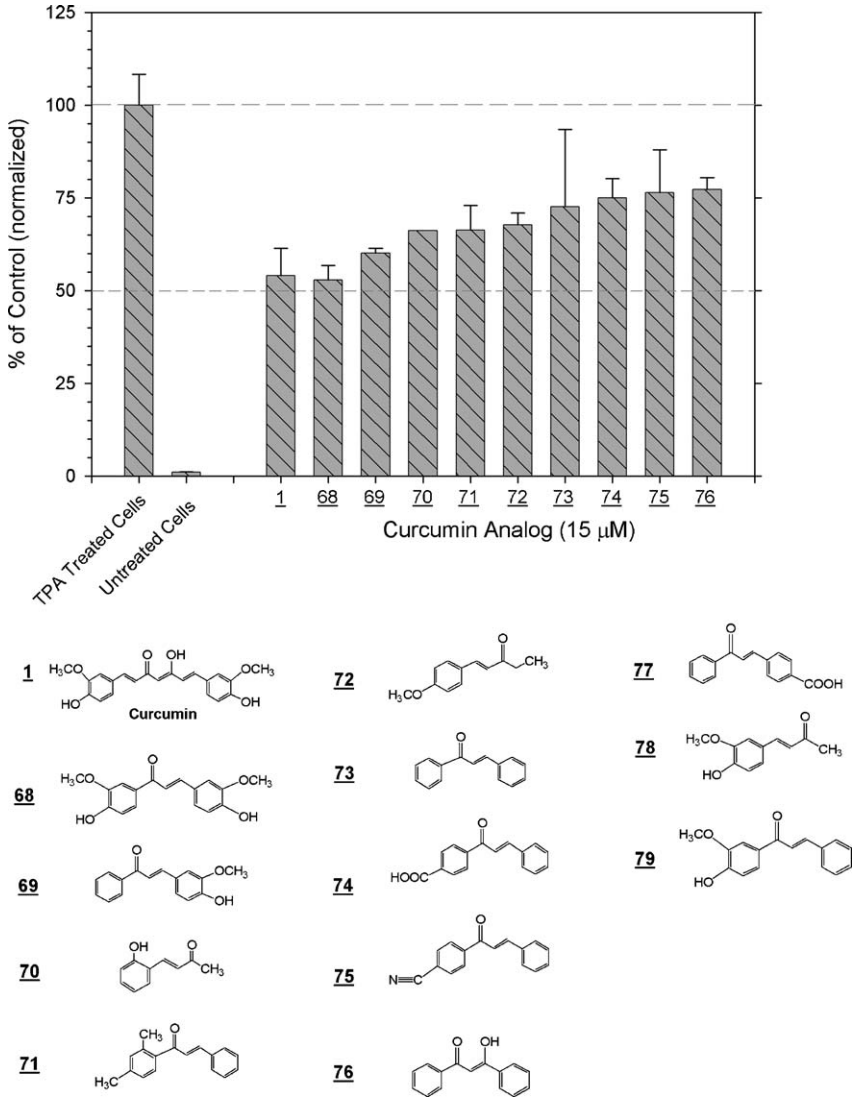


Fig. 7 – Inhibition of the TPA-induced activation of AP-1 by analogs in the 3-carbon series. Conditions were as described in Fig. 3.

80 and 81 were more active than curcumin; analog 83 was inactive. By comparison, analogs 84–87 produced modest enhancements of activity. Clearly, the enone functionality is not essential for biological activity.

3.5. *Dose–response curves and determination of IC_{50} values*

Analogs of curcumin in the 7-carbon and 5-carbon series that were more active than curcumin were analyzed further by dose–response studies to obtain IC_{50} values. Representative dose–response plots are shown in Fig. 9 and the IC_{50} values are summarized in Table 1 along with the anti-oxidant properties of the active analogs. Many of the active analogs do not show anti-oxidant activity. The most active analogs, such as 25, are an order of magnitude more potent than curcumin as inhibitors of the TPA-induced activation of AP-1. Michael adduct 80 is one of the more active analogs. Even analogs with heterocyclic rings such as 27 are more active than curcumin. We conclude, therefore, that biological activity can be retained with wide variations in structures, some of which are only remotely related to curcumin. Numerous analogs can be viewed as lead compounds for the development of inhibitors of the activation of AP-1.

3.6. *Inhibition of COX-2 expression in microglial cells*

To determine whether the effects of curcumin and its analogs in inhibiting the activation of AP-1 extend beyond the 293/AP-1-luc reporter cell line used for screening, curcumin and analogs 27 and 80 were compared using microglial BV-2 cells. This cell line has been shown to express COX-2 in response to LPS stimulation by an AP-1-dependent pathway that is inhibited by curcumin [46]. BV-2 cells stimulated with LPS showed a strong induction of COX-2 mRNA (Fig. 10) that was markedly suppressed by 15 μM curcumin and 50% inhibited by 3 μM curcumin. Analog 27 and analog 80 at 3 μM concentrations were as effective as 15 μM curcumin, con-

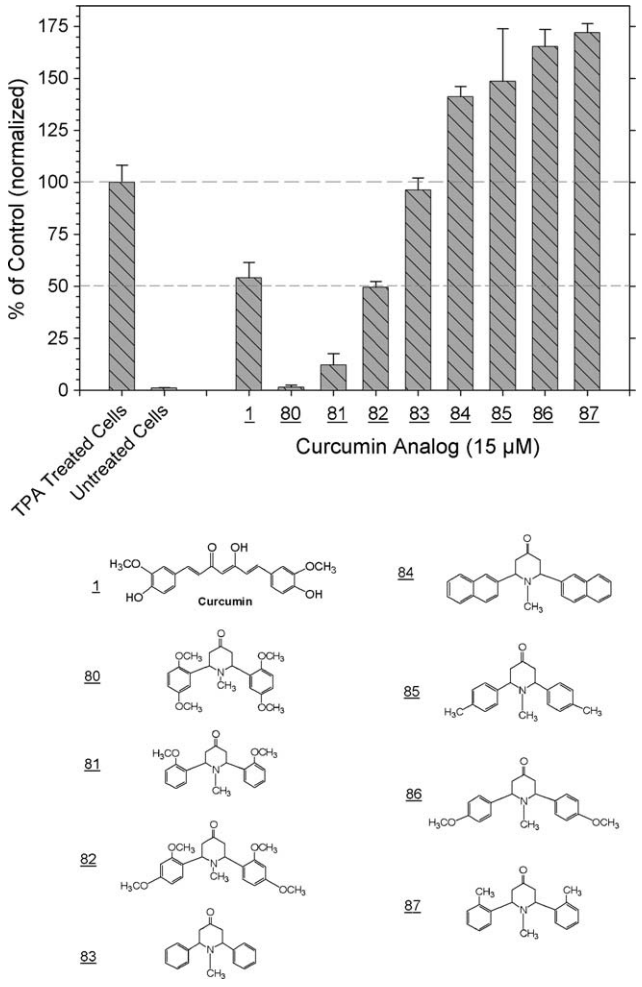


Fig. 8 – Inhibition and enhancement of the TPA-induced activation of AP-1 by Michael adducts. Conditions were as described in Fig. 3. The piperidones are the products of double Michael addition of methylamine to selected analogs from the 5-carbon series. Analogs 80 and 81 were selected for further study (Table 1).

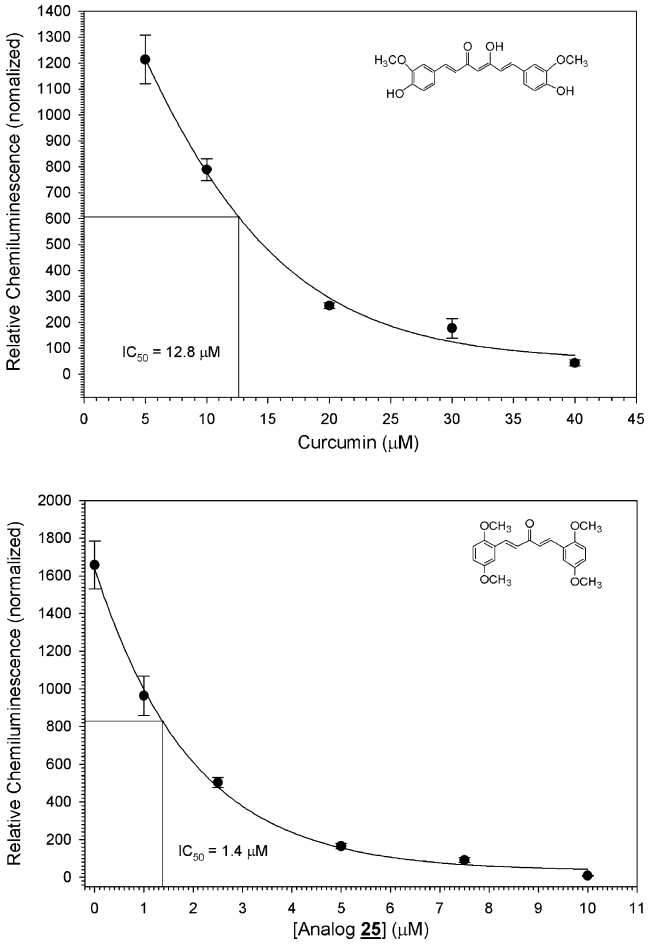


Fig. 9 – Representative dose–response plots. Curcumin and analogs were analyzed in triplicate. Error bars represent standard deviations.

sistent with the conclusions from Table 1 that these two analogs are more potent than curcumin. Moreover, these results indicate that analogs lacking anti-oxidant activity and analogs that are not Michael acceptors are potent inhibitors of AP-1-regulated endogenous gene expression. We conclude, therefore, that the results from the screening studies that use 293/AP-1-luc cells are applicable to other cells and that analogs 27 and 80 can be viewed as promising lead compounds for development of inhibitors of the activation of AP-1.

4. Discussion

The ability of curcumin to suppress the constitutive activation of AP-1 or to inhibit the stress-induced activation of AP-1 has been reported in a number of studies. In addition to suppression of LPS-induced COX-2 expression in microglial cells by inhibiton of the activation of AP-1 [46], curcumin

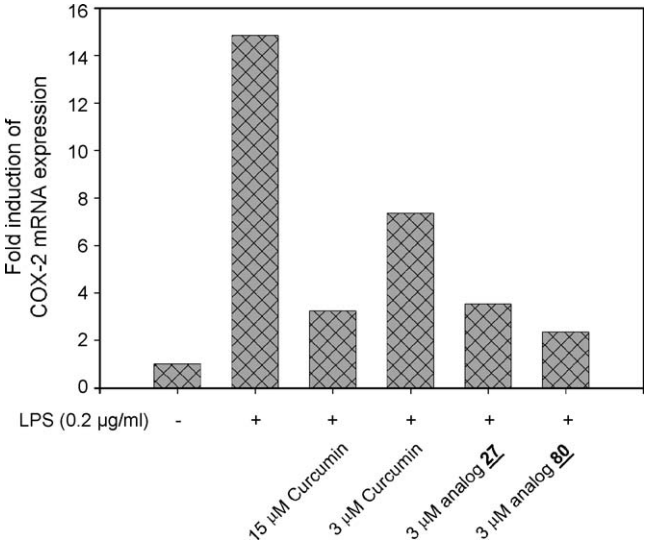
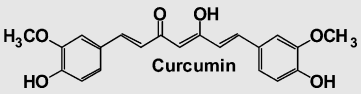
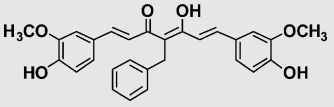
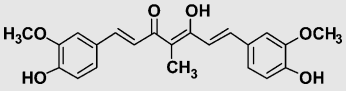
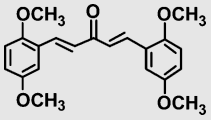
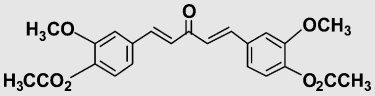
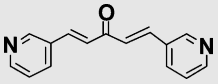
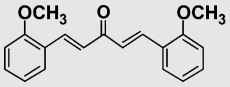
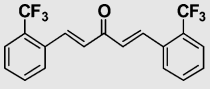
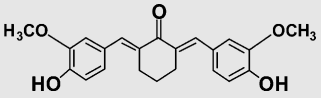
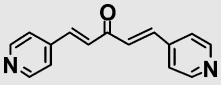
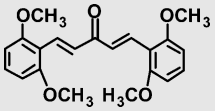
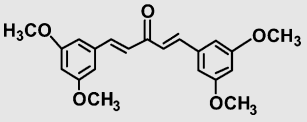
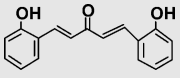
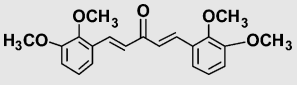
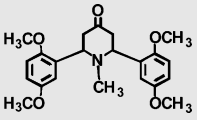
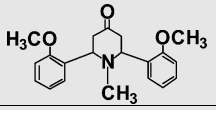


Fig. 10 – Representative plot of the inhibitory effects of curcumin and analogs 27 and 80 on LPS-induced expression of COX-2 mRNA in BV-2 microglial cells.

Table 1 – IC_{50} values and anti-oxidant activities for inhibition of the TPA-induced activation of AP-1 by curcumin and analogs

	Structure	IC_{50} (μm)	Anti-oxidant assay	
			TRAP	FRAP
1	 Curcumin	12.8 ± 0.5	+	+
2		5.3 ± 0.7	+	–
3		6.0 ± 0.2	+	+
25		1.4 ± 0.2	–	–
26		8.3 ± 0.6	–	–
27		4.1 ± 0.02	–	–
28		6.6 ± 0.2	–	–
29		7.1 ± 0.3	–	–
30		7.3 ± 0.4	+	+
31		8.2 ± 0.3	–	–
32		4.1 ± 0.3	–	–
33		4.8 ± 0.2	–	+
34		11.4 ± 1.0	+	–
35		6.0 ± 0.4	–	–
80		3.8 ± 0.5	–	–
81		5.7 ± 0.5	–	–

suppresses AP-1 activity in HeLa cells [47], inhibits the expression of COX-2 in UVB-irradiated keratinocytes by inhibiting activation of AP-1 [48], suppresses the constitutive activation of AP-1 in HTLV-1-infected T-cells [49] and abolishes bile acid-induced hepatocyte apoptosis by inhibiting activation of AP-1 [50]. Moreover, synthetic curcumin analogs have been reported to exhibit biological activities and to be active as inhibitors of the activation of AP-1. For example, curcumin analogs that inhibit the activation of AP-1 have been shown to reduce tumor angiogenesis [51]. Many studies of analogs of curcumin have reported biological activities of these analogs, including anti-inflammatory activity, inhibition of angiogenesis, induction of apoptosis, anti-tumor activity, anti-oxidant activity and antibacterial activity [52–65]. These studies did not focus on a possible role for AP-1 in these multiple biological activities, although it is reasonable to suggest that AP-1 may play a role.

These multiple studies of the biological activities of curcumin and its analogs do not address the question whether the anti-oxidant activity of curcumin or its analogs is required for the reported biological activity or whether the intrinsic reactivity of curcumin and many of its analogs as Michael acceptors is essential for activity. AP-1 is one of a number of redox-sensitive transcription factors whose activity appears to be regulated either directly or indirectly through reversible oxidation-reduction of critical cysteine residues [66,67]. c-Jun and c-fos have single conserved cysteine residues in their DNA-binding domains that undergo reversible redox reactions that alter their DNA-binding properties [68]. In addition, there are a number of AP-1-associated proteins, such as thioredoxin, jun activation domain-binding protein 1 (Jab1), and redox factor-1 (Ref-1), that contribute to regulation of the redox state of AP-1 [69,70]. Other studies suggest that direct covalent modification of the reactive cysteine residues in AP-1 may be important. For example, direct inhibition of AP-1 by 15-deoxy-delta-12,14-prostaglandin J2, which is a cyclopentenone prostaglandin capable of Michael addition, has been demonstrated [71]. Recent studies with curcumin and analogs, however, suggest that inhibition of AP-1 by these Michael acceptors does not involve direct modification of AP-1 [72].

In the present study, we have demonstrated that the anti-oxidant properties of curcumin are not essential for inhibition of the activation of AP-1. This was demonstrated with analogs in the 7-carbon, 5-carbon and 3-carbon series where many of the most active analogs were devoid of anti-oxidant activities. Most of the active analogs, however, retained the α,β-unsaturated ketone functionality and might function as Michael acceptors. It was noteworthy, therefore, that analogs in the piperidone family (Fig. 8) could retain activity, either as inhibitors or enhancers of the TPA-induced -activation of AP-1. Analog **80** is particularly noteworthy since this analog is no longer a Michael acceptor, is devoid of anti-oxidant activity, and is more active that curcumin both in screening with 293/AP-1-luc cells (Table 1) and in studies of COX-2 expression in BV-2 microglial cells (Fig. 10). We view analog **80** as an attractive lead compound for further development of inhibitors of AP-1.

This study did not address the question of the actual targets of curcumin and analogs that result in either inhibition or enhancement of AP-1 activity in response to TPA-induced activation of the 293T/AP-1-luc cells. There are numerous upstream targets, especially protein kinases, that might be targets, as well as AP-1 itself or AP-1-DNA interactions. Curcumin is known to inhibit the activation of transcription factor NF-κB as well as AP-1 [73]. Our earlier studies of curcumin and analogs as inhibitors of the TNFα-induced activation of the transcription factor NF-κB [29], which used a similar Panomics cell line for screening and included many of the analogs used in the present study, suggested that there may be multiple targets, based upon pharmacophore analysis of the data. Interestingly, a number of the most active analogs identified in the current study (Table 1) were also identified in our study of NF-κB, suggesting that there may be common targets for both the AP-1 and NF-κB pathways that are inhibited by some of these analogs. These targets remain to be identified. It also remains to be explained how some of the analogs function as enhancers of the TPA-induced activation of AP-1.

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