

Effects of cyclohexanone analogues of curcumin on growth, apoptosis and NF- κ B activity in PC-3 human prostate cancer cells

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Abstract. Curcumin is a non-nutritive yellow pigment found in the spice turmeric, which is derived from the rhizome of the plant *Curcuma longa* Linn. Six cyclohexanone analogues of curcumin (A₁-A₆) were investigated for their effects on growth and apoptosis in PC-3 human prostate cancer cells. The ability of these compounds to inhibit NF- κ B activity in PC-3 cells was also determined. Five out of the six curcumin analogues (A₂-A₆) had stronger inhibitory effects compared to curcumin on the growth of cultured PC-3 cells. Compounds A₂-A₆ also had stronger stimulatory effects on apoptosis in PC-3 cells than curcumin, and these curcumin analogues more potently inhibited NF- κ B activity than curcumin. The inhibitory effects of these compounds on NF- κ B activity correlated with their effects on growth inhibition and apoptosis stimulation in PC-3 cells. The results of the present study provide a rationale for *in vivo* studies with A₂-A₆ using suitable animal models of prostate cancer.

Introduction

Curcumin is a non-nutritive yellow pigment found in the spice turmeric, which is derived from the rhizome of the plant *Curcuma longa* Linn. Curcumin lacks toxicity in humans (1), and extensive research over several decades has revealed that curcumin possesses anticancer, anti-inflammatory, antioxidant, antiviral and anti-bacterial activities (2,3). Curcumin suppressed cell proliferation or induced apoptosis in cultured prostate cancer cells and other types of cancer cells (4-10). Curcumin also inhibited prostate carcinogenesis (11). Studies from our laboratory and those of other authors have demonstrated enhanced anticancer activities of curcumin when combined with other anticancer agents (12-14). Findings of earlier studies showed that curcumin exerts a wide range of anticancer effects by modulating a diversity of signaling pathways, including nuclear factor- κ B (NF- κ B) and other pathways (15-20). Curcumin has entered clinical trials for certain types of human cancer (21-23). However, the clinical efficacy of curcumin is limited, which is likely to be due to its low bioavailability (21-23). It was suggested that the β -diketone moiety of curcumin causes instability and poor metabolic properties (24-26). Enhanced stability was found in curcumin analogues by deleting the β -diketone moiety of the molecule (27). Recently, it was demonstrated that the cyclohexanone analogues of curcumin have enhanced stability in biological medium compared to curcumin (28). The cyclohexanone-containing curcumin analogue 2,6-bis[3-(3-methoxy-4-hydroxyphenyl)methylene]-cyclohexanone was found to be more potent than curcumin for inhibiting NF- κ B in human breast cancer cells *in vitro* (29).

In an earlier study, we synthesized a series of cyclohexanone curcumin analogues and determined their inhibitory effect on the activity of aldose reductase (30). In the present study, we investigated the effects of these curcumin analogues on the growth and apoptosis of human prostate cancer PC-3 cells. We also determined the inhibitory effect of these analogues on the activation of NF- κ B in PC-3 cells using the

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luciferase reporter assay. Results of our study demonstrated that compounds A₂-A₆ have stronger effects for inhibiting growth and stimulating apoptosis in PC-3 cells compared to curcumin. We also found that these curcumin analogues have stronger effects than curcumin for inhibiting NF- κ B activity in PC-3 cells.

Materials and methods

Chemistry. A series of cyclohexanone curcumin analogues were synthesized by coupling the appropriate substituted benzaldehyde with cyclohexanone as previously described (30). Characterization of the compounds, 2,6-bis(4-hydroxybenzylidene)-cyclohexanone (A₁), 2,6-bis(3,4-dihydroxybenzylidene)-cyclohexanone (A₂), 2,6-bis(4-hydroxy-3-methoxybenzylidene)-cyclohexanone (A₃), 2,6-bis(3,5-di-tert-butyl-4-hydroxybenzylidene)-cyclohexanone (A₄), 2,6-bis(3,4-dimethoxybenzylidene)-cyclohexanone (A₅) and 2,6-bis(4-hydroxy-3,5-dimethoxybenzylidene)-cyclohexanone (A₆), was previously described in detail (30).

Cell culture and reagents. PC-3 cells were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). Curcumin was obtained from Sigma-Aldrich (St. Louis, MO, USA). The RPMI-1640 tissue culture medium, penicillin-streptomycin, L-glutamine and fetal bovine serum (FBS) were obtained from Gibco (Grand Island, NY, USA). The PC-3 cells were maintained in RPMI-1640 culture medium containing 10% FBS supplemented with penicillin (100 U/ml)-streptomycin (100 μ g/ml) and L-glutamine (300 μ g/ml). Cultured cells were grown in a humidified atmosphere of 5% CO₂ at 37°C, and were passaged twice a week. Curcumin and its analogues were dissolved in DMSO and the final concentration of DMSO in all experiments was 0.1%.

MTT assay. PC-3 cells were seeded at a density of 0.2x10⁵ cells/ml in medium in 96-well plates (0.2 ml/well) and incubated for 24 h. The cells were then treated with various concentrations (0.5-10 μ M) of the different curcumin analogues for 72 h. Following treatment, 200 μ l 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide (0.5 mg/ml in PBS) was added to each well of the plate and incubated for 2 h. The plate was then centrifuged at 1,000 rpm for 5 min at 4°C. Following removal of the medium, 0.1 ml DMSO was added to each well. The absorbance was recorded on a microplate reader at 540 nm. The effect of different curcumin analogues on cell growth was assessed as the percentage cell growth compared to DMSO-treated cells.

Determination of the number of viable cells. The number of viable cells following each treatment was determined using the trypan blue exclusion assay (31). In brief, 80 μ l of cell suspension was mixed with 20 μ l of 0.4% trypan blue solution and incubated for 2 min. The cells were then examined under a light microscope (Nikon Optiphot, Japan). Blue cells were counted as dead cells and cells that did not absorb dye were counted as live cells.

Assessment of apoptotic cells by morphology and activation of caspase-3. Apoptotic cells were determined by morphological

assessment in cells stained with propidium iodide (32,33). Cytospin slides were prepared following each experiment and cells were fixed with acetone/methanol (1:1) at room temperature for 10 min, followed by 10 min of propidium iodide staining (1 μ g/ml in PBS), and were then analyzed using a fluorescence microscope (Nikon Eclipse TE200, Japan). Apoptotic cells were identified by classical morphological features, including nuclear condensation, cell shrinkage and formation of apoptotic bodies (32,33).

Caspase-3 activation was measured using an EnzoLyte AMC Caspase-3 Assay Fluorimetric kit (AnaSpec, Fremont, CA, USA) according to the manufacturer's instructions (34). A total of 1x10⁵ cells were plated in triplicate in a flat-bottomed 96-well plate. Cells were treated with different curcumin analogues for 72 h. Following treatment, caspase-3 substrate was added to each well. Plates were incubated at room temperature for 30 min. Fluorescence intensity was measured in a Tecan Infinite M200 plate reader (Tecan US Inc., Durham, NC, USA).

NF- κ B-dependent reporter gene expression assay. NF- κ B transcriptional activity was measured using the NF- κ B-luciferase reporter gene expression assay (35). An NF- κ B luciferase construct was stably transfected into PC-3 cells and a single stable clone, PC-3 C4 (35), was used. PC-3 C4 cells were treated with different curcumin analogues for 24 h, and the NF- κ B-luciferase activities were measured using luciferase assay kits from Promega (Madison, WI, USA). Following treatment, the cells were washed with ice-cold phosphate-buffered saline (PBS), and harvested in 1X reporter lysis buffer. Following centrifugation, 10 μ l aliquots of the supernatants were measured for luciferase activity using a Luminometer from Turner Designs Inc., (Sunnyvale, CA, USA). The luciferase activity was normalized against known protein concentrations, and expressed as the percentage of luciferase activity in the control cells, which were treated with DMSO solvent. The protein level was determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions.

Statistical analysis. The analysis of variance (ANOVA) with the Tukey-Kramer multiple comparison test was used for the comparison of growth inhibition as determined by the trypan blue assay and determination of the NF- κ B-luciferase activities in cultured PC-3 cells that were treated with different curcumin analogues.

Results

Inhibitory effect of curcumin and its analogues on the growth of PC-3 cells. The inhibitory effects of curcumin and its analogues A₁-A₆ on the growth of cultured PC-3 cells were determined using the MTT assay. For each experiment, curcumin was evaluated as the positive control. The inhibitory effects of curcumin did not significantly vary between different experiments. Data from the curcumin incubations were averaged (Fig. 1). Curcumin and its analogues A₁-A₆ inhibited the growth of PC-3 cells in a concentration-dependent manner (Fig. 1). A₄ was the strongest curcumin analogue for inhibiting the growth of PC-3 cells, as determined by the

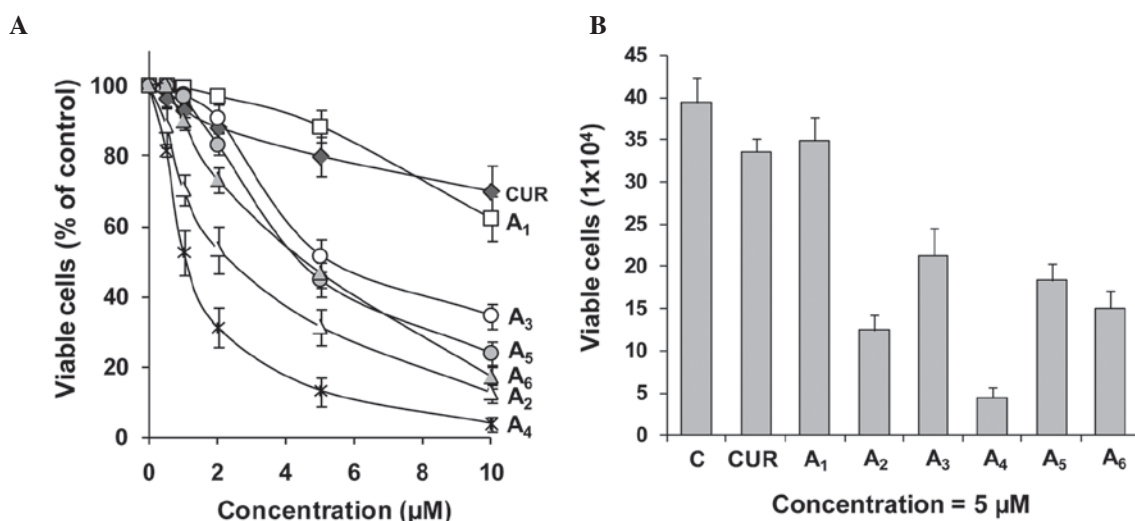


Figure 1. Effects of curcumin analogues on the growth of human prostate cancer PC-3 cells. PC-3 cells were seeded at a density of 0.2×10^5 cells/ml of medium in 96-well plates (0.2 ml/well) and incubated for 24 h. The cells were then treated with various concentrations (0.5-10 μ M) of the different compounds for 72 h. (A) Effects of the different compounds on the growth of PC-3 cells were determined by the MTT assay and (B) the trypan blue exclusion assay. Each value is the mean \pm SD from three experiments. C, control; CUR, curcumin.

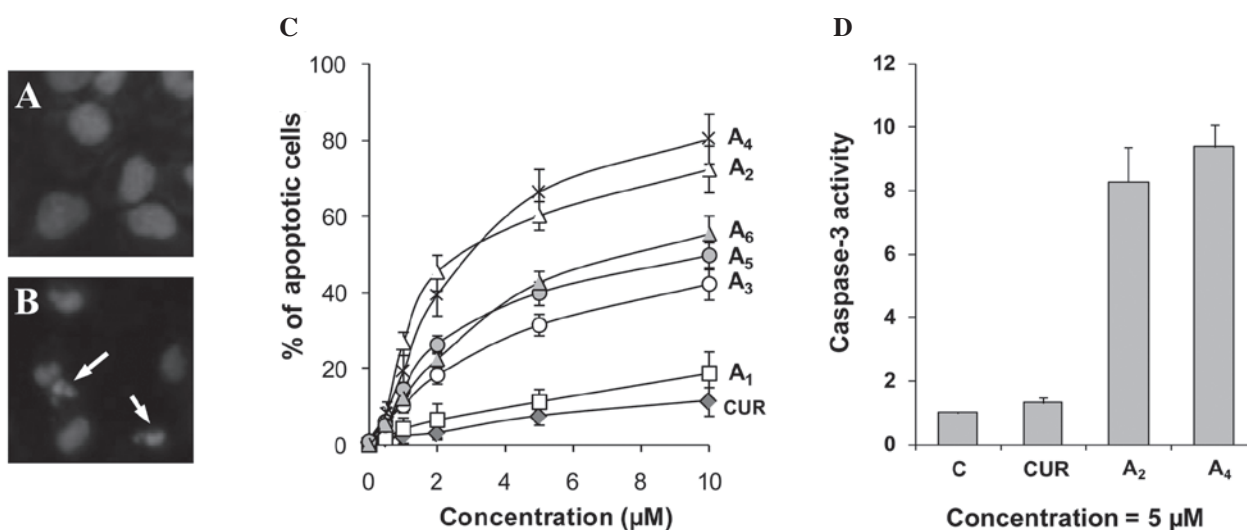


Figure 2. Effects of curcumin analogues on apoptosis. PC-3 cells were seeded at a density of 0.2×10^5 cells/ml of medium in 35-mm tissue culture dishes (2 ml/dish) and incubated for 24 h. The cells were then treated with various concentrations (0.5-10 μ M) of the different compounds for 72 h. (A and B) Representative micrographs of propidium iodide-stained controls and A₄ (5 μ M)-treated PC-3 cells. Arrows indicate apoptotic cells. (C) Percentage of apoptotic cells as determined by morphological assessment in PC-3 cells treated with the various compounds. (D) Caspase-3 activities in PC-3 cells treated with curcumin, A₂ and A₄. Each value is the mean \pm SD from three experiments. C, control; CUR, curcumin.

MTT assay, followed by A₂, A₆, A₅, A₃ and A₁ (Fig. 1A). In additional experiments, the effects of different curcumin analogues on cell growth were determined by the trypan blue exclusion assay. Compounds A₂-A₆ were more potent for decreasing the number of viable PC-3 cells as compared to curcumin (Fig. 1B). Statistical analysis using ANOVA with the Tukey-Kramer test demonstrated that the differences in the number of viable cells between the curcumin-treated group and any curcumin analogue-treated group (except the A₁-treated group) were statistically significant ($P < 0.001$). The number of viable cells was significantly lower in the A₄-treated group than in the curcumin-treated or any other

curcumin analogue-treated group ($P < 0.05$ compared to the A₂-treated group; $P < 0.001$ compared to other curcumin analogue-treated groups).

Stimulatory effect of curcumin analogues on apoptosis in PC-3 cells. Effects of the curcumin analogues A₁-A₆ on apoptosis in PC-3 cells were determined by morphological assessment of apoptotic cells. Apoptotic cells were identified by classical morphological features, including nuclear condensation, cell shrinkage and formation of apoptotic bodies. Morphologically distinct apoptotic cells from representative samples are shown in Fig. 2B. Treatment of PC-3 cells with curcumin resulted

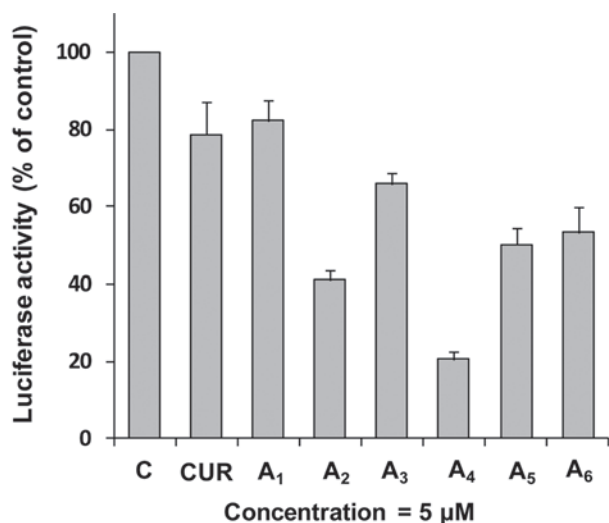


Figure 3. Effects of curcumin and its analogues A₁-A₆ on NF-κB transcriptional activity in PC-3 cells. PC-3 C4 cells were seeded at a density of 0.2×10^5 cells/ml of medium in 35-mm culture dishes (2 ml/dish) and incubated for 24 h. The cells were then treated with different compounds (5 μM) for 24 h. The NF-κB transcriptional activity was measured by a luciferase activity assay. Each value is the mean \pm SD from three experiments. C, control; CUR, curcumin.

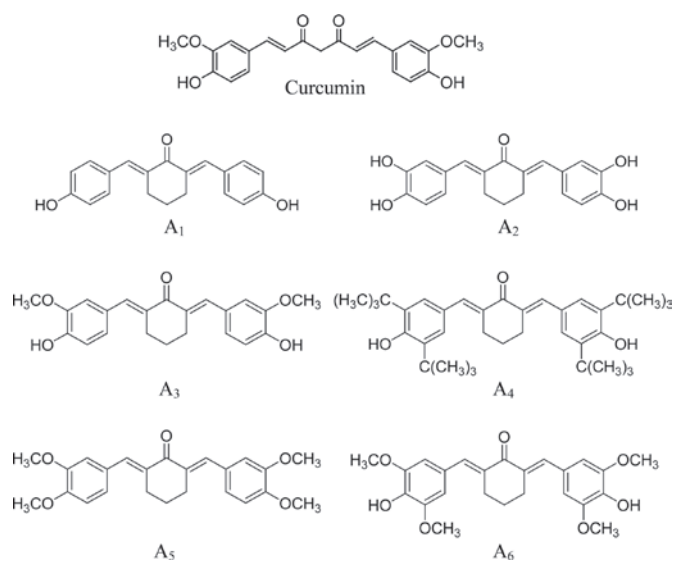


Figure 4. Structures of curcumin and its analogues A₁-A₆.

in a small increase in apoptotic cells (Fig. 2C). Treatment with compounds A₁-A₆ stimulated apoptosis in PC-3 cells in a concentration-dependent manner (Fig. 2C). Compounds A₂ and A₄ demonstrated stronger stimulatory effects on apoptosis in PC-3 cells compared to the other compounds. The effect of the two strongest compounds A₂ and A₄ on activation of caspase-3 in comparison to curcumin was determined. Treatment of PC-3 cells with curcumin caused only a small increase in caspase-3 activity, while treatment with A₂ and A₄ caused an 8.2- and 9.3-fold increase in caspase-3 activity, respectively (Fig. 2D). Our results identified A₂ and A₄ as the two curcumin analogues that had the greatest effect for stimulating apoptosis in PC-3 cells.

Effect of curcumin analogues on NF-κB activity. To investigate the effect of A₁-A₆ on activation of NF-κB activity, we used an NF-κB-luciferase reporter gene expression assay in PC-3 C4 cells. PC-3 C4 is a cell line derived from the stable transfection of PC-3 cells with an NF-κB luciferase construct (35). In these experiments, PC-3 C4 cells were treated with different concentrations of curcumin and its analogues A₁-A₆ for 24 h. Treatment of PC-3 C4 cells with curcumin or A₁ (both 5 μM) caused only modest decreases in the activity of NF-κB (Fig. 3). Treatment with A₂-A₆ (all at 5 μM) caused a further decrease in NF-κB transcriptional activity. Statistical analysis using ANOVA with the Tukey-Kramer test demonstrated that NF-κB activity was significantly lower in the A₄-treated group than in any other treated group ($P < 0.01$ compared to the A₂-treated group; $P < 0.001$ compared to other curcumin analogue-treated groups). There were good correlations between inhibition of NF-κB activity and cell growth inhibition ($r = 0.97$), and between inhibition of NF-κB activity and apoptosis stimulation ($r = 0.96$) in the PC-3 cells treated with all compounds at 5 μM.

Analysis of structure-activity correlation. Six curcumin analogues (A₁-A₆) that contain a five-carbon linker with a mono-carbonyl group (cyclohexanone linker) were evaluated for anticancer activities in human prostate cancer PC-3 cells. All of the curcumin analogues, with the exception of A₁, had stronger inhibitory effects on cell growth and stronger stimulatory effects on the apoptosis of PC-3 cells compared to curcumin. Although the structures of A₃ and curcumin are the same, with the exception of their middle linker (Fig. 4), the anticancer activity of A₃ was stronger than that of curcumin (Fig. 1) suggesting that a cyclohexanone linker increases anticancer activity. A comparison of the six curcumin analogues (all with the same mono-carbonyl linker) revealed that anticancer activity was significantly influenced by substituents on the benzene rings. The presence of a methoxy group on both sides of the *p*-phenol group markedly increased activity compared to a compound with a methoxy group on only one side (A₃ vs. A₆). Tert-butyl substituents on both sides of the *p*-phenol group (A₄) had the strongest anticancer effect among all of the studied compounds. Comparison of A₁ and A₂ suggested that *o*-dihydroxyl substituents on both benzene rings (A₂) had stronger activity than an analogue with a single hydroxyl group on each side (A₁).

Discussion

In the present study, we demonstrated that a series of cyclohexanone curcumin analogues (A₂-A₆) had stronger anticancer activities than curcumin in cultured human prostate cancer PC-3 cells. Among the curcumin analogues, A₄ demonstrated a stronger inhibitory effect on the growth of PC-3 cells than any of the other curcumin analogues. Compounds A₂ and A₄ were stronger than the other compounds for stimulating apoptosis. In addition, we found that all curcumin analogues tested (except for A₁) were more potent inhibitors of NF-κB in PC-3 cells than curcumin. A₄ was the most potent compound among the six curcumin analogues tested for inhibiting the activation of NF-κB.

Extensive studies have shown that curcumin exerts a wide range of antitumor effects through modulation of significant

signaling pathways, including transcription factor NF- κ B and other pathways (15-20,36). Of those involved in antitumor effects, NF- κ B is generally regarded as an important target of curcumin (16,37). NF- κ B has been linked to cell proliferation, invasion, angiogenesis, metastasis, suppression of apoptosis and chemoresistance in multiple tumors (38,39). In addition, evidence suggests that NF- κ B is significant in the growth and radio/chemoresistance of prostate cancer (40-44). Curcumin is able to suppress NF- κ B activation by an Akt-dependent or Akt-independent inhibition of IKK (15,16,45). Certain curcumin analogues, including 3,5-bis(2-fluorobenzylidene) piperidin-4-one (EF24) have been found to have a potent inhibitory effect on NF- κ B (46). In the present study, we identified that 5 out of 6 cyclohexanone curcumin analogues tested had a more potent inhibitory effect than curcumin on activation of NF- κ B in PC-3 cells. The effects of these curcumin analogues on growth inhibition and apoptosis stimulation were associated with their inhibitory effect on activation of NF- κ B. This result indicates that inhibition of NF- κ B activation may be involved in growth inhibition and apoptosis induction in PC-3 cells treated with these curcumin analogues.

Based on the analysis of the correlation between the structures of curcumin analogues and their effects on the growth and apoptosis of human prostate cancer PC-3 cells, analogues with a cyclohexanone linker between the two benzene rings enhance anticancer effects. Substituents on the benzene rings of the analogues also affect their activities. The analogue with a tert-butyl substituent on both sides of the *p*-phenol group (A₄) demonstrated stronger anticancer activity than the other analogues, suggesting that the introduction of more hydrophobic groups on both sides of the *p*-phenol group may be an important strategy for the development of more potent compounds with anti-prostate cancer activity.

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