

Activation of apoptotic protein in U937 cells by a component of turmeric oil

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Aromatic (ar)-turmerone from turmeric oil displays anti-tumorigenesis activity that includes inhibited cell proliferation. This study investigated ar-turmerone-mediated apoptotic protein activation in human lymphoma U937 cells. Ar-turmerone treatment inhibited U937 cell viability in a concentration-dependent fashion, with inhibition exceeding 84%. Moreover, the treatment produced nucleosomal DNA fragmentation and the percentage of sub-diploid cells increased in a concentration-dependent manner; both are hallmarks of apoptosis. The apoptotic effect of ar-turmerone was associated with the induction of Bax and p53 proteins, rather than Bcl-2 and p21. Activation of mitochondrial cytochrome c and caspase-3 demonstrated that the activation of caspases accompanied the apoptotic effect of ar-turmerone, which mediated cell death. These results suggest that the apoptotic effect of ar-turmerone on U937 cells may involve caspase-3 activation through the induction of Bax and p53, rather than Bcl-2 and p21. [BMB reports 2009; 42(2): 96-100]

INTRODUCTION

Stereotypical features of a cell undergoing apoptosis include cell shrinkage, blebbing, phosphatidylserine externalization, DNA fragmentation and nuclear condensation (1). Early in the apoptotic process, mitochondria release cytochrome c, which enters the cytosol and forms a complex with Apaf-1 and a pro-form of caspase-9 (2). These complexes induce the activation of other caspases, which are responsible, either directly or indirectly, for cleavage of several intracellular proteins that characteristically lyse during apoptosis (3, 4). Additionally, p53 protein functions, in part, by responding to DNA damage and inducing apoptosis, which is likely to be a crucial aspect of the function of p53 as a tumor suppressor (5, 6). Wild-type p53 protein arrests DNA-damaged cells in G1 phase by inducing the cyclin-dependent kinase (CDK) inhibitor p21WAF1/

CIPI (7), and non-repaired cells might be eliminated during apoptosis by inducing Bax and repressing Bcl-2 factors (8, 9). The pro-apoptotic protein, Bax, induces the release of cytochrome c and the activation of caspase, resulting in apoptosis. Apoptosis can be blocked by the anti-apoptotic proteins Bcl-2 and Bcl-xL (10). In many cancer therapies, the induction of apoptosis in tumor cells has been shown to be the generalized rule for anti-cancer mechanism conjuncts.

Aromatic turmerone (ar-turmerone), is the major compound present in turmeric volatile oil from *Curcuma longa* L. Curcumin is a major component of this plant; ar-turmerone, which is a sesquiterpene, is also an effective component of *Curcuma longa* L. (11,12). Previously, we reported on the isolation of ar-turmerone and determined its anti-proliferative action on various cancer cell lines (13). However, the functional mechanisms of this process remain unclear.

In the present study, we investigated the influence of ar-turmerone-mediated apoptotic protein activation on the growth of human histiocytic lymphoma U937 cells. Our findings suggest that ar-turmerone-mediated apoptosis is not associated with changes in the levels of p21 or Bcl-2 expression. However, the levels of p53 and Bax expression are dramatically increased in a concentration-dependent manner. We also observed a release of cytochrome c and activation of caspase-3.

RESULTS

Inhibition of cell viability by ar-turmerone

Ar-turmerone showed a dose dependent inhibitory effect in U937 cell proliferation. Cell proliferation is determined by the activity of mitochondrial dehydrogenase in living cell by MTT assay (14,15). The viability was inhibited 61, 68, 81 and 84% at 40, 80, 120 and 160 µg/ml of ar-turmerone for 48 h incubation, respectively. The viability of U937 cells for 24 hr incubation was inhibited 40, 66, 79 and 81% at each concentrations of ar-turmerone. Anti-proliferative action of 40 µg/ml ar-turmerone for 48 h is increased more than that of 40 µg/ml ar-turmerone for 24 h incubation. The inhibition of ar-turmerone on the viability of U937 cells for 24 h incubation in higher concentrations were a little bit less than those of 48 h incubation (Fig. 1A). These results suggest that ar-turmerone has anti-proliferative effect in U937 cells.

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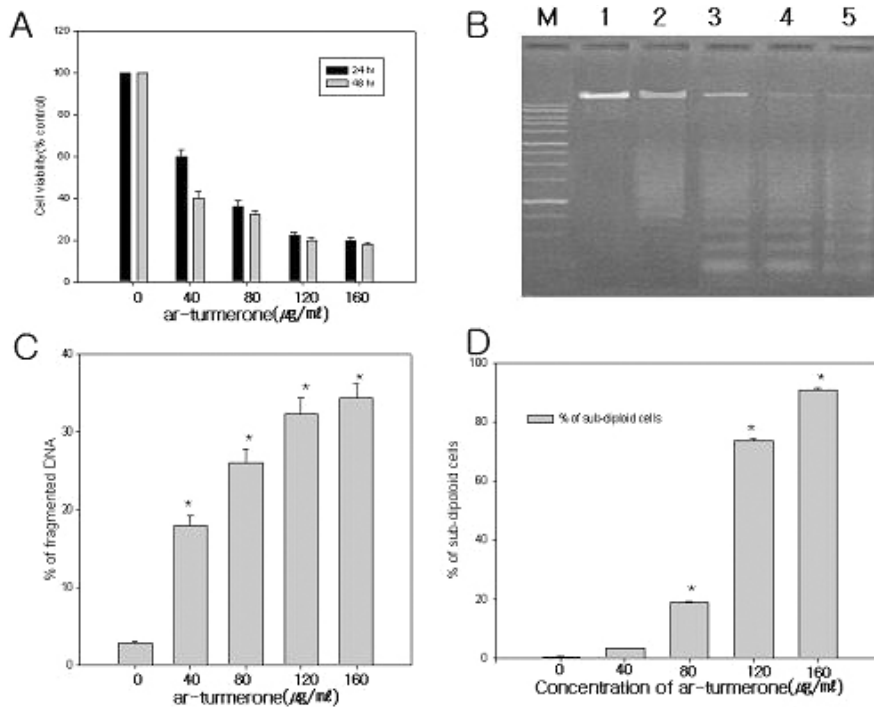


Fig. 1. Decreased cell viability and analysis of apoptosis. (A) After a 24 or 48 h treatment of U937 cells with ar-turmerone, cell viability was assessed by MTT assay. Results are expressed as the percent change of the control condition in which the cells were grown in drug-free medium. Data points represent the mean values of four replicates with the bars indicating standard error of the mean (SEM). (B) Effect of 48 h ar-turmerone treatment on DNA fragmentation of U937 cells. Lane M: DNA marker; Lane 1: control; Lane 2: 40 µg/ml ar-turmerone; Lane 3: 80 µg/ml ar-turmerone; Lane 4: 120 µg/ml ar-turmerone; Lane 5: 160 µg/ml ar-turmerone. (C) Quantitation of DNA fragmentation by [³H] thymidine incorporation. Data were presented as percentage of counts per minute (cpm) of fragmented DNA compared to total cpm. Data represent the mean values of four replicates with bars indicating SEM. *P < 0.05 compared to control. (D) After 48 h incubation with ar-turmerone, cells were stained with PI and the number of sub-diploid cells was determined using FACScan flow cytometry. Data represents mean values of three replicates, with bars indicating SEM. *P < 0.05 compared to control.

Effect of ar-turmerone on apoptosis of U937 cells

A characteristic nucleosomal DNA fragmentation pattern, which is the biochemical hallmark of apoptosis, was detected 48 h after exposure to 40, 80, 120 and 160 µg/ml of ar-turmerone (Fig. 1B). The percentage of DNA fragmentation showed the same results in the quantitative analysis of fragmented DNA using [³H]-thymidine incorporation test (Fig. 1C). Those results were also confirmed by the quantitation of apoptotic sub-diploid cells. As shown in Fig. 1D, the percentage of sub-diploid cells was increased to 18.9, 73.6, and 90.8% with increasing concentrations of ar-turmerone at of 80, 120, and 160 µg/ml, respectively, in U937 cells. These results suggest that ar-turmerone induces clear apoptosis in U937 cells in the range of 80-160 µg/ml. These results suggest that, following ar-turmerone treatment, U937 cells show apoptosis and that there is a good correspondence between the extent of apoptosis and growth inhibition.

Induction of Bax by ar-turmerone

Cellular proteins and total RNAs were isolated after 48 h incubation with ar-turmerone, and immunoblotting and RT-PCR were performed against Bcl-2 and Bax in order to examine the expression of Bcl-2 family in ar-turmerone-induced apoptosis. As shown in Fig. 2, ar-turmerone did not affect the levels of Bcl-2 mRNA and protein, whereas the levels of Bax mRNA and protein were significantly induced after ar-turmerone treatment in a concentration-dependent manner. We also examined the expression of tumor suppressor p53 and cdk inhibitor

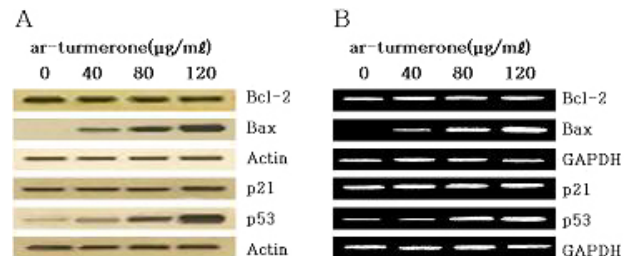


Fig. 2. Effects of ar-turmerone on protein and mRNA levels. Protein and mRNA levels of Bcl-2, Bax, p53, and p21 were examined in U937 cells after 48 h incubation. (A) Total protein was extracted and analyzed by immunoblot using the relevant antibody. Actin was used as an internal control. (B) Total RNA was isolated and RT-PCR was performed using the indicated primers after 48 h incubation of U937 cells with ar-turmerone.

p21 in U937 cells treated with ar-turmerone. The levels of the protein and mRNA of p21 remained unchanged, but the both levels of p53 were significantly increased after treatment of ar-turmerone. These results suggest that the apoptotic effects of ar-turmerone in U937 cells are caused by induction of the proteins and mRNAs of Bax and p53 rather than that of the proteins and mRNAs of Bcl-2 and p21.

Cytochrome c release and caspase-3 activation

The activation of caspases is regulated by the release of cytochrome c from mitochondria to the cytosol (16,17). The pres-

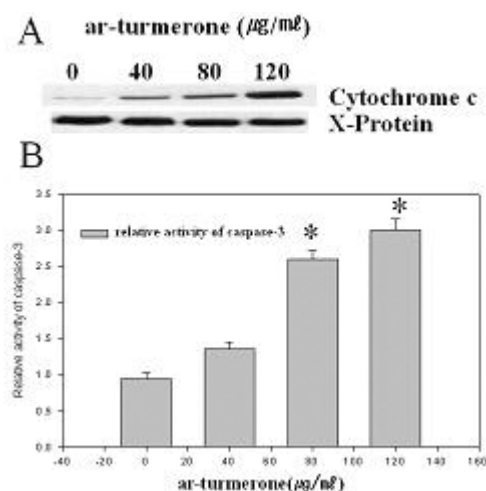


Fig. 3. Induction of cytochrome c release and caspase-3 activity by ar-turmerone. U937 cells were treated with each concentration of ar-turmerone for 48 h. (A) Mitochondrial cytochrome c was detected using anti-cytochrome c monoclonal antibody. The aggregated cytochrome c, X-protein bands were used to normalize the protein loading. (B) Caspase-3 activity was measuring fluorescence. Data represents relative activity of caspase-3 after normalization with defined quantities of protein. Data represents the mean values of three replicates, with bars indicating SEM. * $P < 0.05$ compared to control.

ent study showed that cytochrome c release was markedly induced after treatment of ar-turmerone for 48 h (Fig. 3). We confirmed these results using a caspase-3 activity assay. As shown in Fig. 3, caspase-3 activities were increased 1.4-fold, 2.6-fold, and 3.0-fold after treatment of ar-turmerone 40, 80, and 120 µg/ml, respectively. These results suggest that ar-turmerone induces apoptosis through the release of mitochondrial cytochrome c; then a complex form with Apaf-1 and a pro-form of caspase-9 may subsequently activate caspase-3.

DISCUSSION

The present results clearly demonstrate ar-turmerone-mediated apoptotic protein activation in human lymphoma U937 cells in culture, which explains the previously demonstrated anti-proliferative activity (13). The induction of apoptotic cell death by ar-turmerone was confirmed by the observations of DNA fragmentation and increased numbers of sub-diploid cells (Fig. 1).

Apoptosis is a systematically regulated process that involves the expression of many gene products. Of the major genes that regulate apoptosis, the anti-apoptotic Bcl-2 and pro-apoptotic Bax genes are of particular interest. The pro-apoptotic Bax protein translocates to mitochondria upon exposure to apoptotic stimuli (18), and induces cytochrome c release and caspase activation, resulting in apoptosis that can be blocked by the anti-apoptotic proteins Bcl-2 and Bcl-xL (12). Bcl-2 resides on the cytoplasmic face of the mitochondrial outer membrane, endoplasmic reticulum and nuclear envelope, where it protects

against various cytotoxic insults that lead to cell death (9,18,19). Thus, it has been suggested that the ratio of the level of pro-apoptotic Bax to that of anti-apoptotic Bcl-2 determines whether a cell responds to an apoptotic signal. In this study, no change was observed after treatment of ar-turmerone for Bcl-2 levels in U937 cells, but the levels of pro-apoptotic gene, Bax, were significantly increased in a concentration-dependent manner, resulting in a decrease in the ratio of Bcl-2/Bax (Fig. 3). Additionally, the expression levels of p21 mRNA and protein were not changed after ar-turmerone treatment, but the expression levels of p53 mRNA and protein were increased in a concentration-dependent manner. Therefore, the apoptotic effect of ar-turmerone is likely associated with increased expression of the pro-apoptotic Bax gene and p53 gene without an alteration in the expression of Bcl-2 or p21.

The pro-survival Bcl-2 subfamily and pro-apoptotic Bax subfamily can oppositely regulate apoptosis through the control of cytochrome c release from mitochondria, resulting in caspase activation (9, 20-22). For example, caspase-3 cleaves Bcl-2 and Bcl-xL, which destroys the anti-apoptotic function of these proteins and releases C-terminal fragments that are pro-apoptotic. Ar-turmerone treatment of U937 cells produces a concentration-dependent activation of caspase-3, one of the main executors of the apoptotic process (23, 24). Our data also shows that activation of caspase-3 is regulated by the release of cytochrome c from mitochondria to the cytosol (Fig. 3), indicating that the pathway for apoptosis by ar-turmerone exists, in part, due to the increased expression of Bax, p53 and caspase-3 proteins.

Taken together, these findings suggest that ar-turmerone activates apoptotic protein through the pathway that upregulates a Bax protein and then Bax activates caspase-3 via cytochrome c release. Further studies are needed to identify additional active proteins that confer anti-cancer activity of ar-turmerone.

MATERIALS AND METHODS

Isolation and identification of ar-turmerone

Powdered *Curcuma longa* (200 g) was extracted with methanol. The methanol extract (57 g) was then suspended in distilled water and partitioned with hexane. The hexane fraction (25 g) was loaded on a silica gel column and eluted with a hexane-acetone gradient (30:1 to 1:1) to afford 27 fractions. Fraction S5 (6.3 g) was further separated using a silica gel column chromatography with an elution of a hexane-acetone gradient (50:1 to 1:1), and 16 fractions were obtained. Fraction S5-5 (1.0 g) was further fractionated with silica gel column chromatography, which gave ar-turmerone (248 mg) (25).

MTT assay

This is based on the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to MTT-formazan by mitochondrial enzymes as previously described (13). U937 cells were seeded at a density of 5×10^4 cells per well in 24-well plates and incubated for 24 h and 48 h. Ar-turmerone

was dissolved in DMSO and added to the culture media at concentrations of 0-160 µg/ml range, and the cells were incubated for 48 h. 120 µl of stock MTT solution was added into each well under the dark condition, and plates were incubated at 37°C for 4 h. After centrifugation, 1 ml of the diluted DMSO with ethylalcohol (1:1) was added, which was performed to dissolve formazan. After shaking for 10 minutes at room temperature, 100 µl of each solution was transferred to 96-well plates, and the absorbance value of each well was read at 540 nm using ELISA reader (Model 550 Microplate Reader, Bio-Rad, USA).

DNA isolation and electrophoresis

After being treated with or without ar-turmerone for 48 h, the cells were washed twice with ice-cold PBS and lysed with lysis buffer (10 mM Tris-Cl, pH 7.4, 20 mM EDTA and 0.5% Triton X-100) at 4°C for 30 min (26). DNA was isolated with phenol-chloroform extraction, and treated with 100 ng/µl RNase A (Sigma). Electrophoresis of the DNA was performed on a 1.5% agarose gel in a TAE buffer, and photographed under UV light after staining the gel with ethidium bromide.

Quantitative analysis of fragmented DNA

U937 cells were incubated in growth medium for 4 h with 1 µCi/ml [³H]-thymidine (Amersham Pharmacia Biotech., UK). Then the cells were washed twice with PBS and incubated for 48 h after treatment of ar-turmerone. The cells were washed and lysed with lysis buffer (10 mM Tris-HCl, pH 8.0 1 mM EDTA, 0.2% Triton X-100) (27). Low and high molecular weight DNA were separated by centrifugation and the amount of [³H]-thymidine of each supernatant was determined by liquid scintillation counter (Beckmann, USA). The percent change of DNA fragments was calculated as follows: % Fragments = [c.p.m. of small DNA / (c.p.m. of small DNA + c.p.m. of large DNA) × 100].

RNA extraction and reverse transcription-PCR (RT-PCR)

Following treatment of ar-turmerone, total RNA was isolated with TRI Reagent (GIBCO/BRL). cDNA synthesized from 5 µg of total RNA with 200 units of MMLV-RT (Promega Co., Madison, WI, USA) and 500 ng of oligo-dT primer. For amplification of cDNAs, oligonucleotide primers and cycle parameters for PCR were designed as like this: Bax (sense, 5'-ATG GAC GGG TCC GGG GAG-3'; anti-sense, 5'-TGG AAG AAG ATG GGC TGA-3'), Bcl-2 (sense, 5'-CAG CTG CAC CTG ACG-3'; anti-sense, 5'-GCT GGG TAG GTG GTG CAT-3'), p53 (sense, 5'-GCT CTG ACT GTA CCA CCA TCC-3'; anti-sense, 5'-CTC TCG GAA CAT CTC GAA GCG-3') p21, (sense, 5'-CTC AGA GGA GGC GCC ATG-3'; anti-sense, 5'-GGG CGG ATT AGG GCT TCC-3') and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (sense, 5'-CGG AGT CAA CGG ATT TGG TCG TAT-3'; anti-sense, 5'-AGC CTT CTC CAT GGT GGT GAA GAC-3'). PCR products were electrophoresed on 1% agarose gel, and photographed under UV light, after staining with ethidium bromide.

Preparation of cytosolic extracts and immunoblotting

After treatment of ar-turmerone, the cells were collected and resuspended in 500 µl of extraction buffer (50 mM Pipes-KOH, 220 mM mannitol, 68 mM sucrose, pH 7.4, 50 mM KCl, 5 mM EGTA, 2 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, and protease inhibitors). After 30 min incubation on ice, cells were homogenized using a glass dounce and a tight pestle (50 strokes). Cell homogenates were centrifuged and 10 µl of protein was loaded on 15% SDS-polyacrylamid gels (10). Mitochondrial cytochrome c was detected with anti-cytochrome c monoclonal antibody (PharMingen).

Caspase-3 assay

After treatment of ar-turmerone, U937 cells were harvested, washed twice with ice-cold PBS, and resuspended in lysis buffer (10 mM HEPES, pH 7.4, 2 mM EDTA, 0.1% CHAPS, 5 mM DTT, 1 mM PMSF, 10 µg/ml aprotinin, 20 µg/ml leupeptin). The rest of the protocol followed the manufacturer's instruction (Bio-Fad Lab., Hercules, CA, USA). The fluorescence was measured in a microplate reader (BIO-TEK Instruments, Winooski, VT, USA) using 360 nm excitation and 530 nm emission. Data were expressed fold-induction of caspase-3 activity compared to that of control cells.

Immunoblot analysis

U937 cells were treated with ar-turmerone for 48 h and lysed with lysis buffer (40 mM Tris-HCl 7.4, 10 mM EDTA, 120 mM NaCl, 1 mM dithiothreitol, 0.1% Nonidet P-40, and protease inhibitors). Fifty µg of total protein were electrophoresed using 15% SDS-polyacrylamide gels and used for immunoblot analysis using anti-Bcl-2, anti-actin, anti-caspase-3 polyclonal antibodies and anti-p21 monoclonal antibody (Santa Cruz Inc., Santa Cruz, CA, USA). Monoclonal anti p53 and polyclonal anti-Bax antibodies were purchased from Calbiochem (Cambridge, MA, USA).

Flow cytometry analysis

After treatment with ar-turmerone, the cells were washed with cold PBS and resuspended in PBS. DNA contents of cells were measured using a DNA staining kit (CyleTest Plus DNA Reagent Kit, Becton Dickinson). Propidium iodide (PI)-stained nuclear fractions were obtained by following the kit protocol. Cells with a subdiploid DNA content (>5% of G₀ content) were considered to be apoptotic. Data were acquired using CellQuest Software with a FACScalibur (Beckman Coulter) flow cytometry system using 20,000 cells per analysis. Cell cycle distributions were calculated using ModFit LT 2.0 software (Verity Software House, Topsham, ME, USA).

Statistical analysis

Data were expressed as mean standard error of the (S.E.M.) and were analyzed using one way analysis of variance and Student's t test for individual comparisons. P values less than

0.05 are considered to be statistically significant.

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