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Supercritical Carbon Dioxide Extraction of Aromatic Turmerone from *Curcuma longa* Linn. Induces Apoptosis through Reactive Oxygen Species-Triggered Intrinsic and Extrinsic Pathways in Human Hepatocellular Carcinoma HepG2 Cells

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ABSTRACT: The mechanisms underlying the antiproliferative and antitumor activities of aromatic turmerone (ar-turmerone), a volatile turmeric oil isolated from *Curcuma longa* Linn., have been largely unknown. In this study, 86% pure ar-turmerone was extracted by supercritical carbon dioxide and liquid−solid chromatography and its potential effects and molecular mechanisms on cell proliferation studied in human hepatocellular carcinoma cell lines. Ar-turmerone exhibited significant antiproliferative activity, with 50% inhibitory concentrations of 64.8 ± 7.1, 102.5 ± 11.5, and 122.2 ± 7.6 μg/mL against HepG2, Huh-7, and Hep3B cells, respectively. Ar-turmerone-induced apoptosis, confirmed by increased annexin V binding and DNA fragmentation, was accompanied by reactive oxygen species (ROS) production, mitochondrial membrane potential dissipation, increased Bax and p53 up-regulated modulator of apoptosis (PUMA) levels, Bax mitochondrial translocation, cytochrome *c* release, Fas and death receptor 4 (DR4) augmentation, and caspase-3, -8, and -9 activation. Exposure to caspase inhibitors, Fas-antagonistic antibody, DR4 antagonist, and furosemide (a blocker of Bax translocation) effectively abolished ar-turmerone-triggered apoptosis. Moreover, ar-turmerone stimulated c-Jun N-terminal kinase (JNK) and extracellular signal-related kinase (ERK) phosphorylation and activation; treatment with JNK and ERK inhibitors markedly reduced PUMA, Bax, Fas, and DR4 levels and reduced apoptosis but not ROS generation. Furthermore, antioxidants attenuated ar-turmerone-mediated ROS production; mitochondrial dysfunction; JNK and ERK activation; PUMA, Bax, Fas, and DR4 expression; and apoptosis. Taken together, these results suggest that ar-turmerone-induced apoptosis in HepG2 cells is through ROS-mediated activation of ERK and JNK kinases and triggers both intrinsic and extrinsic caspase activation, leading to apoptosis. On the basis of these observations, ar-turmerone deserves further investigation as a natural anticancer and cancer-preventive agent.

KEYWORDS: *ar-turmerone, Curcuma, hepatocellular carcinoma cells, apoptosis, reactive oxygen species*

INTRODUCTION

Hepatocellular carcinoma is now the second leading cause of cancer mortality in Taiwan, with an overall 5-year survival rate of <5%. The poor prognosis of liver cancer is attributable to its tendency for late presentation, aggressive local invasion, early metastases, and poor response to chemotherapy.[1] Currently, sorafenib has been approved as the first target chemotherapeutic agent for the treatment of advanced hepatocellular carcinoma, as it has been shown to increase median survival by 3 months in such patients.[2] However, sorafenib treatment results in an objective tumor response rate of <10% and only a marginal survival advantage and is associated with multiple adverse events.[3] Treatment options for patients with advanced hepatocellular carcinoma are extremely limited; thus, it is crucially important to develop better therapeutic strategies for the management of this disease.

Natural products, herbs, and spices have long been used in Asian countries as remedies for various diseases, including cancer, and are important factors in counteracting or preventing human cancers.[4] It is currently estimated that >50% of all patients diagnosed with cancer explore complementary and alternative medicine, especially herbal medicine. *Curcuma longa* Linn., commonly known as turmeric, is a perennial herb

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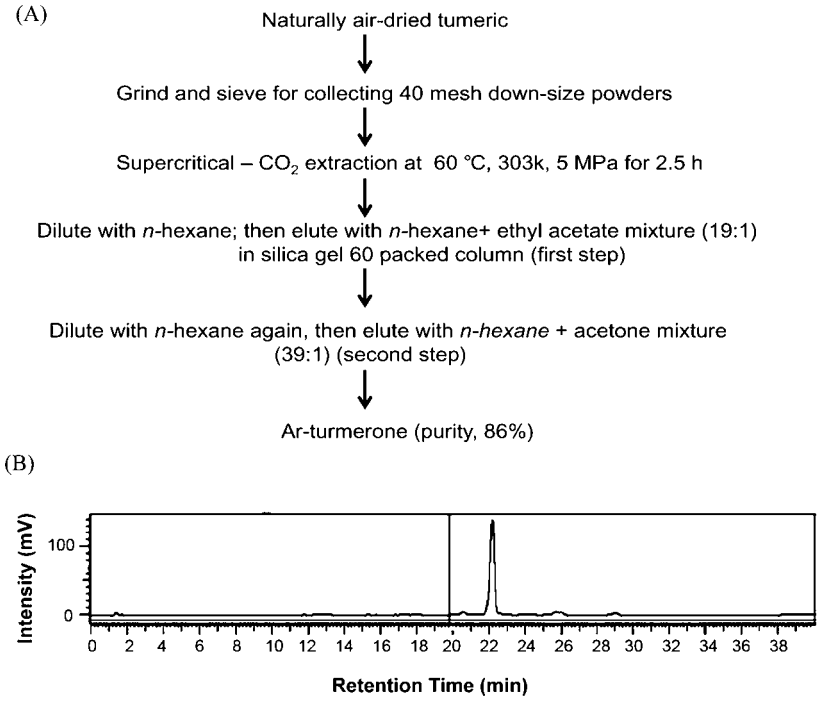

Figure 1. Extraction and purification of ar-turmerone from the root of *C. longa*: (A) flowchart of the extraction and isolation procedure; (B) HPLC chromatographic spectrum of purified ar-turmerone. Naturally air-dried turmeric (*C. longa*) was ground and sieved through an International Tayler 40 mesh sieve (0.42 metric), and then the ar-turmerone was extracted and separated from turmeric powder by supercritical carbon dioxide extraction, liquid–solid chromatography, and HPLC.

belonging to the family Zingiberaceae. The rhizomes and oils of *C. longa* have been extensively used as a spice in domestic cooking, as natural dyes for textiles, and in confectionary, cosmetics, and pharmaceuticals[5] and have been also advocated for use in liver disorders.[6] Curcumin and the volatile oil are two active components that are at least in part responsible for the biological actions of *C. longa*.[7] Curcumin is a major component of *C. longa*; extensive in vitro and in vivo studies over the past half century and particularly in recent years have provided interesting insights into the multiple mechanisms by which curcumin may mediate chemotherapeutic and chemopreventive effects on cancers.[8] Aromatic turmerone (ar-turmerone), the major compound present in turmeric volatile oil, is also an effective component of *C. longa*[9] and has been shown to have several pharmacological properties, including anti-inflammatory,[10] antimicrobial,[11] antifungal,[12] hepatic protection,[13] antiarthritic, and antitumor activities.[14−16] Although the antihepatoma activity of turmeric oil has been studied by several groups,[17] the mechanisms underlying the action of turmeric oil against hepatocellular carcinoma cells are not yet known. In this study, we carried out supercritical carbon dioxide extraction and liquid–solid chromatography to isolate ar-turmerone from *C. longa* and explored the molecular basis of the anticancer activity of ar-turmerone in the human hepatocellular carcinoma cell line, HepG2. We found that ar-turmerone induced apoptosis through reactive oxygen species (ROS)-provoked intrinsic (mitochondrial) and extrinsic (death receptor) death signaling pathways.

MATERIALS AND METHODS

Chemicals and Reagents. Propidium iodide (PI), 4′,6′-diamindino-2-phenylindole (DAPI), dimethyl sulfoxide (DMSO), ribonuclease (RNase), ascorbic acid (ASC), Triton X-100, *N*-acetylcysteine (NAC), PD98059, and furosemide were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Hoechst 33342, 2′,7′-dichlorofluoroscein diacetate acetyl ester (DCFH-DA), and dihydroethidine (HE) were purchased from Molecular Probes (Eugene, OR, USA). Fetal calf serum (FCS), glutamine, and Dulbecco's modified Eagle's medium (DMEM) were purchased from GIBCO BRL (Gaithersburg, MD, USA). Antibodies against p38, phospho-p38, cytochrome oxidase IV, TRAIL, Bcl-2, Bcl-xL, and Bax antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-TNF-related apoptosis inducing ligand (TRAIL) death receptor 4 (DR4) was purchased from Upstate Biotechnology (New York, NY, USA). Antiphospho-ERK1/2, -ERK1/2, and -PUMA antibodies and SP600125 were purchased from Millipore (Billerica, MA, USA). Anti-Fas, -FasL, and -cytochrome *c* antibodies were purchased from BD Pharmingen (San Diego, CA, USA). Anti-β-actin antibody was purchased from Oncogene Science Inc. (Uniondale, NY, USA). Antiphospho-JNK and -JNK antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Anti-Fas (clone ZB4) antagonistic antibody was purchased from Upstate Biotechnology. Anticaspase-8 and -9 and active caspase-3 antibodies were purchased from Santa Cruz Biotechnology and Abcam (Cambridge, MA, USA). TUNEL assay kit was purchased from Roche Diagnostics (Mannheim, Germany). Caspase activity assay kits were purchased from R&D Systems (Minneapolis, MN, USA). Caspase-3 inhibitor (Z-DEVD-FMK), casepase-8 inhibitor (Z-IETD-FMK), caspase-9 inhibitor (Z-LEHD-FMK), and DR4:Fc (a DR4 antagonist) were purchased from KAMIYA (Seattle, WA, USA).

Extraction, Purification and Quantification of Ar-tumerone. Ar-turmerone isolated from the root of *C. longa* Linn. was provided by Professor Chieh-Ming J. Chang, Department of Chemical Engineering, National Chung-Hsing University, with supercritical carbon dioxide extraction and liquid–solid chromatography isolation as described elsewhere.[18,19] The purity of ar-turmerone is 86%. Briefly, turmeric powder was subjected to supercritical carbon dioxide extraction conditions at 303 K and 5 MPa, and the extraction time was 2.5 h. Following the extraction, the oil-laden CO_2 was led into a Pyrex glass type collection vial and expanded through a micrometering valve to release the turmeric oil. Next, the supercritical carbon dioxide-

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extracted oil was diluted with *n*-hexane and then loaded into a middle-pressure Pyrex glass column (300 mm L × 25 mm i.d., Rei-E, Taiwan) that was packed with 130 g of silica gel resin (LiChroprep-Si-60, 40–63 mm, Merck, Darmstadt, Germany) to perform a two-stepwise elution process (Figure 1A). In the course of chromatography, the eluting solvent comprises *n*-hexane/ethyl acetate (19:1) and *n*-hexane/acetone (39:1) in the first and second steps, respectively. The flow rate for loading, washing, and elution was constantly kept at 0.5 mL/min. The eluted samples were automatically collected in 10 mL vials using an intelligent fractional collector (2SF-2120, Advantec, Japan). Nearly 30 fractions were collected, and each fraction containing 10 mL was checked by a thin-layer chromatography (TLC) analysis. After the solvent had been dried out, the concentrated ar-turmerone was obtained. Next, HPLC quantification was performed using a reverse-phase column (Purospher RP-18, 5 μm, 125 mm × 4 mm, Merck) that was linked to a Hitachi L-4200 UV–vis detector. The column temperature was maintained at 313 K. UV detection of samples was performed at a wavelength of 254 nm, and the injection volume was 20 mL. The gradient mobile phase used for analysis was mixed by solvent A, 0.0025% TFA solution, and solvent B, acetonitrile. A flow rate of 1 mL/min under the initial condition of 80:20 (A/B) was held for 5 min, brought to 48% solvent B for 5 min, to 60% solvent B and held for 10 min, and brought to 100% solvent B for 10 min. Ar-turmerone was completely separated by the above gradient elution (Figure 1A).

Cell Culture and Cytotoxicity Assay. Human hepatocellular carcinoma HepG2 cells were maintained in DMEM, containing 10% FCS, antibiotics (100 U/mL penicillin and 100 U/mL streptomycin), and 1% L-glutamine, at 37 °C in a humidified atmosphere with 5% CO_2, and culture medium was changed each 2 days. For the cytotoxicity assay, cells were treated with various concentrations of ar-turmerone, and for indicated time points, the viable cells were evaluated by trypan blue dye exclusion method.

Annexin V/PI Staining Assay. HepG2 cells were treated with various concentrations of ar-turmerone for 16 h. Then, the cells were collected, washed, and stained with annexin V FITC kit according to the manufacturer's protocol (Trevigen, Gaithersburg, MD, USA). Briefly, cells were incubated in 100 μL of labeling solution containing 1 μL of FITC-conjugated annexin V and 10 μL of PI in the dark at room temperature for 15 min. The level of fluorescence intensity of the cells was detected by flow cytometry (Becton Dickinson, USA).

Terminal Deoxynucleotidyl Transferase dUTP Nicked-End Labeling (TUNEL) Assay. Apoptotic cells were determined by TUNEL assay according to the manufacturer's instructions (Boehringer Mannheim). Briefly, after treatment, cells were washed with PBS and fixed with 2% paraformaldehyde for 20 min with gentle vortex. Fixed cells were washed with PBS twice, and then the cells were incubated in 500 μL of Triton X-100 (0.1% v/v) for 30 min and then washed with PBS. Adding TdT enzyme (terminal deoxynucleotidyl transferase and FITC-dUTP (FITC-deoxyuridine triphosphate) containing solution for 2 h in the dark, cells were stained with Hoechst 33342 for nuclear staining and then washed with PBS and sealed with 10% glycerol mounting solution. Labeled cells were examined and photographed by Nikon Coolpix 995 (Nikon Corp. Imaging Co., Tokyo, Japan). TUNEL-positive green fluorescence cells were shown as apoptotic cells. The TUNEL positive cells were also analyzed by flow cytometry.

DNA Fragmentation Analysis. Cells were harvested and maintained in lysis buffer (50 mM Tris, pH 7.5, 10 mM EDTA, and 0.3% Triton X-100) for 30 min, and the supernatant was collected and incubated with DNA extraction buffer (0.1 mg/mL proteinase K and 0.2 mg/mL RNase) for 1 h, at 55 °C, and then extracted with phenol/chloroform (1:1) and separated in 2% ethidium bromide-contained agarose gel. Fragmented DNA was visualized under a UV light box and photographed.

Caspase Activity Assay. Caspase activities were measured according to the manufacturer's instructions (R&D Systems). Briefly, cell lysates obtained from ar-turmerone-treated or untreated cells were incubated with the caspase-specific peptide substrates for 2 h at 37 °C, and then the fluorescence intensity was assessed with a fluorescent microplate reader (Fluoroskan Ascent, Labsystem, Finland), excited at 400 nm and emitted fluorescence at 505 nm.

Total Protein Preparation and Western Blot Analysis. Cells were harvested and washed twice with ice-cold PBS and lysed in modified RIPA buffer containing protease inhibitors. The cell lysates were centrifuged at 12000*g* for 30 min at 4 °C, the supernatant was collected, and total protein content was determined according to the Bradford method. For Western blot analysis, equal amounts of proteins were separated onto SDS–polyacrylamide gels and then electrophoretically transferred from the gel onto a PVDF membrane (Millipore, Billerica, MA, USA). After blocking, the membrane was reacted with specific primary antibodies overnight at 4 °C. The membrane was then incubated with horseradish peroxidase-conjugated secondary antibody for 1 h, and the blots were visualized using the ECL-Plus detection kit (PerkinElmer Life Sciences, Inc., Boston, MA, USA).

Protein Subcellular Fractionation. Cells were washed and harvested, and then incubated on ice for 30 min in sucrose buffer (250 mM sucrose, 2 mM EDTA, 2 mM EGTA, 10 mM DTT). Lysates were centrifuged at 1300*g* for 10 min at 4 °C; the soluble cytosolic fraction was collected after further centrifugation of the raw cytosolic fraction at 100000*g* for 30 min at 4 °C. Mitochondria were purified from heavy membrane fraction by sucrose density-gradient centrifugation; briefly, heavy membrane pellet was resuspended in 1 mL of sucrose buffer and laid on the top of 1.2 and 1.5 M sucrose buffer gradient before being centrifuged at 1300*g* for 30 min at 4 °C. Sucrose-density-gradient-purified mitochondria-enriched fractions were collected at 1.2 and 1.5 M interphases, respectively, washed with PBS, and dissolved in RIPA lysis buffer. All fractions were disrupted by sonication and prepared before Western blot analysis.

Determination of Intracellular Reactive Oxygen Species Level and Mitochondrial Membrane Potential ($\Delta\Psi$m). To assess the intracellular ROS level, cells were incubated with ar-turmerone for the indicated periods and then incubated with 10 μM 2,7-dichlorodihydrofluorescein diacetate (DCF-DA; Molecular Probes Inc.) or 10 μM dihydroethidium (HE; Molecular Probes Inc.) for 30 min prior to harvesting. For mitochondrial membrane potential analysis, lipophilic fluorochrome 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzamidazolyl carbocyanine iodide (JC-1, Molecular Probes) was applied 30 min prior to harvesting. Then the levels of fluorescence intensity of the cells were analyzed by flow cytometry (Becton Dickinson FACScan) under an excitation wavelength of 488 nm and emission wavelengths of 530 nm for green fluorescence and 585 nm for red fluorescence.

Statistical Analyses. Each experiment was performed in triplicate and repeated three times. The data were expressed as the mean ± SD. Statistical comparisons were made by means of one-way analysis of variance (ANOVA) with post-Tukey test, and significant difference between the control and tested groups was using Student's *t* test. The statistic significance was presented as $*$, $p < 0.05$; $**$, $p < 0.01$; and $***$, $p < 0.001$.

RESULTS

Preparation of Ar-turmerone. Ar-turmerone was isolated, purified, and identified from turmeric by supercritical carbon dioxide extraction, liquid–solid chromatography, and high-performance liquid chromatography (HPLC) quantification. Figure 1A presents a flow diagram of extraction and isolation procedures. Next, an HPLC fingerprint chromatogram was established for the quantification and quality control of the isolated sample (Figure 1B), which showed only one major peak (retention time = 22.1 min). The peak was identified as ar-turmerone. The purity of ar-turmerone in the eluted sample was 86%.

Ar-turmerone Induces Apoptosis. To examine the effect of ar-turmerone on the cell viability of human hepatocellular carcinoma cells, HepG2, Huh-7, and Hep3B cell lines were treated with various concentrations of ar-turmerone for 24 h. As

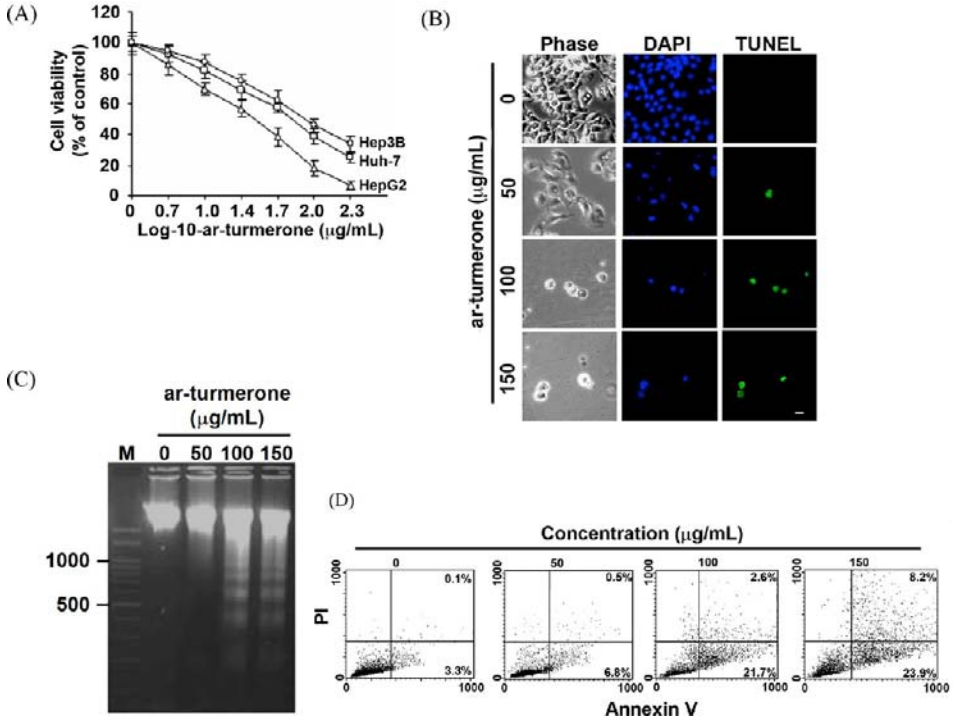

Figure 2. Ar-turmerone induces apoptosis. Human hepatocellular carcinoma HepG2, Huh-7, and Hep3B cell lines were treated with various concentrations of ar-turmerone (0, 5, 10, 25, 50, 100, and 200 μg/mL) for 24 h. (A) Cell viability was determined by the trypan-blue exclusion method. Data are expressed as the mean ± SD from three independent experiments. HepG2 cells were treated with different concentrations of ar-turmerone for 48 h followed by (B) TUNEL assay (magnification ×100; scale bar, 25 μm) and (C) DNA fragmentation analysis as described under Material and Methods. (D) For annexin V/PI double staining, HepG2 cells were treated with 0, 50, 100, and 150 μg/mL ar-turmerone for 16 h. The cells were double-stained with FITC-conjugated annexin V and PI to identify apoptotic cells using flow cytometry analysis.

shown in Figure 2A, ar-turmerone showed significant cytotoxic activity in all three tested hepatocellular carcinoma cell lines in a concentration-dependent manner; the resulting 50% inhibitory concentration (IC_{50}) values of ar-turmerone against HepG2, Huh-7, and Hep3B cells were 64.8 ± 7.1, 102.5 ± 11.5, and 122.2 ± 7.6 μg/mL, respectively. Among the tested cell lines, ar-turmerone exhibited the most potent inhibitory effect on HepG2 cell viability. Ar-turmerone exhibited a cytostatic effect at low concentrations (<50 μg/mL) and caused cytotoxicity at relatively higher concentrations (>50 μg/mL), as evidenced by the fact that treatment of these three hepatocellular carcinoma cell lines with a high dose of ar-turmerone caused morphological shrinkage and membrane blebbing (data not shown), which are hallmarks of apoptosis. To confirm this possibility, the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, DNA fragmentation analysis, and the annexin V/PI double-staining assay were performed in HepG2 cells. Data from the TUNEL assay showed an increase in TUNEL-positive cells (apoptotic cells) upon ar-turmerone treatment (Figure 2B). Similarly, internucleosomal DNA fragments were observed after ar-turmerone treatment (Figure 2C). The annexin V/PI double-staining analysis showed that the cell apoptosis rates of ar-turmerone-treated cells (lower and right quadrants in Figure 2D) were significantly higher than those in the control group. These results suggest that the ar-turmerone-induced cytotoxic effect is partly through an apoptotic pathway.

Caspase Activation Contributes to Ar-turmerone-Induced Apoptosis. The activation of caspase is a widely recognized feature of apoptosis; thus, the effect of ar-turmerone on caspase activity in HepG2 cells was examined. As indicated in Figure 3A, exposure to ar-turmerone significantly increased the activity of caspase-3, -8, and -9; besides, Western blot also revealed the cleaved caspase-8, caspase-9, and caspase-3 upon ar-turmerone treatment (Figure 3B). Pretreatment with caspase inhibitors prior to ar-turmerone administration effectively diminished ar-turmerone-triggered apoptosis (Figure 3C), suggesting that caspase activation is indeed involved in ar-turmerone-induced apoptosis.

Regulation of Bcl-2 and Fas/FasL Family Molecules by Ar-turmerone. The activation of caspase-3, -8, and -9 during ar-turmerone-induced apoptosis suggests the possibility that both extrinsic and intrinsic apoptotic pathways might be involved.[20] Therefore, the regulation of apoptosis-associated proteins such as those of the Bcl-2 family (representing intrinsic pathways) and the Fas/FasL family (representing extrinsic pathways) were examined in untreated and ar-turmerone-treated HepG2 cells. As depicted in Figure 4A, the levels of Bax, p53 up-regulated modulator of apoptosis (PUMA), Fas, FasL, TRAIL, and death receptor 4 (DR4) were increased, whereas those of Bcl-2 and Bcl-xL proteins were lower in ar-turmerone-treated cells than in untreated controls. Moreover, cleaved poly(ADP-ribose) polymerase (PARP), a caspase-3 downstream target, was also observed in ar-turmerone-treated cells. Translocation of Bax from the cytosol to the mitochondrial membrane is considered a critical step in the release of mitochondrial cytochrome *c* to prime intrinsic apoptosis;[21] therefore, to investigate the subcellular localization of Bax, HepG2 cells were incubated with or without 100 μg/mL ar-turmerone for the indicated time points, and then cell lysates were fractionated and subjected to Western blot analysis. Actin and cytochrome oxidase IV were used as cytosolic and

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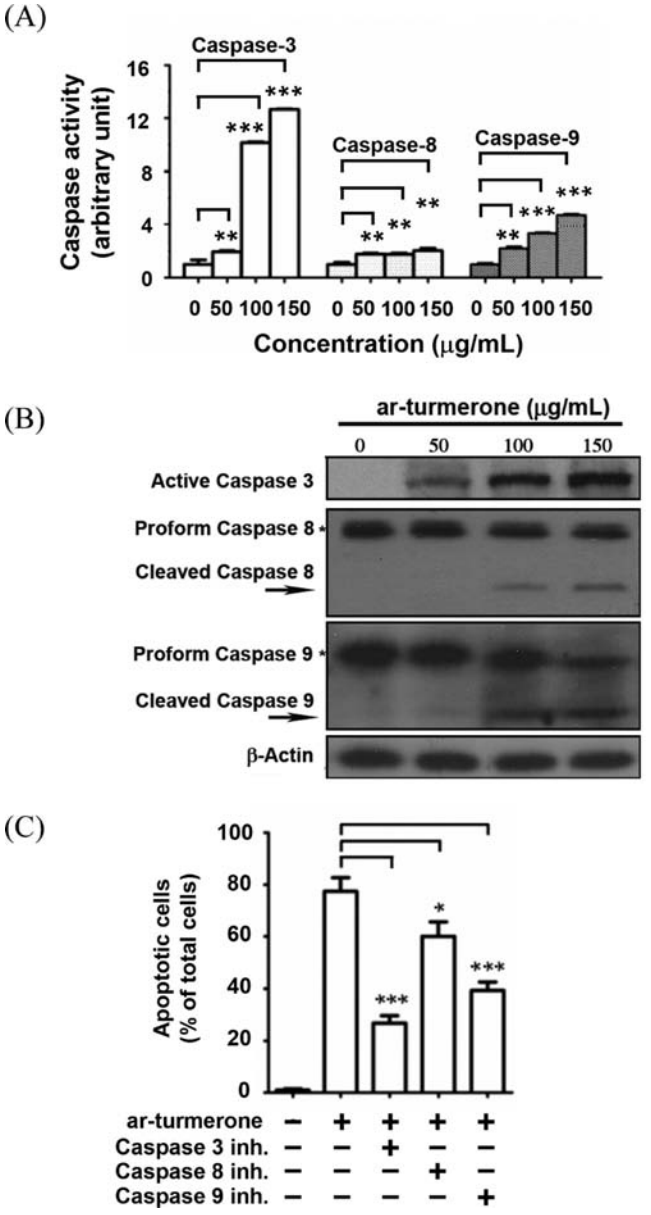

Figure 3. Ar-turmerone-elicited apoptosis is caspase-dependent. (A) HepG2 cells were treated with different doses of ar-turmerone for 24 h, followed by analysis of caspase-3, -8, or -9 activities using fluorogenic peptide substrates. (B) HepG2 cells were treated with various concentrations of ar-turmerone for 24 h; total proteins were collected and analyzed by Western blot using antibodies against caspase-8 and -9 and active caspase-3, respectively. (C) HepG2 cells were pretreated with 50 μM caspase inhibitor for 1 h followed by treating with 100 μg/mL ar-turmerone for an additional 24 h; apoptotic cells were identified by the TUNEL assay. Data are expressed as the mean ± SD from three independent experiments (*, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$).

mitochondrial markers, respectively. In Figure 4B, exposure to ar-turmerone not only increased the protein level of Bax but also induced its translocation from the cytosol to the mitochondria; in contrast, cytochrome *c* was released from the mitochondria to the cytosol. The mitochondrial membrane permeability transition pore (MPTP) is the major target regulated by the Bcl-2/Bax interaction on the mitochondrial membrane.[22,23] Therefore, the effect of ar-turmerone on MPTP was investigated. Exposure to ar-turmerone resulted in a time-dependent dissipation of the mitochondrial membrane potential; the percentage of depolarized cells was increased from 4.9 to 30.3 and 71.1% after 16 and 24 h of incubation, respectively (Figure 4C). In addition, ar-turmerone-mediated apoptosis was drastically reduced by furosemide (a Bax translocation blocker), Fas blocking antibody, and DR4:Fc (DR4 death signaling antagonist) (Figure 4D). These results indicate that signaling pathways activated by both Bcl-2 family proteins and death receptors contribute to ar-turmerone-induced apoptosis in HepG2 cells.

ROS Production Is Required for Ar-turmerone-Induced Apoptosis. Dysregulation of the cellular redox status contributes to mitochondrial damage that may facilitate the further release of ROS into the cytoplasm,[24] which is considered a crucial factor in priming cellular apoptosis.[13,25] To address the possibility that ar-turmerone-triggered apoptosis can be attributed to ROS generation, HepG2 cells were exposed to 100 μg/mL ar-turmerone for the indicated time periods, and changes in ROS levels were examined using the specific fluorescence probes DCF-DA and HE. Detailed analysis of the time course for ROS generation, as shown in Figure 5A, revealed that the fluorescence started to increase at 5 min, with a peak at 30 min after stimulation with 100 μg/mL ar-turmerone. Moreover, pretreatment of HepG2 cells with the antioxidants NAC (0.5 mM) and ascorbic acid (ASC; 0.1 mM) for 1 h followed by the addition of ar-turmerone significantly attenuated ar-turmerone-mediated ROS production (Figure 5B), mitochondrial membrane dysfunction (Figure 5C), Bax, PUMA, Fas, DR4, and active caspase-3 regulation (Figure 5D), and apoptosis (Figure 5E). These results imply that the generation of ROS upon ar-turmerone treatment might be an essential upstream factor in the regulation of apoptosis.

Extracellular Signal-Related Kinase (ERK) and c-Jun N-Terminal Kinase (JNK) Participate in Ar-turmerone-Mediated Apoptosis. Several studies have demonstrated that mitogen-activated protein kinase (MAPK) family molecules, including ERK, JNK, and p38, play roles in the regulation of the ROS-induced apoptotic cascade and related downstream molecules.[26] To explore whether MAPKs are involved in ar-turmerone-triggered apoptosis, the levels of total and phosphorylated ERK, JNK, and p38 (which correlate with the activation of these kinases) were evaluated in untreated and ar-turmerone-treated HepG2 cells. As shown in Figure 6A, the phosphorylation of ERK1/2 and JNK was detectable as early as 30 min after the start of ar-turmerone treatment and persisted for at least 4 h of treatment, whereas the levels of total ERK, JNK, p38, and phosphorylated p38 were not affected by ar-turmerone treatment. These results indicate that the ERK1/2 and JNK pathways are activated in response to ar-turmerone in HepG2 cells. To address whether the activation of ERK and JNK caused by ar-turmerone was associated with apoptosis, cells were treated with inhibitors of ERK (PD98059), JNK (SP600125), and p38 (SB203580) prior to ar-turmerone administration. As shown in Figure 6B, pharmacological inhibition of ERK and JNK activity, but not of p38 activity, markedly attenuated ar-turmerone-induced apoptosis. Moreover, pretreatment with ERK and JNK inhibitors effectively abolished the phosphorylation of ERK and JNK 1 h after administration of ar-turmerone and that of PUMA, Bax, Fas, DR4, and active caspase-3 after ar-turmerone treatment (Figure 6C). These results suggest that activation of ERK and JNK is involved in ar-turmerone-mediated regulation of apoptosis-related molecules, thereby leading to apoptosis.

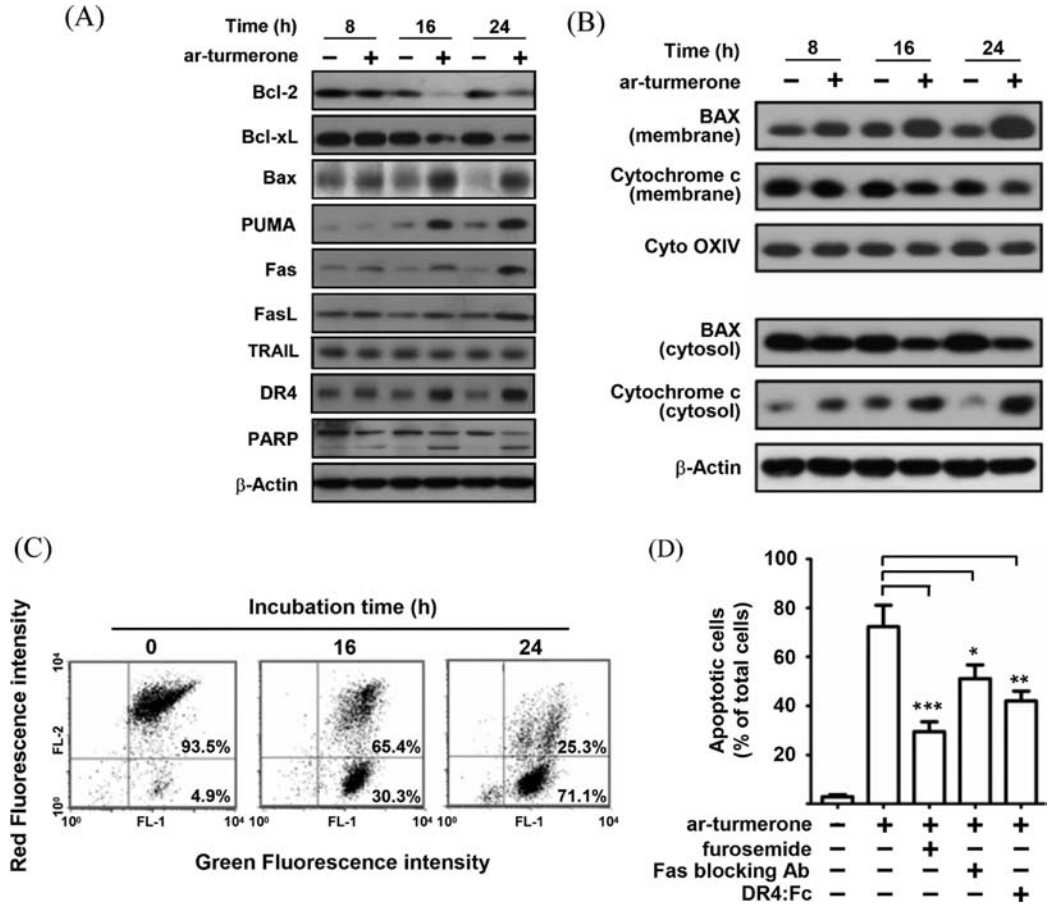

Figure 4. Both intrinsic and extrinsic signaling pathways are involved in ar-turmerone-triggered apoptosis. (A) Regulation of apoptosis-related molecules. HepG2 cells were treated without or with 100 μg/mL ar-turmerone for 8, 16, and 24 h followed by Western blot analysis using specific antibodies against, PUMA, Bax, Bcl-2, Bcl-xL, Fas, FasL, TRAIL, DR4, and PARP. β-Actin was used as a loading control. (B) Bax and cytochrome *c* translocation. HepG2 cells were treated without or with 100 μg/mL ar-turmerone for 8, 16, and 24 h; the subcellular distribution of Bax and cytochrome *c* was examined by cellular fractionation and Western blot. β-Actin and cytochrome oxidase IV (Cyto OX IV) were used as loading controls for the cytosolic and mitochondrial fractions, respectively. (C) HepG2 cells were treated with 100 μg/mL ar-turmerone for 16 and 24 h; the cells were then incubated with the JC-1 dye and subjected to flow cytometry to evaluate the mitochondrial membrane potential. (D) HepG2 cells were pretreated with 200 mM furosemide, 1 mg/mL Fas blocking antibody, or 100 ng/mL DR4:Fc (an antagonist of DR4 death signaling) for 2 h and then treated with 100 μg/mL ar-turmerone for another 24 h. Apoptotic cell death was determined by the TUNEL assay. Data are expressed as the mean ± SD from three independent experiments (*, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$).

ROS Acts as an Upstream Regulator of ERK and JNK Activation. Our observations indicate that ROS generation started at 5 min following ar-turmerone treatment, whereas the phosphorylation of ERK1/2 and JNK was detected at 30 min in response to ar-turmerone. To characterize whether ROS production was required for the phosphorylation of ERK1/2 and JNK, HepG2 cells were pretreated with antioxidants (0.5 mM NAC and 0.1 mM ASC), and the levels of phosphorylated ERK and JNK were determined by Western blot. As expected, the presence of NAC and ASC diminished the levels of phosphorylated ERK1/2 and JNK but not total ERK1/2 and JNK protein levels (Figure 7A). However, pretreatment with either SP600125 or PD98059 did not inhibit the ROS generation induced by ar-turmerone (Figure 7B), indicating that ROS production initiated ar-turmerone-mediated ERK and JNK phosphorylation and activation.

DISCUSSION

C. longa Linn. has been used as an important nutritional flavoring agent and natural remedy for many ailments for centuries in ancient systems of medicine such as Chinese and Indian medicine. Many active components have been isolated from *C. longa*, including curcumin and turmeric oil. A literature search has revealed that several additional studies have been recently carried out related to the anticancer activities of curcumin. However, only a few pharmacological effects of turmeric oil have been studied. Only a few studies have examined the medicinal properties of turmeric oil and provided some description of its anticancer effects. Ar-turmerone is a major bioactive compound that exists naturally in turmeric oil and has been shown to inhibit melanogenesis in B16F10 melanoma cells.[27] A previous study demonstrates that ar-turmerone induces Bax-associated apoptosis in human lymphoma U937 cells.[28] In this study, we extracted and isolated ar-turmerone from the root of *C. longa* by supercritical carbon dioxide extraction, liquid–solid chromatography, and HPLC (Figure 1). Moreover, we report for the first time that ar-turmerone induces ROS-mediated ERK and JNK activation, which triggers both intrinsic (mitochondria-mediated) and extrinsic (death receptor-mediated) apoptotic cell death in human hepatocellular carcinoma (HepG2) cells.

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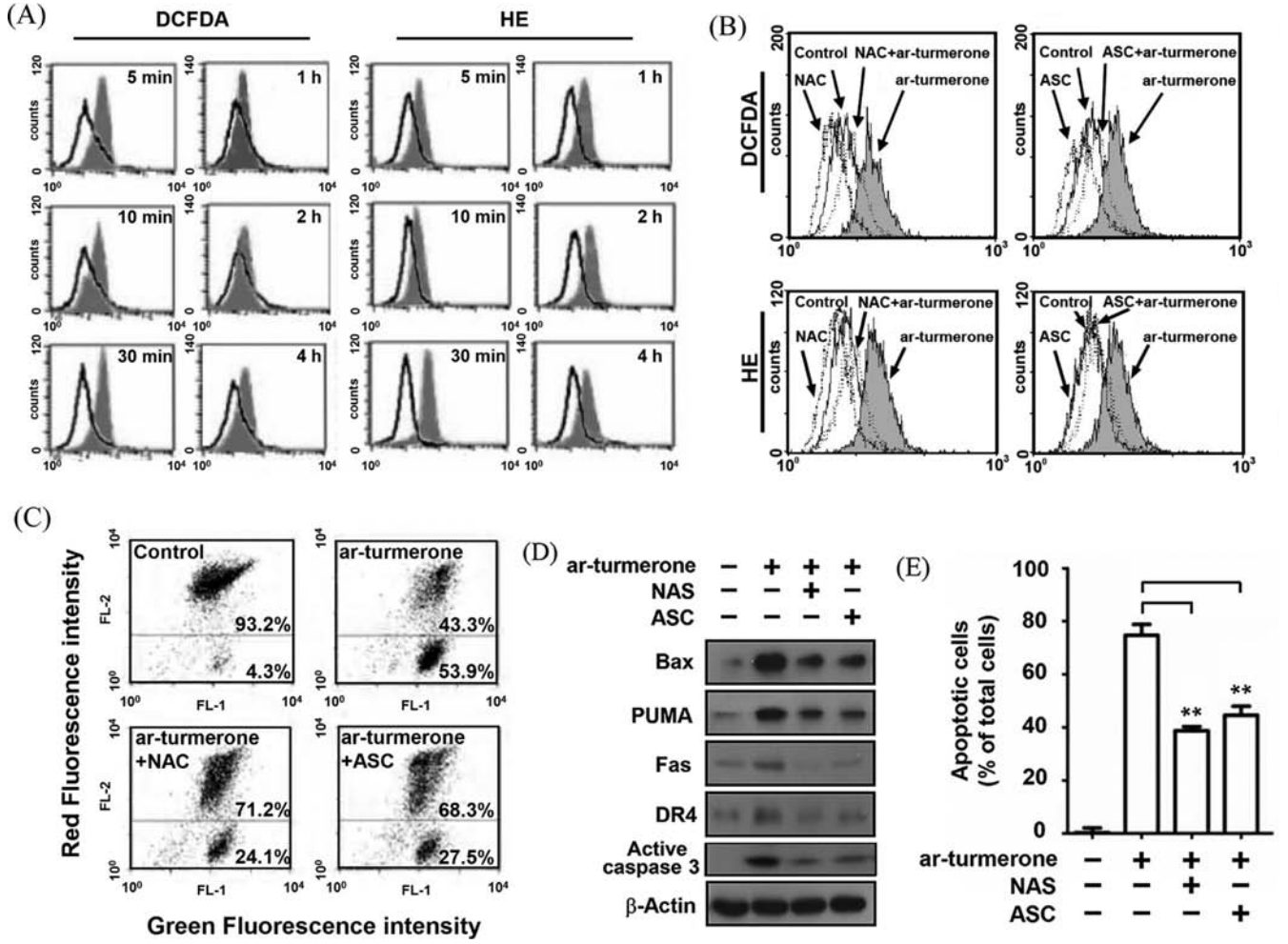

Figure 5. Generation of ROS is required for ar-turmerone-induced apoptosis. (A) ROS generation. HepG2 cells were untreated (white) or treated with 100 μg/mL of ar-turmerone (gray) for 5, 10, and 30 min and 1, 2, and 4 h; the cells were then incubated with DCF-DA or HE and subjected to fluorescence-activated cell sorting (FACS) analysis to examine the intracellular levels of hydroxyl and superoxide radicals, respectively. HepG2 cells were pretreated with the antioxidants NAC (0.5 mM) and ASC (0.1 mM) for 1 h, followed by 100 μg/mL of ar-turmerone treatment. (B) ROS production was evaluated after 30 min of ar-turmerone treatment; different treatments are labeled and marked by arrow. (C) The mitochondrial membrane potential was examined after 16 h of ar-turmerone treatment. (D) The levels of PUMA, Bax, Fas, DR4, and active caspase-3 were determined by Western blot 24 h after ar-turmerone treatment. (E) Apoptotic cells were evaluated by the TUNEL assay after 24 h of ar-turmerone treatment. Data are expressed as the mean ± SD from three independent experiments (**, $p < 0.01$ versus the ar-turmerone-treated group).

The pro-apoptotic and anti-apoptotic members of the Bcl-2 family of proteins have opposing regulatory effects on apoptosis through the control of cytochrome *c* release from mitochondria, which in turn induces caspase-9 and -3 activation.[29,30] In this study, treatment of HepG2 cells with ar-turmerone resulted in the concentration-dependent activation of caspase-3, -8, and -9 (Figure 3A,B). Furthermore, the addition of ar-turmerone caused an increase in the expression levels of Bax and PUMA and a decrease in those of Bcl-2 and Bcl-xL (Figure 4A). The translocation of Bax from the cytosol to the mitochondrial membrane, followed by the disruption of the mitochondrial membrane potential, was also observed in the presence of ar-turmerone (Figure 4B,C). These results reveal that the addition of ar-turmerone not only increases the protein level of Bax but also induces its translocation from the cytosol to the mitochondrial membrane (Figure 4A,B), indicating the existence of multiple mechanisms of Bax regulation mediated by ar-turmerone. Furthermore, the inhibition of Bax translocation by furosemide significantly reduced ar-turmerone-induced cell death (Figure 4D). All of these findings suggest that a Bax-mediated mitochondria-dependent intrinsic apoptotic pathway was provoked by ar-turmerone in HepG2 cells. Similarly, a previous study reported that ar-turmerone-induced mitochondria-dependent apoptosis occurred through Bax translocation and caspase activation in human lymphoma U937 cells.[31] In anticancer therapy, another key signaling mechanism known to play a role in the execution of apoptosis is the death receptor system.[32] This extrinsic pathway is initiated by the tumor necrosis factor (TNF) receptor superfamily, including Fas, the TNF receptor, and the TNF-related apoptosis-inducing ligand (TRAIL) receptors DR4 and DR5, with sequential activation of the initiator caspase-8 and of the effector caspase-3.[32] This is the first study to demonstrate that caspase-3 and -8 are activated and that Fas and DR4 are significantly increased in ar-turmerone-treated HepG2 cells (Figures 3A and 4A). Furthermore, Fas blocking antibody and DR4 antagonist effectively abolished ar-turmerone-induced apoptosis (Figure 4D), indicating that Fas and DR4 played important roles in ar-turmerone-triggered apoptosis. These results suggest that ar-turmerone-elicited apoptosis is mediated

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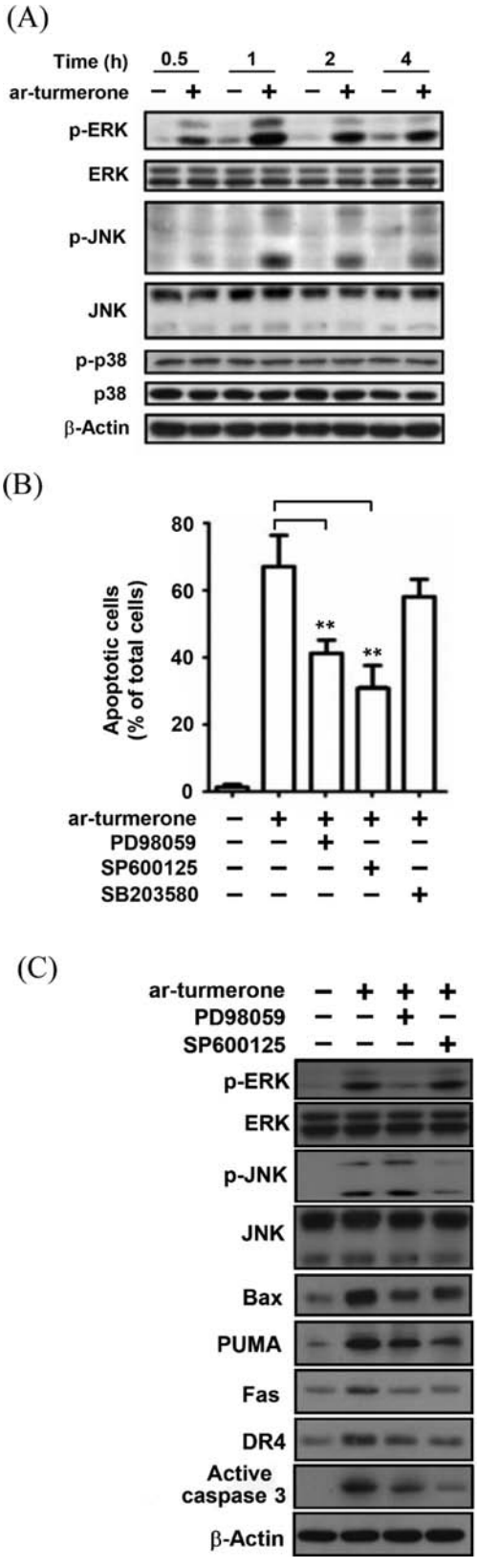

Figure 6. Involvement of ERK and JNK activation in ar-turmerone-induced apoptosis. (A) ERK and JNK are activated by ar-turmerone. HepG2 cells were treated without or with 100 μg/mL of ar-turmerone for 0.5, 1, 2, and 4 h. After treatment, cell lysates were extracted and analyzed by Western blot using the indicated antibodies (p-ERK, ERK, p-JNK, JNK, p-p38, and p38). β-Actin was used as a loading control. (B) Inhibition of ERK and JNK activity protects cell against ar-turmerone-mediated cytotoxicity. Cells were pretreated with PD98059 (20 μM, ERK inhibitor) and SP600125 (5 μM, JNK inhibitor) for 1 h and then treated with 100 μg/mL ar-turmerone for an additional 24 h; the apoptotic cells were then determined by the TUNEL assay. (C) For modulation of apoptosis-related molecules, cells were pretreated with PD98059 (20 μM, ERK inhibitor) and SP600125 (5 μM, JNK inhibitor) for 1 h and treated with 100 μg/mLar-turmerone for another 1 h (to detect the levels of p-ERK, ERK, p-JNK, and JNK proteins) and for another 24 h (to detect the levels of PUMA, Bax, Fas, DR4, and active caspase-3 proteins). β-Actin was used as a loading control.

through both intrinsic and extrinsic pathways. Besides, the Fas/FasL and signaling pathway plays an important role in tumorigenesis; its impairment in cancer cells may lead to apoptosis resistance and contribute to tumor progression. Thus, the development of effective therapies targeting the Fas/FasL system may play an important role in the fight against cancer. Our results indicate that ar-turmerone might be worthy of serving as an anticancer or chemoprevention agent for treating hepatocellular carcinoma cells.

Accumulating evidence has shown that ROS play an important role in the induction of apoptosis.[13] Many anticancer drugs are able to produce intracellular ROS that subsequently activate mitochondrial permeability transition pores followed by a disruption in the mitochondrial membrane potential.[33] Therefore, the generation of ROS is part of the mechanism by which most chemotherapeutic agents or ionizing radiations kill cancer cells.[34] A recent paper demonstrates that many cancers have decreased levels or activities of important antioxidant enzymes, including superoxide dismutase and catalase.[35] This situation makes cancer cells less efficient at removing ROS. They are therefore under higher levels of oxidative stress, which can make them more vulnerable to ROS-generating compounds than are normal cells.[34] In this study, one of the striking features of ar-turmerone-induced apoptosis in HepG2 cells was the accumulation of intracellular ROS (Figure 5A). The importance of ROS production in ar-turmerone-induced apoptosis was confirmed by the ability of the antioxidants NAC and ASC to inhibit turmeric oil-induced ROS generation (Figure 5B), suppress ERK and JNK phosphorylation and activation (Figure 7A), decrease PUMA, Bax, Fas, DR4 and active caspase-3 expression (Figure 5D), and rescue HepG2 cells from ar-turmerone-induced apoptosis (Figure 5E). Overall, our data indicate that ar-turmerone is a potent inducer of oxidative stress, which plays a primary role in triggering the progression of the apoptotic process through the activation of both intrinsic and extrinsic pathways in HepG2 cells.

Previous studies provide evidence of ROS as potential inducers of MAPK signal pathways.[36] The MAPK family, composed of ERK, JNK/stress-activated protein kinase (SAPK), and p38-MAPK, are important mediators of signal transduction and respond to a variety of stimuli, such as growth factors, cellular stresses, and ROS.[36] Many studies have concluded that in general, activation of the ERK pathway drives cell growth and survival,[37] whereas, JNK and p38-MAPK are weakly activated by growth stimuli but significantly activated by stress signals followed by the induction of apoptosis.[23,38] JNK and p38-MAPK are also predominately activated in response to cellular redox stress.[39,40] It has been reported that inhibition of ERK signaling leads to the increased

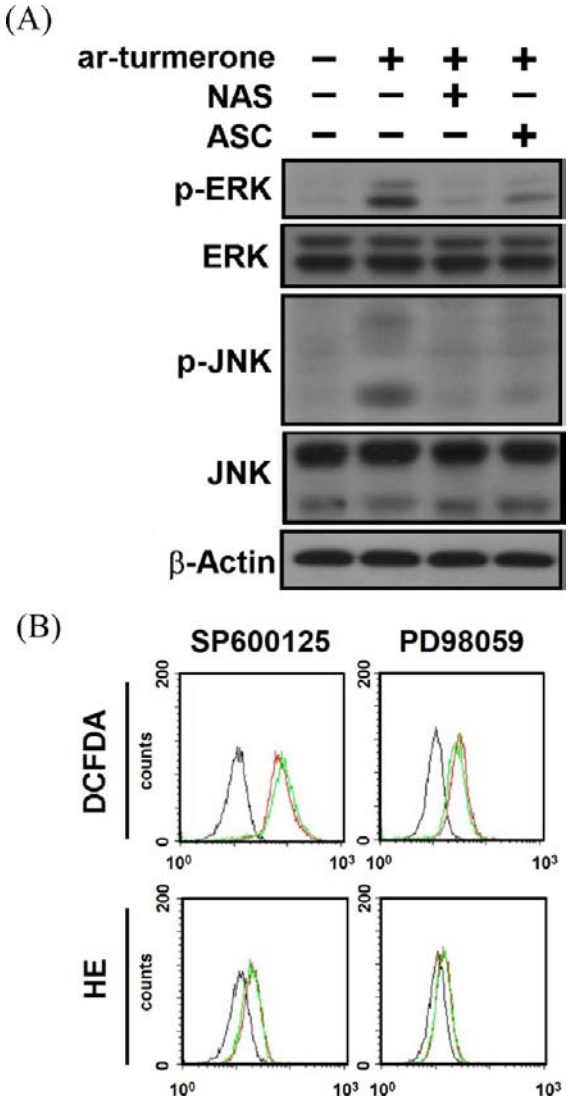

Figure 7. ROS acts as an upstream regulator of ERK and JNK activation. HepG2 cells were pretreated with the antioxidants NAC (0.5 mM) and ASC (0.1 mM) for 1 h, followed by 100 μg/mL ar-turmerone treatment. (A) The levels of phosphorylated ERK, JNK, and total ERK and JNK were examined after 1 h of ar-turmerone treatment. (B) HepG2 cells were pretreated with either PD98059 (20 μM, MEK/ERK inhibitor) or SP600125 (5 μM, JNK inhibitor) for 1 h, treated with 100 μg/mL ar-turmerone for an additional 30 min, incubated with DCF-DA or HE, and then subjected to FACS analysis to examine the intracellular levels of hydroxyl and superoxide radicals, respectively: untreated control, black line; ar-turmerone-treated culture, red line; PD98059 + ar-turmerone or SP600125 + ar-turmerone treatment, green line.

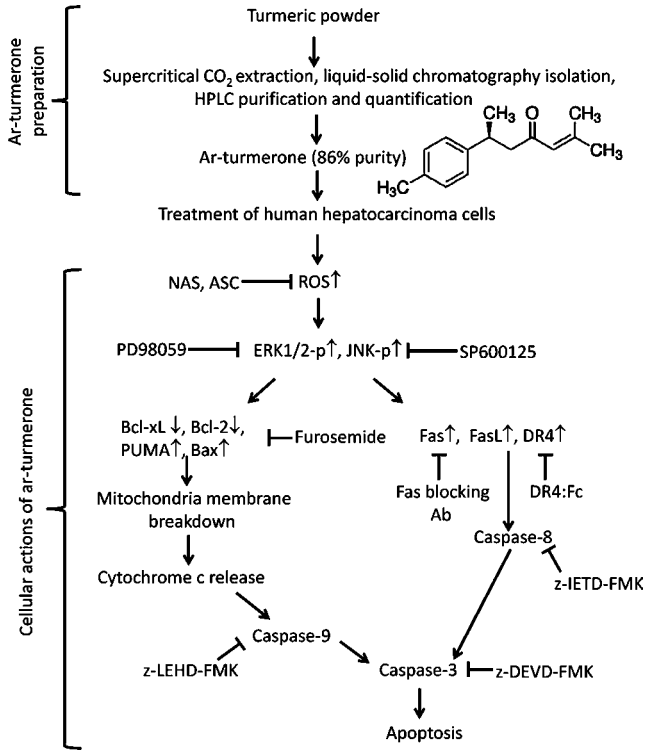

Figure 8. Schematic representation of ar-turmerone preparation and ar-turmerone-induced apoptosis.

sensitivity of ovarian cancer cell lines to cisplatin.[41] However, in this study, we have provided evidence that ERK activation is important for the induction of ar-turmerone-induced apoptosis in HepG2 cells. Ar-turmerone treatment resulted in high and sustained activation of ERK in these cells, and pharmacological inhibition of ERK by PD98058 significantly blocked ar-turmerone-mediated apoptosis (Figure 6B). There is increasing evidence that, in different cell types such as HeLa cells, osteosarcoma cells, neuroblastoma cells, lung cancer cells, and mouse embryonic fibroblasts, activation of ERK by means of different agents such as cisplatin, paclitaxel, adriamycin, etoposide, and gemcitabine is involved in the induction of apoptosis and cell cycle arrest.[42] Previous papers demonstrate that apoptosis mediated by JNK and/or p38-MAPK is accompanied by Bcl-2 phosphorylation, Bax subcellular translocation, and subsequently the disruption of the mitochondrial membrane potential, release of cytochrome *c*, and activation of caspases, indicating that JNK and/or p38-MAPK act as upstream regulators in Bax-mediated mitochondria-dependent apoptosis.[38,43] The present study shows for the first time that ar-turmerone causes the activation of ERK and JNK, but not p38-MAPK, in HepG2 cells (Figure 6A). Pharmacological inhibition of ERK and JNK drastically attenuated ar-turmerone-induced expression of PUMA, Bax, Fas, DR4, and active caspase-3, as well as HepG2 cell apoptosis (Figure 6B,C). Our observations reveal that ERK and JNK act as upstream regulators of PUMA, Bax, Fas, and DR4 in ar-turmerone-induced cell death.

In conclusion, our findings show that ar-turmerone purified by supercritical carbon dioxide extraction and liquid−solid chromatography isolation from the root of *C. longa* can provoke intracellular ROS generation, which subsequently induces ERK and JNK activation and increases PUMA, Bax, Fas, and DR4 expression. Up-regulation of Fas and DR4 by ar-turmerone elicits the extrinsic apoptotic pathway by activating caspase-8 and caspase-3; moreover, increasing PUMA and Bax, as well as decreasing Bcl-2 and Bcl-xL, initiate the intrinsic apoptotic pathway by promoting the release of cytochrome *c* and the subsequent activation of caspase-9 and caspase-3 (Figure 8). Our study clearly establishes an underlying mechanism for ar-turmerone-mediated cell death in human hepatocellular carcinoma HepG2 cells; these observations might reveal the molecular basis of ar-turmerone-induced anticancer action.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

ROS, reactive oxygen species; PUMA, p53 up-regulated modulator of apoptosis; DR, death receptor of TRAIL; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-related kinase; HLPC, high-performance liquid chromatography; IC_{50}, 50% inhibitory concentration; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; PARP, poly ADP-ribose polymerase; MPTP, membrane permeability transition pore; DCF-DA, dichlorofluorescein diacetate; HE, dihydroethidine; NAC, *N*-acetylcysteine; ASC, ascorbic acid; MAPK, mitogen-associated protein kinase; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand.

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