

## RESEARCH COMMUNICATION

# Curcumin Induces Apoptosis Involving bax/bcl-2 in Human Hepatoma SMMC-7721 Cells

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### Abstract

Curcumin is a major active component of *Curcuma aromatica* salisb, which has been shown to inhibit proliferation of a wide variety of tumor cells. In this study, the molecular mechanisms of curcumin inducing apoptosis in human hepatoma SMMC-7721 cells were examined. We find that curcumin inhibits the growth of SMMC-7721 cells significantly in a concentration-dependent manner, with typical apoptotic morphological changes of cellular nuclei. Annexin-V/PI double staining detected by flow cytometry and expression of the relative apoptotic proteins (Bax, Bcl-2 and caspase-3) revealed a strong apoptosis-inducing competent of curcumin in SMMC-7721 cells. Curcumin increased the expression of bax protein while decreasing that of bcl-2 protein significantly. The results suggest that curcumin induction of apoptosis involves modulation of bax/bcl-2 in SMMC-7721 cells and provide a molecular basis for the development of naturally compounds as novel anticancer agents for human hepatomas.

**Keywords:** Curcumin - human hepatoma SMMC-7721 cells - apoptosis - bcl-2 - bax

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### Introduction

Hepatocellular carcinoma (HCC) is a frequent malignancy worldwide with high prevalence and transitivity (Jia et al., 2007). Although surgical operations or non-surgical therapeutic modalities have been employed, treatment for HCC is rarely curative and shows low rates of survival. Besides, the crowd ill with hepatitis and cirrhosis have a higher potential risk of developing symptoms of HCC. Therefore, it is urgent to develop more effective drugs of this cancer.

Apoptosis is a physiological process responsible for the removal of cells that have already completed their specific functions, or are harmful to the growth and development of organism. Two typical pathways leading to apoptosis have been identified, named the extrinsic and the intrinsic pathway (Ziegler and Kung, 2008). The extrinsic pathway is initiated through the interaction of ligands and surface receptors (Klein et al., 2005), such as tumor necrosis factor receptor (TNFR) and death receptors (DR). The surface receptors deliver a death signal from the extracellular microenvironment to the cytoplasm before leading to apoptosis. The intrinsic pathway is initiated by stimuli such as cytotoxic drugs, DNA damage and cellular distress. The apoptotic signals are transmitted dependent on mitochondria and Bcl-2 protein family (Green, 2005). Cell apoptosis plays a central role in controlling cell proliferation and hence is pivotal to the prevention of tumor development (Ghobrial et al., 2005). The understanding of apoptosis process has provided

the theoretical basis for novel, effective therapies, such as selectively inducing cell death in carcinoma cell lines, and enhancing the cytotoxic effects of established chemotherapeutic agents in cancer cells (Pettersson et al., 2001).

Resistance to apoptosis of cancer cells is an inherent part of the carcinogenic process and is also associated with resistance to chemotherapeutic drugs (Johnstone et al., 2002). Therefore, new drugs are required to improve efficacy to cancer cells without toxicity to normal cells. Based on these goals of treatment for cancer, recent attention has focused on phytochemical as anticancer agents. Curcumin, also known as turmeric yellow or diferuloylmethane, is a phenol derived from the herb *Curcuma aromatica* salisb (Adhikari et al., 2007). In the past 10 years, several studies in vitro and in vivo have reported that curcumin possesses significant pharmacological actions, including antioxidant, anti-inflammatory, anti-angiogenic, anti-proliferative and wound healing (Sharma et al., 2005), without cytotoxic effects on healthy cells (Syng-Ai et al., 2004). Curcumin has also shown noticeable chemopreventive efficacy against various malignancies. Several studies have suggested that curcumin induced apoptotic cell death in some malignant cells (Bakhshi et al., 2008; Cheng et al., 2010).

The broad range of activities of curcumin has been attributed to the ability affecting multiple targets of curcumin. However, how curcumin actually achieves this broad-spectrum activity is unknown. In this study, we have

investigated whether curcumin could induce apoptosis in human hepatoma SMMC-7721 cells for the first time. Our results suggest that the potent anticancer activity of curcumin is involved with the concerted modulation of the expression of two key proteins, bax/bcl-2, which are critical to the apoptosis induction through intrinsic pathway.

## Materials and Methods

### Reagents

Curcumin (C<sub>21</sub>H<sub>20</sub>O<sub>6</sub>, (1E,6E)-1,7-bis (4-hydroxy-3-methoxyphenyl) -1,6-hepta-diene-3,5-dione, purity ≥95%) (Figure 1) was extracted from *Curcuma aromatica salisb* according to the protocols reported previously (Kim et al., 2005). In the experiments, the stock solution of curcumin were dissolved with dimethyl sulfoxide (DMSO, equivalent to <1% of the final volume), and stored at -20°C protected from light.

### Cell Culture

Human hepatoma cell line SMMC-7721 cells were purchased from Cell Bank of Shanghai Institute of Biochemistry & Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI-1640 medium (GIBCO, Invitrogen, Inc.), and supplemented with 10% heat-inactivated fetal bovine serum (Sijiqing, Hangzhou, China), 100 U/ml penicillin G, and 100 µg/ml streptomycin at 37 °C with 5% CO<sub>2</sub>.

### Cell Growth Inhibition

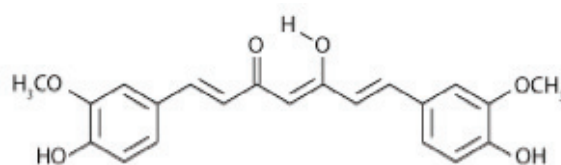
Logarithmic SMMC-7721 cells were detached to prepare 1.0 × 10<sup>4</sup> /ml cell suspension, and partitioned into 96-well plates at 100 µl/well for 24 h at 37 °C. Then cells were treated with curcumin at different concentrations (5 µM ~ 75 µM) at 100 µl/well. After 24 h incubation, 5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich, St. Louis, Mo) solution (20 µl/well) was added and cultured for another 4 h. Then the supernatant was discarded and DMSO was added (100 µl/well). The suspension was placed on micro-vibrator for 5 min and the absorbance (A) was measured at 570 nm by the Universal Microplate Reader (EL800, BIO-TEK instruments, Inc.). The inhibitory ratio was calculated by the following formula:

$$\text{Inhibitory ratio}\% = \left( \frac{A_{\text{control}} - A_{\text{treated}}}{A_{\text{control}}} \right) \times 100\%$$

$A_{\text{treated}}$  and  $A_{\text{control}}$  were the average absorbance of three parallel experiments from the treated and blank control groups, respectively. The results were presented as mean ± SD.

### Cell Morphological Assessment

Human hepatoma cell line SMMC-7721 cells were seeded in 6-well tissue culture plates at a concentration of 1 × 10<sup>5</sup> cells/well and treated with curcumin (10, 20 and 40 µM) for 24 h. At the end of incubation, the morphology of cells was monitored under an inverted light microscope. All floating and attached cells were harvested with 0.02% (w/v) EDTA and 0.25% (w/v) trypsinase. Then the cell



**Figure 1. Molecular Structure of Curcumin (C<sub>21</sub>H<sub>20</sub>O<sub>6</sub>, (1E,6E)-1,7-bis (4-hydroxy-3-methoxyphenyl) -1,6-hepta-diene-3,5-dione, MW: 368)**

suspension was fixed with icecold 4% paraform for 20 min, permeabilized with 0.1% Triton X-100 for 25 min and washed with ice-cold PBS (2000 rpm × 5 min). At last the cells were stained with fluorochrome dye DAPI (1 µg/ml) (4',6-diamidino-2-phenylindole; Santa Cruz, CA) for 25 min and observed under a fluorescence microscope (Olympus IX51, Japan) with a peak excitation wave length of 340 nm.

### Annexin-V/PI double-staining assay

1 × 10<sup>6</sup> cells were seeded in 6-well tissue culture plates and incubated for 24 h at 37 °C. Curcumin (10, 20 and 40 µM) was directly added into the 6-well plates and incubated with cells for an additional 24 h. Then cells were harvested and resuspended with PBS. Apoptotic cells were identified by FITC-conjugated Annexin-V/PI double supravital staining, according to the manufacturer's instructions of the Annexin V-FITC Apoptosis Detection kit (KeyGen, Nanjing, China). Apoptotic cell death was examined by FACSCalibur flow cytometry (Becton Dickinson, San Jose, CA).

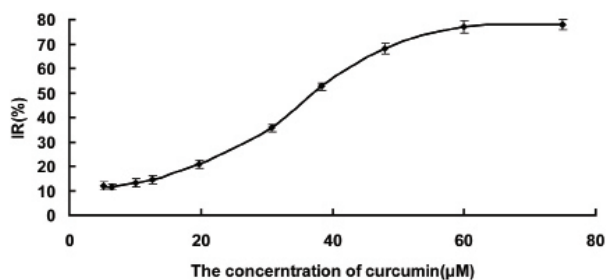
### Western-blotting for Bax, Bcl-2 and caspase-3 proteins

After SMMC-7721 cells were incubated with curcumin (10, 20 and 40 µM) for 24 h, the total proteins in cells were lysed and isolated by lysis buffer (100 mM Tris-Cl, pH 6.8, 4% (m/v) SDS, 20% (v/v) glycerol, 200 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 1 g/ml aprotinin). The concentration of proteins was measured using the BCA assay method with Varioskan spectrofluorometer and spectrophotometer (Thermo, Waltham, MA) at 562 nm.

Protein samples were separated with 10% SDS-polyacrylamide gel (SDS-PAGE) and transferred onto the PVDF membranes (Millipore, Billerica, MA). Immune complexes were formed by incubation of albumin from bovine serum (BAS, Sigma-Aldrich, St. Louis, Mo) for 2 h, and then the proteins with primary antibodies, rabbit anti-caspase 3, rabbit anti-Bax, rabbit anti-Bcl-2, and mouse anti-actin (Santa Cruz, CA, diluted 1:500) overnight at 4°C. Blots were washed and incubated for 1 hour with IRDye™800 conjugated anti-mouse and anti-rabbit second antibodies (Santa Cruz, CA diluted 1:15000). Immunoreactive protein bands were detected with an Odyssey Scanning System (LI-COR Inc., Superior St., Lincoln, NE).

### Statistical Evaluation

All results shown represent the means ± SD from triplicate experiments performed in a three parallel manner unless otherwise indicated. Statistical analyses were



**Figure 2. Inhibitory Effects of Curcumin on the Growth of SMMC-7721 Cells for 24 h by MTT Assay.** The results are the mean of three parallel experiments for each concentration point. Data were shown as means ± SD.

performed using an unpaired, two-tailed Student’s t-test. All comparisons are made relative to untreated controls and significance of difference is indicated as \*P < 0.05 and \*\*P < 0.01.

**Results**

*Curcumin inhibits the growth of SMMC-7721 cells*

Human hepatoma SMMC-7721 cells were cultured in the absence and presence of varying concentrations of curcumin for 24 h. The effects of curcumin on cell growth were assessed by MTT assay. No significant growth inhibition was observed when curcumin was applied at concentrations lower than 10 μM (data not shown). However, potent cytotoxicity against the growth of SMMC-7721 cells was observed when curcumin was upon higher concentrations (Figure 2). The IC50 value of curcumin against SMMC-7721 for 24 h was 35μM.

*Cell morphological assessment after curcumin treatment*

The morphological changes of cells by optical microscope after treatment with curcumin (10, 20 and 40 μM) for 24 h. Upon the treatment of curcumin, SMMC-7721 cells were severely distorted, grew slowly and some cells turned round in shape. The untreated cells displayed normal, healthy shape demonstrated by the clear skeletons (data not shown).

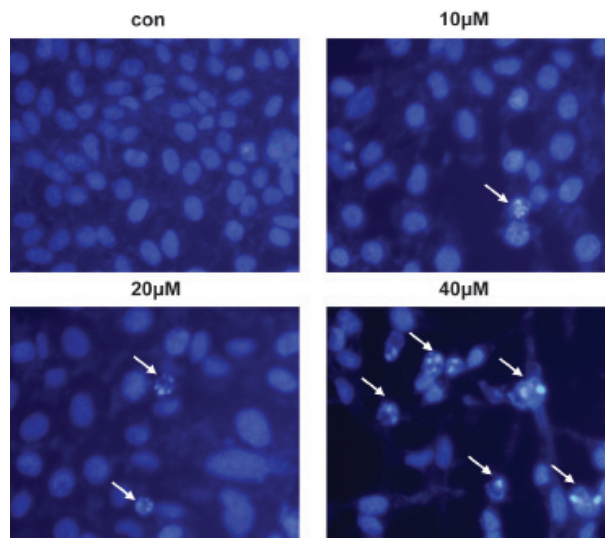
Under the fluorescent microscope, the nucleolus changes of SMMC-7721 were observed (Figure 3). Untreated SMMC-7721 cells were stained equally blue fluorescence which showed that the chromatin equally distributed in nucleolus. After curcumin treatment, the cells displayed chromatin congregated and nucleolus pyknosis, which emitting bright fluorescence, the early phenomena of apoptosis.

*Curcumin induces apoptosis of SMMC-7721 cells*

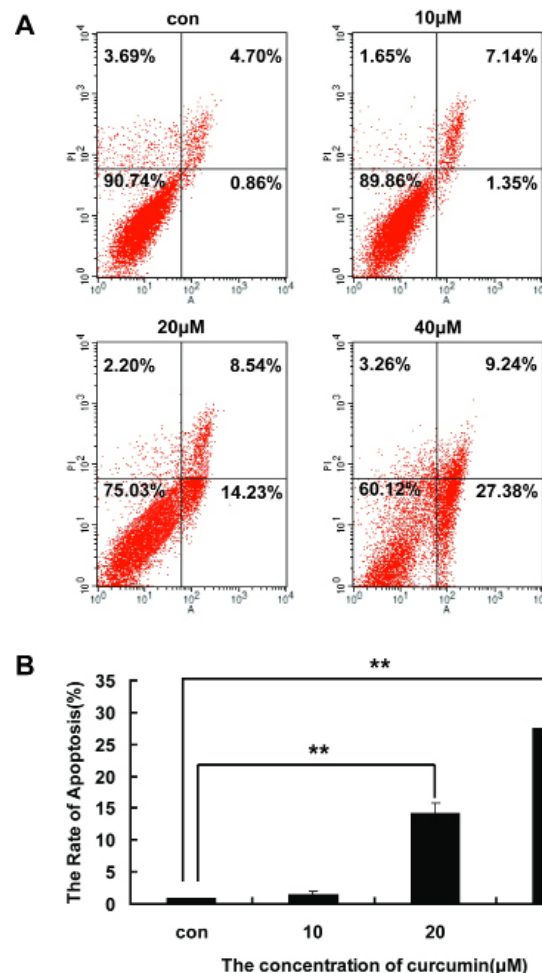
SMMC-7721 cells treated with 10 μM curcumin had no significant changes compared to the control group. However, the percentage of early apoptotic cells induced by 20 μM and 40 μM curcumin increased to 14.23% and 27.38%, separately (Figure 4). The percentage of late apoptotic cells treated with 20 μM and 40 μM increased less than 2%.

*Expression of Bax, Bcl-2 and caspase-3 proteins*

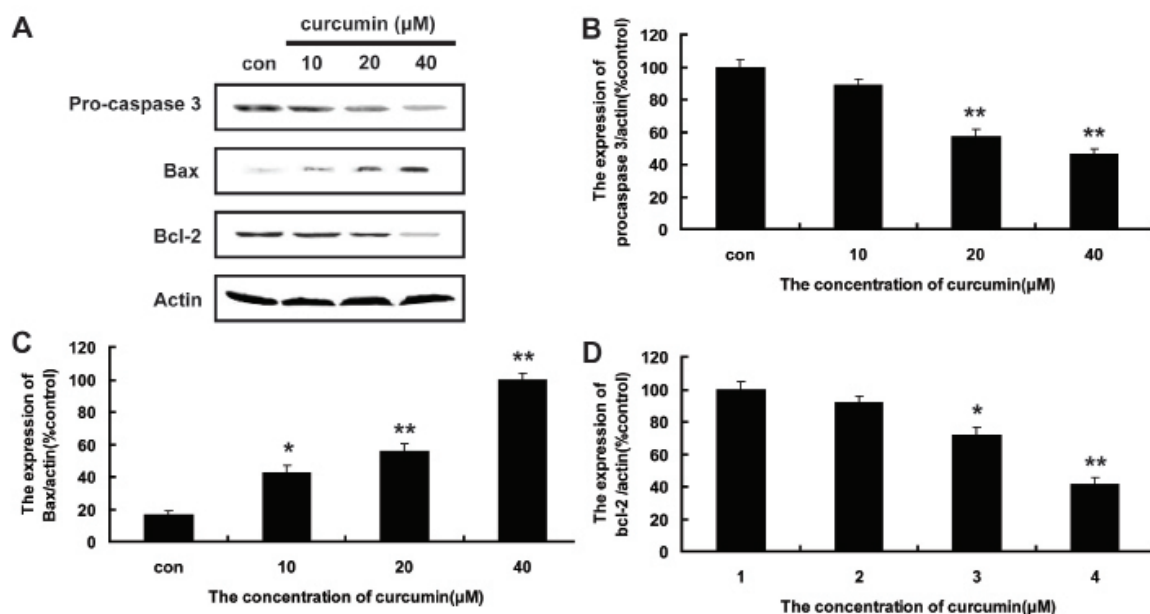
After incubation with curcumin (10, 20 and 40 μM) for 24 h, the expression of bcl-2 protein decreased



**Figure 3. Nucleolus Morphologic Changes by Curcumin under Fluorescent Microscopy.** Treatment (0, 10, 20 and 40 μM) for 24 h - white arrows indicate the apoptotic cells with nuclear fragments



**Figure 4. Apoptotic-inducing Effects of Curcumin by Annexin-V/PI Double-staining Assay.** (A) After SMMC-7721 cells were treated with curcumin (0, 10, 20 and 40 μM) for 24 h, collected cells were incubated with Annexin V and PI for 30 min at 37 °C and then subjected to flow cytometry analysis. (B) Histograms quantized the early apoptosis rates in SMMC-7721. Asterisks (\*P < 0.05 or \*\*P < 0.01) indicate significant difference compared with control



**Figure 5. Effect of Curcumin on the Expression of Procaspase 3, Bax and bcl-2 in SMMC-7721 Cells.** (A) After cells were treated with curcumin (0, 10, 20 and 40  $\mu\text{M}$ ) for 24 h, the expression of procaspase 3, Bax and bcl-2 proteins were detected by western blotting. (B-D) Densitometric analysis gave the relative ratios of procaspase 3, Bax and bcl-2 compared with control. Asterisks (\* $P < 0.05$  or \*\* $P < 0.01$ ) indicate significant difference compared with control

dramatically, whereas the expression of bax protein increased significantly in SMMC-7721 cells. The levels of bcl-2 and bax protein expression in SMMC-7721 were clearly correlated to the concentration of wogonin applied as shown in Figure 5B. The procaspase-3 cleaved to active form in SMMC-7721 cells after treatment with 20  $\mu\text{M}$  and 40  $\mu\text{M}$  curcumin (Figure 5A).

## Discussion

More and more attentions have been focused on phytochemical to search for new anticancer agents with more efficacy to cancer cells and less toxicity to normal cells. Curcumin, derived from the root of the plant *Curcuma longa*, which has been used as a dietary factor and as a herbal medicine with broad-spectrum activity for centuries in several south-eastern countries. Recently, curcumin has been demonstrated to induce cell cycle arrest and apoptosis in a wide variety of cancer cells (Wu et al., 2010; Wang et al., 2011). These suggest that curcumin is a competitive candidate as a novel anti-cancer agent. The proposed mechanisms of its anti-cancer properties include direct cytotoxicity (Jana et al., 2004), immune modulatory effects (Su et al., 2008) and normalization of carcinogen (Xu and Thornalley, 2001). However, the studies on apoptotic activity of curcumin are still limited and remain to be uncovered. In this study, we reported the involvement of bax/bcl-2 as the molecular mechanism of curcumin induced apoptosis in human hepatoma SMMC-7721 cells. Our results demonstrated that the curcumin could inhibit the growth of SMMC-7721 cells in a concentration dependent manner. The value of  $\text{IC}_{50}$  at 24 h was determined to be 35  $\mu\text{M}$ . SMMC-7721 cells treated with curcumin displayed typical apoptotic characteristics, including cytoplasmic shrinkage, plasma membrane blebbing, nuclear chromatin condensation, chromosomal DNA cleavage, and fragmentation of the

cells into membrane-enclosed vesicles or apoptotic bodies (Kerr et al., 1972).

Phosphatidylserine (PS), normally located in the inner wall of cellular plasma membrane, was translocated and exposed to the outer wall at the beginning of apoptosis, therefore providing a "molecular marker" on apoptotic cells (Martin et al., 1995). Annexin-V has a high affinity for PS and binds to cells with exposed PS. Use of Annexin-V in combination with PI allowed the distinction of early apoptotic and necrotic cells from viable cells. Our results revealed that obvious apoptosis of SMMC-7721 cells could be observed upon 20  $\mu\text{M}$  curcumin. The early apoptotic rate reached 27.4% after incubation with 40  $\mu\text{M}$  curcumin.

Apoptosis is a multiple factor-regulated process with two major pathways: the extrinsic pathway involved in death-receptor and the intrinsic pathway involved in mitochondria. Many chemotherapeutic agents have been shown to cause apoptosis through caspase dependent pathway (Shu et al., 2009). Caspase-3 is an executioner caspase, which upon activation can systematically demolish cells through cleaving PARP then lead to DNA fragmentation. Decreased procaspase-3 was observed in curcumin-treated SMMC-7721 cells. Further more, our studies showed that curcumin resulted in an obvious decrease in bcl-2 protein expression and a notable increase in bax protein production in SMMC-7721 cells. The Bcl-2 protein family include proapoptotic members such as bax, bak, bad, bcl-xs, bid, bik, bim, and hrk, and antiapoptotic members such as bcl-2, bcl-xl, bcl-w, bfl-1, and mcl-1 (Tamm et al., 2001). The pro-apoptotic proteins and anti-apoptotic proteins of the Bcl-2 protein family may turn on and off apoptosis because of the formation of heterodimers among these proteins (Kawai-Yamada et al., 2005). Interestingly, the effects of apoptosis induction are more dependent on the ratio between bcl-2 and bax than on the quantity of bcl-2 alone (Pettersson et al., 2002).

Two possibilities have been proposed for the observed relationship between bcl-2 and bax proteins: (i) Bcl-2 induced a pathway maintaining cell survival, with bax serving as a negative regulator of bcl-2; (ii) Bax directly or indirectly generated cell death signals, with bcl-2 being the dominant inhibitor of bax (Sato et al., 1994). Thus, the balance between the expression levels of the protein units bcl-2/bax is critical for cell survival or death. It has been reported that human colon cancer cells lacking Bax resist curcumin-induced apoptosis (Rashmi et al., 2005). Therefore, the high apoptosis rate of SMMC-7721 observed in this study is attributed to the ability of curcumin to lower the ratio of bcl-2/bax in SMMC-7721 cells. Therefore, the apoptotic effect of curcumin is achieved by its ability to modulate the concerted expression of bcl-2/bax proteins.

In conclusion, we have demonstrated that curcumin induces apoptosis in human hepatoma cell line SMMC-7721 cells involved with the modulation of bcl-2/bax protein expression. This finding provided a molecular basis for the development of naturally compounds as novel anticancer agents for HCC.

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