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# Redox modulation and human bile duct cancer inhibition by curcumin

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ABSTRACT

Curcumin, a major component from tumeric and well-known dietary spice, possesses various pharmacological effects. The cancer chemoprevention effect is suggested to act through its pro-oxidant property. The study was to clarify effects of curcumin on cholangiocarcinoma cells, a cancer of the bile duct that refractory to chemotherapeutic drugs. We examined time-course of oxidant formation in relation to antitumor and the adaptive antioxidant response of the cells. Curcumin induced antiproliferation and apoptosis in KKU-M214 CCA cells with concentration- and time- dependent manners. The antiproliferative effect of curcumin was observed at concentrations as low as 3 μM and was not necessarily associated with oxidative stress, while induction of apoptosis required significant production of superoxide anion, suppression of cellular redox and collapse of mitochondrial transmembrane potential. Western blot analysis showed a temporal relationship between the suppression of nuclear NF-κB with Bcl-XL protein levels. Up-regulation of p53 and Bax was associated with marked oxidative stress and apoptosis. Curcumin also induced Nrf2 protein expression with up-regulation of γ-glutamylcysteine ligase mRNA and increased cellular antioxidant, glutathione. The study suggests that curcumin could be developed into an effective chemoprevention against CCA.



## 1. Introduction

Epidemiological and animal studies have shown that phytochemicals present in several diet may be effective for the prevention and treatment of malignancy. Cancer chemoprevention and antitumor activity of various phytochemicals, including curcumin, tea catechin or isothiocyanates may be associated with anti-inflammatory and cytoprotective effects in normal cells, and induction of cell cycle arrest and apoptotic cell death in transformed cells (Nair et al., 2007; Surh, 2003). In particular, curcumin, a product of spice turmeric, *Curcuma longa* Linn., has significant antioxidant, anti-inflammatory, cytoprotective, and antitumor activities *in vitro* and in some preclinical animal models (Barve et al., 2008; Sandur et al., 2007; Sharma et al., 2005; Sompamit et al., 2009). The safety of curcumin is well recognized by the fact that several populations in the world consume curcumin as a dietary spice for centuries (Aggarwal and Sung, 2009; Sharma et al., 2005). Curcumin has been shown to inhibit the proliferation of a wide variety of tumor cells, including non-small cell lung carcinoma cells (Shishodia et al., 2003), mammary epithelial carcinoma cells (Choudhuri et al., 2005), colon adenocarcinoma cells (Rashmi et al., 2005), and pancreatic carcinoma cells (Lee et al., 2005). The antitumor effects have been suggested to depend partly on the suppression of the nuclear factor-kappa B (NF-κB) signaling pathway (Aggarwal and Sung, 2009; Surh, 2003), whereas its downstream genes are ultimately involved with several critical processes leading to carcinogenesis, including proinflammation, proliferation, angiogenesis, antiapoptosis and drug resistance (Aggarwal and Sung, 2009). The target genes down-regulated by curcumin include cyclooxygenase-2, cyclin-D1, c-myc, IAP, Bcl-2, Bcl-XL, VEGF, MMP-9, and ICAM-1. These down-regulated NF-κB gene products of curcumin and other plant polyphenols may lead to the chemosensitizing and radiosensitizing effects (Garg et al., 2005). Curcumin can induce apoptosis through modulation of the mitochondrial permeability transition causing the release of AIF, cytochrome C and finally caspase 3 activation (Thayyullathil et al., 2008). The tumor suppressor p53 protein plays an important

*Abbreviations:* $\triangle\Psi_m$, mitochondrial transmembrane potential; CCA, cholangiocarcinoma; GCL, γ-glutamylcysteine ligase; GSH, reduced glutathione; GSSG, glutathione disulfide; Nrf2, nuclear factor-E2-related factor 2; nuclear factor-kappa B, NF-κB; ROS, reactive oxygen species.

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role in the induction of cell cycle arrest and apoptosis by transactivation-dependent and -independent effects (Vaseva and Moll, 2009). On the other hand, oxidative stress also activates the nuclear factor-E2-related factor 2 (Nrf2), then binds with the cis-acting antioxidant response element (ARE) and modulates downstream antioxidant genes, such as γ-glutamylcysteine ligase (GCL), glutathione transferases, and heme oxygenase-1 (Kensler et al., 2007; Lu, 2009). This adaptive antioxidant response may be implicated in cancer resistance (Chen et al., 2008; Wang et al., 2008). Previous study showed that curcumin induced increased cellular GSH levels (Sandur et al., 2007) and this may be related to antioxidative stress response.

How curcumin mediated anti-inflammatory and proapoptotic effects is the subject of intense research. Curcumin has been suggested to act through its pro-oxidant/ antioxidant effects because formation of reactive oxygen species by curcumin and curcumin derivatives correlates with their apoptotic activity on tumor cells (Mishra et al., 2005; Syng-Ai et al., 2004). The induction of reactive oxygen species leads to change in cellular GSH, the main redox buffer, which may, in turn, modulates several redox-sensitive enzymes and transcription factors leading to growth suppression, apoptotic cell death (Mishra et al., 2005; Syng-Ai et al., 2004). The antiproliferation and induction of apoptosis are usually supposed to be resulted from a similar insult, i.e. oxidative stress. This may not be always accurate. The roles of ROS and redox status in cancer inhibition are needed clarification. Moreover, several of curcumin's effects may depend on cell type; for instance, curcumin has high efficacy in cells with high constitutive NF-κB activity and low expression of Bcl-XL (Aggarwal and Sung, 2009; Jang and Surh, 2004).

Cholangiocarcinoma (CCA), a malignant tumor of the biliary tract, presents a formidable difficulty in diagnosis and treatment (Blechacz and Gores, 2008; Khan et al., 2002). The worldwide incidence and mortality rates of intrahepatic CCA are increasing (Khan et al., 2002). Current adjuvant or palliative chemotherapy has not been shown to substantially improve survival in patients (Blechacz and Gores, 2008; Khan et al., 2005; Lazaridis and Gores, 2005). There is a need to improve chemotherapy to circumvent drug resistance in CCA because it is evidently involved in the expression of multi-drug resistance genes as well as the up-regulation of antiapoptotic genes and the down-regulation of proapoptotic genes (Blechacz and Gores, 2008).

In this study, the effects of curcumin on the induction of antiproliferation, apoptosis and the adaptive antioxidant response in CCA cells were analyzed on time-course association with pro-oxidant activity and modulation of redox-sensitive transcription factors and enzymes to establish characteristics of curcumin induced tumor inhibition.

## 2. Materials and methods

### 2.1. Cell culture and cytotoxicity assay

Cultured cell line, KKU-M214, established in our institute by Dr. B. Sripa of Department of Pathology, Faculty of Medicine, was derived from human intrahepatic cholangiocarcinoma tissues. The CCA cells were routinely cultured in Ham's F12 media supplemented with 4 mM L-glutamine, 1 mM sodium pyruvate, 12.5 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES; pH 7.3), 100 U/mL penicillin, 100 μg/mL streptomycin sulfate, and 10% fetal calf serum as previously described (Buranrat et al., 2007). The media was renewed every 3 days, trypsinized with 0.25% trypsin–EDTA, and subcultured in the same media.

KKU-M214 cells were seeded onto 96-well culture plates at a density of $5 \times 10^3$ cells/well. After an overnight culture, the media was renewed with an addition of curcumin, and the cells were cultured for another 48 h. Cytotoxicity was determined by fluorescent dye staining. Briefly, the cells were washed and incubated in phosphate-buffered saline (PBS) with an addition of 4 μL acridine orange and ethidium bromide (1 μg/mL, each). The cells were examined using a Nikon Eclipse TS100 inverted microscope with 480 nm and 535 nm excitation and emission filters, respectively. The microphotographs were taken of three predetermined areas per well with triplicate wells per concentration using a Nikon Coolpix digital camera, and the number of viable, apoptotic, and necrotic cells were counted. The viable cells were colored green with intact nuclei. Necrotic cells were stained bright orange, and apoptotic cells were stained green with an appearance of cell shrinkage, condensation, and nuclei fragmentation. The number of viable, apoptotic and necrotic cells were counted. The cytotoxicity of curcumin was calculated as percent viable cells in the treatment against the control (no curcumin) at the same period of times.

### 2.2. Determination of superoxide formation and glutathione

The KKU-M214 cells were cultured in 35-mm dishes for overnight, after which the cell cultures were washed and replaced with fresh media supplemented with curcumin. At various times, the cultures were washed with PBS and superoxide formation were counted using the lucigenin-enhanced chemiluminescent method with a luminometer (Model 20/20n, Turner Biosystem) as described previously (Prawan et al., 2009).

The total glutathione assay was performed according to Tietze's methods (Tietze, 1969). Glutathione disulfide (GSSG) was assayed by a previously described method (Somparn et al., 2007) using 1-methyl-2-vinylpyridinium triflate (M2VP) as a glutathione scavenger. The cell cultures were trypsinized, washed three times with cold PBS, centrifuged at 1500g at 4 °C for 10 min, and resuspended in PBS buffer. A 100 μL aliquot of the cell suspension was reacted with M2VP (33 mmol/L in water) or water. The cell suspensions were mixed gently and stored at −20 °C until analysis. An aliquot of the cell suspension was saved for protein determination using Bradford's dye binding assay. To determine the efflux of glutathione from cultured cells, cultured media was collected after incubation of the cultured cells at the indicated times to analyze GSH and GSSG as described above.

### 2.3. RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

The KKU-M214 cells were seeded in 6-well multi-dishes and incubated overnight. The cultured cells were exposed to curcumin for 9 h. Total RNA was extracted using Trizol LS reagent following the manufacturer's instructions. The subsequent procedures followed our previously described method (Buranrat et al., 2007). The total RNA (1 μg) was reverse transcribed in 20 μL containing 0.5 μg oligo(dT)15 primer, 20 units RNasin ribonuclease inhibitor and 200 units Improm-II reverse transcriptase (Promega, Madison, WI) in 5× PCR buffer, 3 mM $MgCl_2$, and 1 mM dNTP. First strand cDNA was synthesized in a thermal cycler at 42 °C for 60 min. The cDNA products were used as a template for PCR. PCR amplification was performed using specific primers for the γ-glutamylcysteine ligase catalytic subunit (GCLC) and β-actin, which was used as an internal control. The PCR primer sequences were as follows: GCLC forward primer, 5′-ATC ATC AAT GGG AAG GAA GGT-3′; GCLC reverse primer 5′-GTC ATT AGT TCT CCA GAT GCT C-3′, GenBank accession number NM_001498; β-actin forward primer, 5′-TGC CAT CCT AAA AGC CAC-3′; β-actin reverse primer, 5′-TCA ACT GGT CTC AAG TCA GTG-3′, GenBank accession number NM_001101. PCR was performed in a final volume of 20 μL containing cDNA template, 0.25 μM of each GCLC and β-actin primer, 0.5 U Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA), 2.5 mM $MgCl_2$, and 0.125 mM dNTP using a Px2 Thermal Cycle (Thermo Electron, Milford, MA). After an initial denaturing step of 95 °C for 5 min, 32 PCR cycles were performed as follows: denaturing for 1 min at 95 °C, annealing for 1 min at 52 °C, and extension for 1 min at 72 °C. A final extension was performed at 72 °C for 5 min. The PCR products were electrophoretically separated on a 2% agarose gel containing ethidium bromide. A negative control was included in every experimental run, where it always showed no amplication product. The gels were visualized and photographed. Band intensity was analyzed using Gel-Pro3 software. The relative amount of GCLC mRNA was expressed in relation to β-actin mRNA.

### 2.4. Measurement of mitochondrial transmembrane potential

To measure the change in mitochondrial transmembrane potential ($\Delta\Psi_m$), the lipophilic cation fluorescent dye JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolcarbocyanine iodide) was used. After treatment with curcumin at varied concentrations for defined period of times, KKU-M214 cells in 96-well plates were loaded with JC-1 (Clayman Chemical, Ann Arbor, Michigan) by incubation for 30 min at 37 °C. After that cultured cells were rinsed, incubated in JC-1 assay buffer and mitochondrial membrane potential was analyzed by a Gemini XPS fluorescent plate reader (Molecular Devices). In healthy mitochondria, JC-1 forms J-aggregates which display strong fluorescent intensity with excitation and emission at 560 and 595 nm, respectively. In depolarized mitochondria, JC-1 exists as monomers which show strong fluorescence with excitation and emission at 485 and 535 nm, respectively. The shift down in ratio of fluorescent intensity of JC-1 aggregates to fluorescent intensity of monomers is used as an indicator of depolarization of $\Delta\Psi_m$.

### 2.5. Western blot analysis of whole cell and nuclear protein extracts

Whole cell lysates and nuclear proteins were prepared as previously described (Jang and Surh, 2004). Treated KKU-M214 cells were washed with PBS, collected, and lysed with cell lysis buffer (20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM

$Na_2EDTA$, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM $Na_3VO_4$, 1 μg/mL leupeptin, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride) at 4 °C with vigorous shaking. Following centrifugation at 10,000 g for 15 min, the supernatant was collected and stored at -80 °C until use. Nuclear protein was prepared by lysing cultured cells with hypotonic buffer A (10 mM HEPES-KOH (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF, and 0.1 mM EGTA) and incubated in an ice bath for 15 min. The lysates were centrifuged at 12,000*g* at 4 °C for 15 min and the nuclear pellet was resuspended in ice-cold buffer B (20 mM HEPES-KOH (pH 7.9), 25% glycerol, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, and 1 mM EGTA), followed by incubation at 0 °C for 45 min. After vortexing, the suspension was centrifuged at 12,000*g* at 4 °C for 30 min. The supernatant containing nuclear proteins was stored at −80 °C until Western immunoblot analysis of NF-κB. The protein samples were mixed with 5× loading dye buffer, heated to 95 °C for 5 min, and the protein was electrophoretically separated on 10% SDS–polyacrylamide gel. The proteins were transferred to polyvinylidene difluoride membranes at 50 V for 2 h. The PVDF membranes were blocked for 1 h at room temperature with 5% (w/v) skimmed milk powder in Tris buffered saline (TBS) containing 0.1% Tween-20. The PVDF membrane was incubated overnight at 4 °C with primary antibodies including rabbit polyclonal IgG against the NF-κB p65 subunit (sc-109, Santa Cruz Biotechnology), diluted 1:500, mouse monoclonal IgG against Bcl-XL (sc-8392), diluted 1:1000, rabbit polyclonal IgG against Bax (sc-493), diluted 1:2000, rabbit polyclonal IgG against cyclin-D1 (sc-718), diluted 1:1500, mouse monoclonal IgG1 against β-actin (sc-8432), diluted 1:2500, rabbit polyclonal IgG against Nrf2 (sc-13032), diluted 1:500, or mouse monoclonal IgG1 against p53 (sc-98), diluted 1:1000 in PBS. The primary antibody was removed and the blots were extensively washed with TBS/Tween-20. The blots were incubated for 2 h at room temperature with 1:5000 dilution of respective horseradish peroxidase-conjugated secondary antibodies (goat anti-mouse or anti-rabbit IgG). After removal of the secondary antibody and TBS buffer washes, the blots were incubated with ECL substrate solution (Supersignal® West Pico Chemiluminescent Substrate). The densities of the specific NF-κB, Bcl-XL, Bax, cyclin D1, and β-actin bands were visualized and captured by Imagequant 400 (GE Healthcare).

### 2.6. Statistical analysis

Data are presented as mean ± SEM. The student's *t*-test and analysis of variance were used to determine significant differences between each experimental group. The ranksum test was also performed for non-parametric test. The level of significance was set at $P < 0.05$.

## 3. Results

### 3.1. Cytotoxicity of curcumin in CCA cells

KKU-M214 cells were tested against curcumin at varied concentrations. The IC50 values for cytotoxicity of curcumin was 5.9 ± 2.6 μM. The antiproliferative effect of curcumin is shown in Fig. 1A. At concentrations as low as 3 μM curcumin, the inhibition of cell proliferation was apparent at 9 h; at higher concentrations, marked growth suppression was observed as early as 3 h (Fig. 1A). An induction of apoptotic cell death was demonstrated at concentrations ⩾10 μM (Fig. 1B), whereas curcumin at the highest concentration (100 μM) induced marked apoptosis within 9 h. This apoptosis declined after 9 h with an appearance of necrotic cell death (Fig. 1C). The induction of necrotic cell death was only observed at very high curcumin concentrations (Fig. 1C).

### 3.2. Reactive oxygen species formation

Curcumin has been suggested to generate reactive oxygen species (ROS) and, in turn, ROS may be responsible for mediating various cellular responses. When KKU-M214 cells were incubated with curcumin, the release of superoxide anion was detected in a dose-dependent and time-dependent manner (Fig. 2). A high concentration of curcumin (30 μM) induced a remarkable production of superoxide, whereas lower concentrations stimulated much lower levels of ROS. The increased formation of superoxide was apparent at 24 h and related to the time-course of the induction of apoptotic cell death.

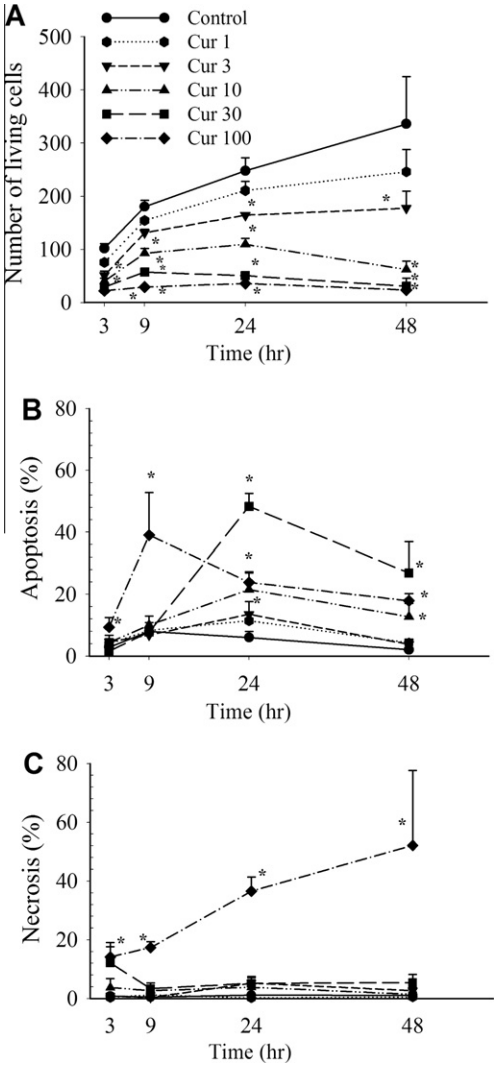


**Fig. 1.** The effects of curcumin on KKU-M214 cells. The cells were cultured with various concentrations of curcumin at 37 °C for 0–48 h. Cell cytotoxicity analysis was based on fluorescent staining, which was counted as the number of viable cells per the defined area (A), % apoptotic cells (B), and % necrotic cells (C). Data represent mean ± SEM from 3 experiments. *$P < 0.05$ compared to the control at the corresponding time point.

### 3.3. Cellular GSH formation, efflux of GSSG, and GCLC expression

Cellular GSH is a major antioxidant responsible for the modulation of various redox-sensitive enzymes and proteins. Curcumin (10 μM) increased total cellular GSH in KKU-M214 cells within 3–9 h; however, there was apparently no change when 30 μM curcumin was used (Fig. 3A and B). On the other hand, GSSG levels were unaltered with 10 μM curcumin, but gradually increased with time after incubation with 30 μM curcumin (Fig. 3C and D). To determine if there was change in the kinetics of cellular glutathione after exposure to curcumin, the medium samples from cell cultures were analyzed. Curcumin induced an efflux of GSH and GSSG, particularly at 30 μM of curcumin (Fig. 4A). The redox status in cell culture medium presented rapidly declined ratios of GSH and GSSG in a dose-dependent manner (Fig. 4B). The concentrations of total GSH and GSSG in fresh culture medium were very low (<0.1 μM).

The increased GSH amount, either intracellular GSH (cells treatment with 10 μM curcumin) or efflux GSH (cells treatment with 30 μM curcumin), may be due to the induction of synthesis of

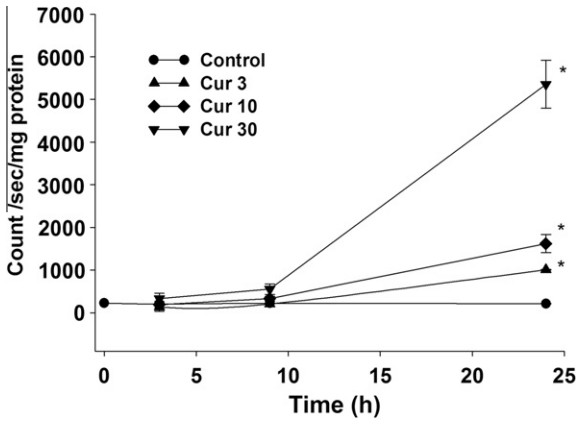


**Fig. 2.** Induction of reactive oxygen species formation in KKU-M214 cells. Cell cultures were incubated with varying concentrations of curcumin (3–10 μM) at the defined time periods. The release of superoxide anion was determined directly from cultured dishes by the lucigenin-enhanced chemiluminescence assay. Data represent mean ± SEM from 3 experiments. $^{*}P < 0.05$ compared to the control at the corresponding time point.

GSH by GCL. GCL is the rate limiting enzyme in *de novo* GSH synthesis. The GCL catalytic subunit (GCLC) was up-regulated within 9 h after incubation and in a dose-dependent manner with the curcumin treatment (Fig. 5).

### 3.4. Disruption of mitochondrial transmembrane potential

Mitochondrial dysfunction is recognized as a critical event in apoptotic cell death. The integrity of the inner mitochondrial membrane can be assessed by monitor of the potential gradient across the membrane using the fluorescent dye JC-1. The mitochondrial transmembrane potential was rapidly depolarized after treatment with curcumin, as shown by the decrease in the ratio of fluorescent intensity of JC-1 aggregates/JC-1 monomers. The decline in $\Delta\Psi_m$ was observed in KKU-M214 cells treated with curcumin as low as 3 μM at 6 h (Fig. 6). The depolarization of $\Delta\Psi_m$ was associated with antiproliferation which was observed at early time (3–9 h), where there were still no changes in superoxide formation and cellular GSH redox ratios. A large drop in $\Delta\Psi_m$ was observed at 24 h of incubation (Fig. 6) concurrently with a surge of superoxide release and apoptotic cell death.

### 3.5. Western analysis of NF-κB and proteins related to survival

Curcumin suppressed NF-κB expression in several cell types, and this may be associated with its several pharmacological activities. In the present study, the p65 subunit of NF-κB was constitutively expressed at the basal state in KKU-M214 cells. Curcumin significantly suppressed NF-κB at the first hour of incubation times (Fig. 7A). The suppression was evident within 30 min (data not shown), reached lowest at 1 h and was bounded back to its basal level within 9 h. The expression of Bcl-XL followed a similar time-course as NF-κB. However, cyclin D1 expression significantly increased and declined to baseline at 24 h and later. On the other hand, proapoptotic protein Bax gradually increased with time and reached a maximum at 9–24 h (Fig. 7B). The expression of Bax was associated with a pattern of antiproliferation and the induction of apoptosis.

The expression of Nrf2 protein, the transcription factor that regulates various antioxidant responsive genes, was relatively low at basal state and induced by curcumin in a concentration-dependent manner (Fig. 7C). Similarly, p53 which may be involved in the modulation of antiapoptotic and proapoptotic genes, had a tendency to increase expression following the treatment with curcumin for 24 h (Fig. 7C), whereas its baseline expression was very

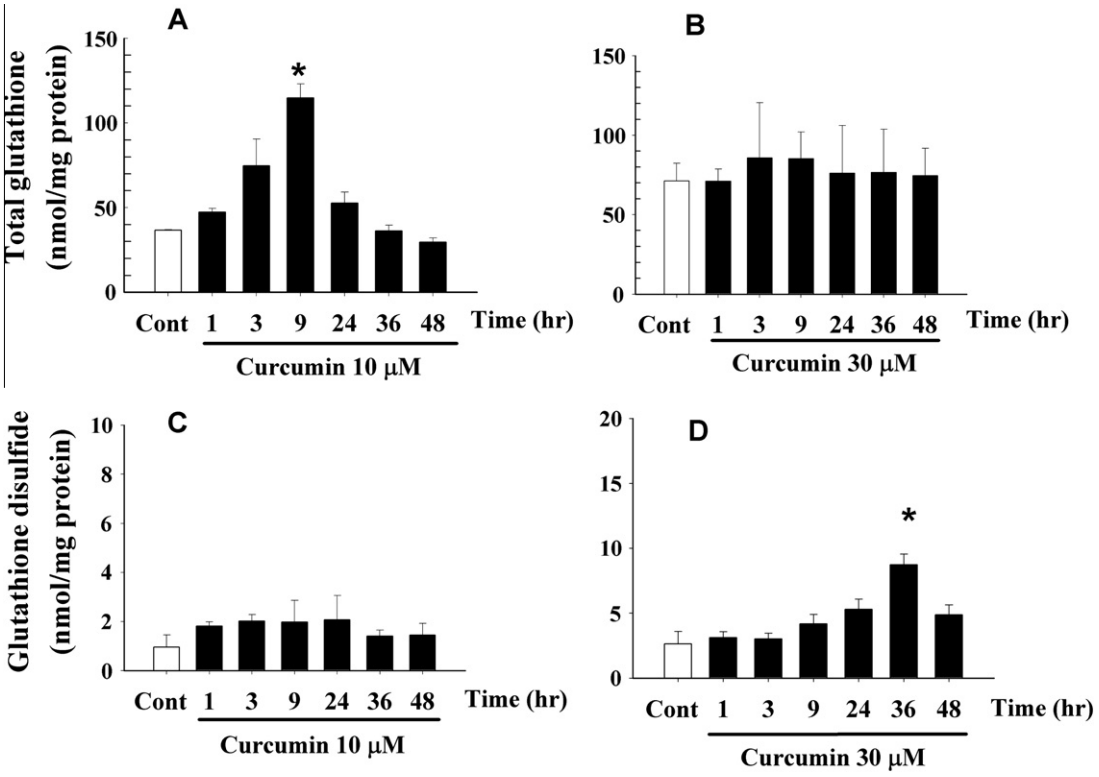


**Fig. 3.** The effect of curcumin on cellular GSH and GSSG levels. KKU-M214 cells were cultured with curcumin for 1–48 h. Cultured cells were removed from the dishes, washed, and aliquoted for the assay of GSSG, total GSH, and protein content. Time-course of changes in total GSH levels when cultured cells were treated with 10 μM (A) or 30 μM (B) curcumin and changes in glutathione disulfide when treated with 10 μM (C) or 30 μM (D) curcumin. Each bar represents mean ± SEM from 3 experiments. $^{*}P < 0.05$ compared with the control.

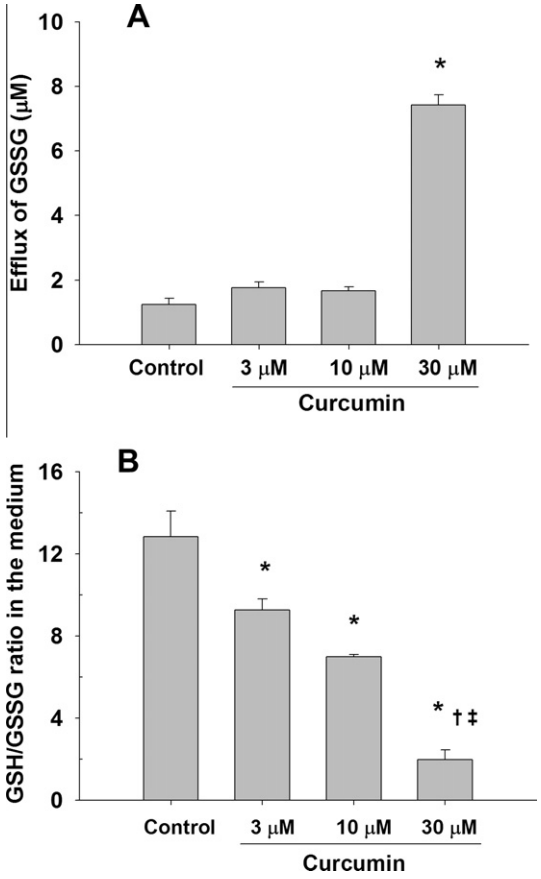


**Fig. 4.** The efflux of GSSG and the GSH redox balance in the medium of curcumin-treated KKU-M214 cells. The cultured KKU-M214 cells were treated with curcumin (3–30 μM) for 24 h and the cultured media were collected for the analysis of GSH and GSSG. Efflux of GSH and GSSG from cultured cells into the medium is presented as GSSG levels (A) and redox ratios of GSH/GSSG (B). Data represent mean ± SEM. *$P < 0.05$ compared to the control group, †$P < 0.05$ compared to 3 μM curcumin group, ‡$P < 0.05$ compared to 10 μM curcumin group.

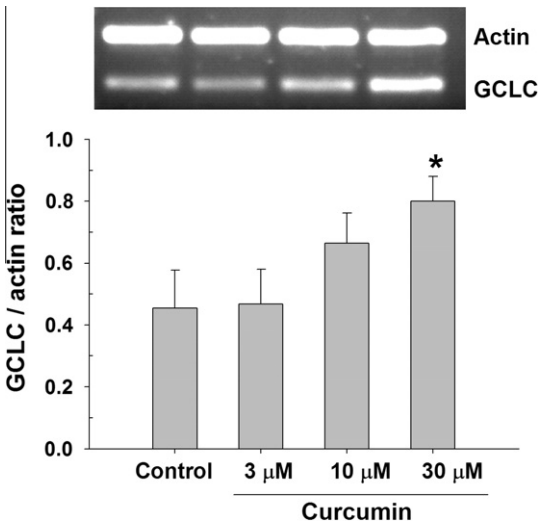


**Fig. 5.** The expression of GCLC mRNA induced by curcumin. KKU-M214 cells were cultured with various concentrations of curcumin (3–10 μM) for 9 h. The culture dishes were washed, scraped, and total RNA were extracted using Trizol reagent. Total RNA was used for the quantification of GCLC by reverse transcription PCR. Each bar represents mean ± SEM. *$P < 0.05$ compared to the control.

low. The induction of p53 protein was concentration-dependent and marked induction (at 10 μM) was associated with induction

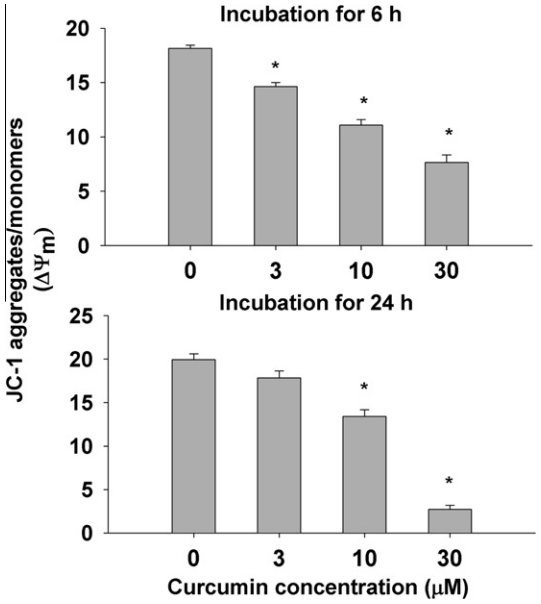


**Fig. 6.** Change in mitochondrial transmembrane potential following treatment with curcumin. KKU-M214 cells were exposed to curcumin (0–30 μM) for 6 or 24 h. The mitochondrial transmembrane potential was determined by incubation of the cells with fluorescent dye, JC-1, and $\Delta\Psi_m$ was assessed by using the ratio of fluorescent intensity of JC-1 aggregates/JC-1 monomers. Each bar represents mean ± SEM. *$P < 0.05$ compared to the control.

of apoptotic CCA cell death. Bax protein was relatively increased, but decreased at high concentration of curcumin.

## 4. Discussion

The chemoprevention of cancer may involve the inhibition of proliferation and the induction of apoptosis in malignant or premalignant cells. Although the aim of chemopreventive agents is to target cancer cells, some of the agents' effects may induce cellular defense mechanisms; for instance, they may induce antioxidant and xenobiotic detoxifying enzymes or inhibit xenobiotic activating enzymes in normal cells (Nair et al., 2007; Surh, 2003). Our study showed that the induction of cell death by curcumin is temporally related to the generation of ROS, declined cellular redox status, however, it is preceded by the loss of $\Delta\Psi_m$ and Bax protein expression. The modulation of NF-κB expression and its downstream genes were apparent early events and related to inhibition of cell proliferation. We also observed a coordinated adaptive oxidative stress response in KKU-M214 cells probably via the Nrf2-antioxidant response element (ARE) pathway with enhanced GCLC expression and GSH formation.

CCA is notorious for being insensitive to most chemotherapeutic agents (Blechacz and Gores, 2008; Lazaridis and Gores, 2005). In this study, curcumin potently induced cytotoxicity in KKU-M214 cells. KKU-M214 cells exhibited constitutive activity of NF-κB in the nuclear compartment, which may be associated with a high expression of Bcl-XL and cyclin D1 and confer resistance to chemotherapy and radiotherapy (Garg et al., 2005; Kunnumakkara et al., 2007). Suppression of the p65 subunit of NF-κB by curcumin may be associated with the inhibition of Bcl-XL and consequent cell growth suppression which was shown as early as within 3 h, although Bcl-XL levels were only transiently suppressed. Bax protein belongs to the BCL-2 family and functions as a critical proapoptotic factor (Chao and Korsmeyer, 1998). The up-regulation of Bax protein was observed at latter time periods (9–24 h) and temporally related to the times at which there was apoptotic cell death

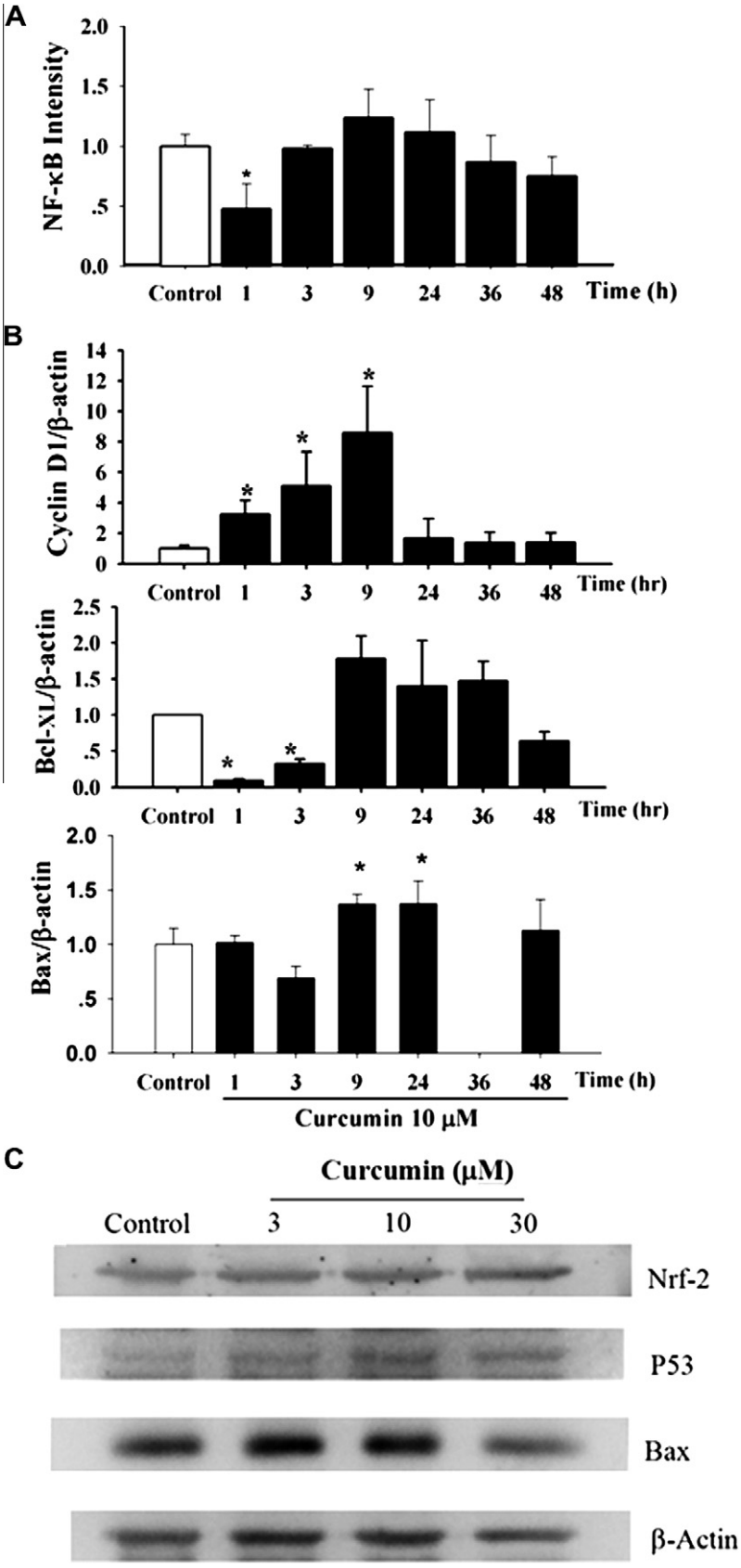


**Fig. 7.** Western blot analysis of nuclear NF-κB and the proapoptotic and antiapoptotic proteins from KKU-M214 cells treated with curcumin. KKU-M214 cells were cultured with curcumin at the indicated concentrations and indicated times. The treatment with curcumin was compared with corresponding controls (Control). (A) Time-course analysis of the NF-κB p65 subunit in the nuclear fraction. (B) Time-course analysis of Bcl-XL, Bax, cyclin D1, and β-actin in the whole cell lysate. Each bar represents mean ± SEM, $n = 3$ experiments. *$P < 0.05$ compared to the control. (C) Concentration response of Nrf2, p53, and Bax from whole cell lysates after treatment with curcumin (0–30 μM) for 24 h, the figure was representative from 2 experiments with similar results.

together with marked cell growth suppression. Several studies in human tumors and experimental animal models suggest an essential role of Bax, Bcl-XL and Bcl-2 in colon, gastric and cervical cancer cells, whereas up-regulation of Bax or down-regulation of Bcl-XL were associated with induction of apoptosis of tumor cells (Ionov et al., 2000; Singh and Singh, 2009). The up-regulation of Bax by curcumin may cause apoptotic cell death, which was observed predominantly at 24 h. Cyclin D1, a critical component of the cell cycle control mechanism, was induced at early time periods probably as an adaptive survival response of the cancer cells and down-regulated during the same period of time where there as a marked cell growth suppression.

Curcumin is a strong antioxidant both *in vitro* and *in vivo* (Sandur et al., 2007; Sharma et al., 2005; Sompamit et al., 2009), however it induces increased pro-oxidants, i.e. ROS and GSSG formation, in a concentration-dependent manner. The pro-oxidant activity may be primarily responsible for the proapoptotic effect of curcumin because the rate of ROS formation correlates with cytotoxicity. Recent reports have shown that the cytotoxic effect was abrogated by exogenous GSH to scavenge the oxidants (Mishra et al., 2005; Sandur et al., 2007). Our study shows a close relationship between the time-course of pro-oxidant formation (formation of ROS, cellular GSSG, and GSSG efflux), and disruption of $\Delta\Psi_m$ with the apoptotic effects. Despite the fact that we did not observe increased ROS release at the early period of time, an increased efflux of GSH and GSSG into the medium suggests that there is an oxidative change in intracellular compartment, and increased efflux GSSG from the cells is to reduce oxidant species inside the cells (Lu, 2009). It should be noted that intracellular redox of GSH could be well maintained despite the presence of curcumin up to 10 μM concentrations (Fig. 3A and C), whereas at the higher concentration (30 μM, Fig 3B and D), the cellular GSH redox maintenance together with $\Delta\Psi_m$ are collapsed and massive ROS is formed. Mitochondria are important intracellular organelles for energy production as well as cell death. A large change or collapse of $\Delta\Psi_m$ is an indicative of the MPT and opening of the permeability transition pore leading to cell death (Tsujimoto et al., 2006). It is conceivable that at the low concentrations of curcumin, oxidant species may be generated, however the cells are still able to maintain cellular redox partly by unloading the oxidants into the extracellular medium. As a result, the antiproliferative effect of curcumin at low concentrations (up to 10 μM) is apparent with modest sign of oxidative stress, while apoptotic and necrotic cell death probably require a significant disruption of cellular redox and $\Delta\Psi_m$. It is conceivable that despite antiproliferative and apoptosis effects are causally related to oxidant formation, antiproliferative effect of curcumin is probably a reversible process, while apoptosis involves an induction of an irreversible injury to the cell organelles. The important determinant between these two processes may be the concentration of and time-course of exposure to curcumin, for instances, 3 mM curcumin shows a strong antiproliferative effect, but no apoptosis (Fig. 1).

The tumor suppressor p53 protein has a major role in the cellular response to a wide and diverse range of stress signals. Oxidant induced by curcumin may play important role in induction of p53 expression observed in this study and it probably modulates mitochondrial membrane permeability transition (MPT) via interaction with BCL-2 family protein, leading to collapse of mitochondrial membrane potential. Our result is consistent with recent reports demonstrate that curcumin induced formation of ROS, increased expression of p53 and p21 and induced cell cycle arrest and apoptosis by release of apoptosis inducing factor (AIF) as well as cytochrome c from mitochondria (Thayyullathil et al., 2008). It is conceivable that mitochondria play key roles in mediation of curcumin effects. The depolarization of $\Delta\Psi_m$ was an early event observed in KKU-M214 cells, while the BCL-2 family proteins which controls mitochondrial outer membrane permeabilization (Vaseva and Moll, 2009), were revealed a dynamic change in Bcl-XL and Bax proteins. Changes in cellular oxidant levels have implicated cysteine thiols present in various transcription factors including p53, NF-κB and Nrf2 (Na and Surh, 2006). The observed increase of GSH efflux even at low concentrations of curcumin treatment may suggest an altered cellular oxidative status with consequence of activation of redox-sensitive transcription proteins.

GCL expression or activity is modulated by oxidants, antioxidants, growth factors, and inflammation (Lu, 2009). GCL gene is regulated by Nrf2 via antioxidant response element (ARE), where Nrf2 is rapidly activated under oxidative stress conditions (Lu, 2009). Our study revealed increased GCLC expression and high GSH formation as an adaptive oxidative stress response of the cells which was observed at early time period, i.e. within 9 h. The antioxidant effects of curcumin may be due to the activation of the Nrf2-ARE signaling pathway. The coordinated adaptive gene response, i.e. induction of various antioxidant, and cytoprotective enzymes such as GCL, superoxide dismutase, NADPH oxidoreductase-1, thioredoxin reductase, and heme oxygenase-1 may paradoxically protect cancer cells from oxidative stress (Chen et al., 2008; Wang et al., 2008) until the pro-oxidant effects prevails over the adaptive survival response of the cells and cell death was ensued.

In conclusion, curcumin exerts cytotoxic effect on CCA cells. The fate of CCA cells may depend on the extent of oxidant stress, cellular redox maintenance, and the modulation of subsequent cellular signallings. The initial effect of curcumin may involve suppression of NF-κB activation, depolarization of $\Delta\Psi_m$ and following with formation of oxidant species. These effects lead to antiproliferation, apoptosis, and on the other hand, induce adaptive antioxidant response which depend on concentration of curcumin and exposure time. The study suggests that curcumin could be developed into an useful chemoprevention against bile duct cancer.

## Conflict of Interest

The authors declare that there are no conflicts of interest.

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## References

Aggarwal, B.B., Sung, B., 2009. Pharmacological basis for the role of curcumin in chronic diseases: an age-old spice with modern targets. Trends Pharmacol. Sci. 30, 85–94.

Barve, A., Khor, T.O., Hao, X., Keum, Y.S., Yang, C.S., Reddy, B., Kong, A.N., 2008. Murine prostate cancer inhibition by dietary phytochemicals–curcumin and phenyethylisothiocyanate. Pharm. Res. 25, 2181–2189.

Blechacz, B., Gores, G.J., 2008. Cholangiocarcinoma: advances in pathogenesis, diagnosis, and treatment. Hepatology 48, 308–321.

Buranrat, B., Prawan, A., Sripa, B., Kukongviriyapan, V., 2007. Inflammatory cytokines suppress arylamine *N*-acetyltransferase 1 in cholangiocarcinoma cells. World J. Gastroenterol. 13, 6219–6225.

Chao, D.T., Korsmeyer, S.J., 1998. BCL-2 family: regulators of cell death. Annu. Rev. Immunol. 16, 395–419.

Chen, H.H., Song, I.S., Hossain, A., Choi, M.K., Yamane, Y., Liang, Z.D., Lu, J., Wu, L.Y., Siddik, Z.H., Klomp, L.W., Savaraj, N., Kuo, M.T., 2008. Elevated glutathione levels confer cellular sensitization to cisplatin toxicity by up-regulation of copper transporter hCtr1. Mol. Pharmacol. 74, 697–704.

Choudhuri, T., Pal, S., Das, T., Sa, G., 2005. Curcumin selectively induces apoptosis in deregulated cyclin D1-expressed cells at G2 phase of cell cycle in a p53-dependent manner. J. Biol. Chem. 280, 20059–20068.

Garg, A.K., Buchholz, T.A., Aggarwal, B.B., 2005. Chemosensitization and radiosensitization of tumors by plant polyphenols. Antioxid. Redox. Signal. 7, 1630–1647.

Ionov, Y., Yamamoto, H., Krajewski, S., Reed, J.C., Perucho, M., 2000. Mutational inactivation of the proapoptotic gene BAX confers selective advantage during tumor clonal evolution. Proc. Natl. Acad. Sci. USA. 97, 10872–10877.

Jang, J.H., Surh, Y.J., 2004. Bcl-2 attenuation of oxidative cell death is associated with up-regulation of gamma-glutamylcysteine ligase via constitutive NF-kappaB activation. J. Biol. Chem. 279, 38779–38786.

Kensler, T.W., Wakabayashi, N., Biswal, S., 2007. Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. Annu. Rev. Pharmacol. Toxicol. 47, 89–116.

Khan, S.A., Taylor-Robinson, S.D., Toledano, M.B., Beck, A., Elliott, P., Thomas, H.C., 2002. Changing international trends in mortality rates for liver, biliary and pancreatic tumours. J. Hepatol. 37, 806–813.

Khan, S.A., Thomas, H.C., Davidson, B.R., Taylor-Robinson, S.D., 2005. Cholangiocarcinoma. Lancet 366, 1303–1314.

Kunnumakkara, A.B., Guha, S., Krishnan, S., Diagaradjane, P., Gelovani, J., Aggarwal, B.B., 2007. Curcumin potentiates antitumor activity of gemcitabine in an orthotopic model of pancreatic cancer through suppression of proliferation, angiogenesis, and inhibition of nuclear factor-kappaB-regulated gene products. Cancer Res. 67, 3853–3861.

Lazaridis, K.N., Gores, G.J., 2005. Cholangiocarcinoma. Gastroenterology 128, 1655–1667.

Lee, K.W., Kim, J.H., Lee, H.J., Surh, Y.J., 2005. Curcumin inhibits phorbol ester-induced up-regulation of cyclooxygenase-2 and matrix metalloproteinase-9 by blocking ERK1/2 phosphorylation and NF-kappaB transcriptional activity in MCF10A human breast epithelial cells. Antioxid. Redox Signal. 7, 1612–1620.

Lu, S.C., 2009. Regulation of glutathione synthesis. Mol. Aspects Med. 30, 42–59.

Mishra, S., Kapoor, N., Mubarak Ali, A., Pardhasaradhi, B.V., Kumari, A.L., Khar, A., Misra, K., 2005. Differential apoptotic and redox regulatory activities of curcumin and its derivatives. Free Radic. Biol. Med. 38, 1353–1360.

Na, H.K., Surh, Y.J., 2006. Transcriptional regulation via cysteine thiol modification: a novel molecular strategy for chemoprevention and cytoprotection. Mol. Carcinog. 45, 368–380.

Nair, S., Li, W., Kong, A.N., 2007. Natural dietary anti-cancer chemopreventive compounds: redox-mediated differential signaling mechanisms in cytoprotection of normal cells versus cytotoxicity in tumor cells. Acta Pharmacol. Sin. 28, 459–472.

Prawan, A., Buranrat, B., Kukongviriyapan, U., Sripa, B., Kukongviriyapan, V., 2009. Inflammatory cytokines suppress NAD(P)H:quinone oxidoreductase-1 and induce oxidative stress in cholangiocarcinoma cells. J. Cancer Res. Clin. Oncol. 135, 515–522.

Rashmi, R., Kumar, S., Karunagaran, D., 2005. Human colon cancer cells lacking Bax resist curcumin-induced apoptosis and Bax requirement is dispensable with ectopic expression of Smac or downregulation of Bcl-XL. Carcinogenesis 26, 713–723.

Sandur, S.K., Ichikawa, H., Pandey, M.K., Kunnumakkara, A.B., Sung, B., Sethi, G., Aggarwal, B.B., 2007. Role of pro-oxidants and antioxidants in the anti-inflammatory and apoptotic effects of curcumin (diferuloylmethane). Free Radic. Biol. Med. 43, 568–580.

Sharma, R.A., Gescher, A.J., Steward, W.P., 2005. Curcumin: the story so far. Eur. J. Cancer 41, 1955–1968.

Shishodia, S., Potdar, P., Gairola, C.G., Aggarwal, B.B., 2003. Curcumin (diferuloylmethane) down-regulates cigarette smoke-induced NF-kappaB activation through inhibition of IkappaBalpha kinase in human lung epithelial cells: correlation with suppression of COX-2, MMP-9 and cyclin D1. Carcinogenesis 24, 1269–1279.

Singh, M., Singh, N., 2009. Molecular mechanism of curcumin induced cytotoxicity in human cervical carcinoma cells. Mol. Cell Biochem. 325, 107–119.

Sompamit, K., Kukongviriyapan, U., Nakmareong, S., Pannangpetch, P., Kukongviriyapan, V., 2009. Curcumin improves vascular function and alleviates oxidative stress in non-lethal lipopolysaccharide-induced endotoxaemia in mice. Eur. J. Pharmacol. 616, 192–199.

Somparn, N., Kukongviriyapan, U., Tassaneeyakul, W., Jetsrisuparb, A., Kukongviriyapan, V., 2007. Modification of CYP2E1 and CYP3A4 activities in haemoglobin E-beta thalassemia patients. Eur. J. Clin. Pharmacol. 63, 43–50.

Surh, Y.J., 2003. Cancer chemoprevention with dietary phytochemicals. Nat. Rev. Cancer 3, 768–780.

Syng-Ai, C., Kumari, A.L., Khar, A., 2004. Effect of curcumin on normal and tumor cells: role of glutathione and bcl-2. Mol. Cancer Ther. 3, 1101–1108.

Thayyullathil, F., Chathoth, S., Hago, A., Patel, M., Galadari, S., 2008. Rapid reactive oxygen species (ROS) generation induced by curcumin leads to caspase-dependent and -independent apoptosis in L929 cells. Free Radic. Biol. Med. 45, 1403–1412.

Tietze, F., 1969. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. Anal. Biochem. 27, 502–522.

Tsujimoto, Y., Nakagawa, T., Shimizu, S., 2006. Mitochondrial membrane permeability transition and cell death. Biochim. Biophys. Acta 1757, 1297–1300.

Vaseva, A.V., Moll, U.M., 2009. The mitochondrial p53 pathway. Biochim. Biophys. Acta 1787, 414–420.

Wang, X.J., Sun, Z., Villeneuve, N.F., Zhang, S., Zhao, F., Li, Y., Chen, W., Yi, X., Zheng, W., Wondrak, G.T., Wong, P.K., Zhang, D.D., 2008. Nrf2 enhances resistance of cancer cells to chemotherapeutic drugs, the dark side of Nrf2. Carcinogenesis.