

Antiproliferative and Apoptosis-inducing Activity of Curcumin against Human Gallbladder Adenocarcinoma Cells

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Abstract. *Aim: Curcumin has potent antitumor activity against many types of human cancers. However, the inhibitory effects and possible mechanisms of curcumin on gallbladder cancer remains to be determined. Materials and Methods: Using HAG-1 human gallbladder adenocarcinoma cells, we investigated the effects of curcumin on cell proliferation, apoptosis, cell-cycle perturbation, and signal proteins for survival, proliferation, and apoptosis. Results: Curcumin exhibited dose-dependent antitumor activity against HAG-1 cells, arresting the cells in G₂/M phase, with progressive expansion of the apoptotic cell population. Upon curcumin treatment, AKT activation was substantially suppressed, with subsequent reduction of activities of mammalian target of rapamycin (mTOR) and its downstream molecules S6 kinase-1 (S6K1) and eIF4E-binding protein-1 (4E-BP1), but constitutive activity of extracellular signal-regulated kinase (ERK1/2) was clearly enhanced. Curcumin reduced the expression and phosphorylation of anti-apoptotic Bcl-2, but did not affect the expressions of pro-apoptotic Bax and anti-apoptotic nuclear factor (NF-κB). Conclusion: These results suggest that curcumin induces G₂/M arrest and apoptosis through multiple mechanisms involving enhanced mitogen-activated protein (MAP) kinase activity, reduced AKT-mTOR activity, and reduced Bcl-2 function. These data provide a mechanistic rationale for the potential use of curcumin in the treatment of gallbladder cancer.*

Carcinoma of the gallbladder is a highly lethal disease among a variety of types of human cancers. The surgical cure rate is extremely low, even if the disease is detected in relatively early stages. Most of the patients with advanced

disease at presentation have no therapeutic options other than systemic chemotherapy, which has minimal impact on survival. Therefore, novel agents are urgently needed for the treatment of advanced gallbladder cancer. One approach would be the search for new agents acting against molecular targets specific for gallbladder cancer. However, the paucity of data for the biological and genetic features of this neoplasm has made it difficult to develop useful therapeutic approaches for this cancer type.

Curcumin is derived from turmeric (*Curcuma longa*) and is a natural polyphenol. Curcumin has long been used as a food, coloring agent, and traditional medicine. It is safe and non-toxic, and has demonstrable anti-inflammatory and antioxidant properties (1). Curcumin has been shown to inhibit the formation of carcinogen-induced cancer of the colon (2), breast (3), stomach (4), liver (5), and lung (6) in experimental animals. Such chemopreventive activities of curcumin are thought to involve up-regulation of cancer-detoxifying enzymes (7) and antioxidants (8), suppression of cyclooxygenase-2 expression (9), and inhibition of NF-κB release (10). Moreover, increasing evidence has indicated that curcumin has anticancer effects against a variety of human tumor cell types through modulation of diverse molecular targets involving cell survival/apoptosis and proliferation (1). Clinically, curcumin administration showed favorable responses in certain patients with pancreatic cancer in a phase II study (11). However, the anticancer activity of curcumin against gallbladder cancer remains to be determined.

In this study, we examined the anticancer effects of curcumin on gallbladder cancer using the well-characterized HAG-1 human gallbladder adenocarcinoma cell line (12). The molecular mechanisms of curcumin-induced growth inhibition and apoptosis were also investigated.

Materials and Methods

Cell culture and chemicals. HAG-1 is a human epithelial cell line derived from a moderately-differentiated adenocarcinoma of the gallbladder, and its cellular and molecular features were described previously (12). Neither mutations nor amplifications of *N*-, *H*-, or *K-RAS* genes have been detected (12). The cells were cultured in

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Dulbecco's modified Eagle's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA), 100 IU/ml penicillin, and 100 µg/ml streptomycin in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. Curcumin (more than 80% purity) was purchased from Sigma-Aldrich (St. Louis, MO, USA). A 1000 mM solution prepared by dissolving original curcumin powder with dimethyl sulfoxide (DMSO, Sigma-Aldrich) were diluted 10-fold with 100% ethanol, making 100 mM stock solutions, and stored at -20°C. This dilution process using 100% ethanol was needed to prevent re-crystallization of curcumin when diluted directly with culture medium. The final concentration of DMSO for all experiments and treatments (including vehicle controls, where no drug was added) was maintained at less than 0.05%. These conditions were found to be non-cytotoxic at least for 72 h.

Determination of growth inhibition and apoptosis assessment by poly-ADP-ribose-polymerase (PARP) cleavage. The anti-proliferative effect of curcumin on HAG-1 cells was assessed by the WST assay, using the manufacturer's instructions (Dojindo, Kumamoto, Japan). The WST assay is a colorimetric method in which the intensity of the dye is proportional to the number of the viable cells. Briefly, 100 µl suspension of HAG-1 cells was seeded into each well of a 96-well plate (BD Falcon, Franklin Lakes, NJ, USA) at a density of 2,000 cells per well. After overnight incubation, 100 µl curcumin solution at different concentrations was added and cells further cultured for 72 hours. Cell viability was then measured by Premix CCK-8 Cell Proliferation Assay System (Dojindo) according to the manufacturer's protocol. Each experiment was performed using six replicate wells for each curcumin concentration and was carried out independently three times. The IC₅₀ value was defined as the concentration needed for a 50% reduction in the absorbance.

Apoptosis was assessed by PARP cleavage detected by western blot using antibody to PARP antibody (9542). PARP is a substrate for certain caspases activated during early stages of apoptosis. These proteases cleave PARP to fragments of approximately 89 kDa and 24 kDa. Detection of the 89 kDa PARP fragment with antibody to PARP thus serves as an early marker of apoptosis.

Cell-cycle analysis and apoptosis measurement. At various times following treatment with or without curcumin, floating and trypsinized adherent cells were combined, and fixed in 100% ethanol and stored at 4°C for up to three days prior to cell cycle analysis. After the removal of ethanol by centrifugation, cells were then washed with phosphate buffered saline (PBS) and stained with a solution containing propidium iodide (PI; Sigma-Aldrich) and RNase A on ice for 30 min. Cell-cycle analyses were performed on a Beckman Coulter Gallios Flow Cytometer using the Kaluza ver. 1.2 software packages (Beckman Coulter, Brea, CA, USA), and the extent of apoptosis was determined by measuring the sub-G₁ population.

Immunoprecipitation and western blot analysis of signaling proteins. The cells were washed twice with ice-cold PBS and scraped into 0.5 ml of lysis buffer [10× Cell Lysis Buffer, 1 mM PMSF]. After removal of cell debris by centrifugation, protein concentrations of the supernatants were determined by using a BCA protein assay kit (Bio Rad, Hercules, CA, USA). Immune complexes were boiled in electrophoresis sample buffer (Bio-Rad).

For western blotting, equal amounts of proteins or immunoprecipitated target proteins were resolved by 4-15% sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and electrotransferred onto a polyvinylidene difluoride (PVDF) membrane (GE Healthcare, Piscataway, NJ, USA). Non-specific binding sites were blocked by incubating the membranes in blocking buffer (Nacalai Tesque, Kyoto, Japan) at room temperature for 30 min. The membranes were then incubated with primary antibodies against either phospho-mTOR (Ser2448), phospho-p44/42 MAP kinase (ERK1/2) (Thr202/Tyr204), phospho-AKT (Ser473), phospho-S6K1 (Thr389), phospho-4E-BP1 (Thr37/46), phospho-Bcl-2 (Ser70), phospho-NF-κB (Ser536), or Bax (2772). The membranes were hybridized with a horseradish peroxidase-conjugated secondary antibody (7074). Immunoblots were developed with the enhanced chemiluminescence (ECL) system (GE Healthcare) and were then quantitated using LAS-3000 Luminescent Image Analyzer (Fuji Film, Tokyo, Japan). The blots were stripped and re-probed with primary antibodies against mTOR (9964), ERK1/2 (9102), AKT (9272), Bcl-2 (2876), NF-κB (8242), and β-actin (4967). All primary and secondary antibodies were purchased from Cell Signaling Technology, Danvers, MA, USA. For reblotting, membranes were incubated in stripping buffer (Thermo, Rockford, IL, USA) for 30 min at room temperature before washing, blocking, and incubating with antibody. Triplicate determinations were made in separate experiments.

Statistical analysis. To determine the significance of observed differences, analysis of variance (ANOVA) was applied to the cell-cycle data using a statistical software (version 12.0.1 for Windows, SPSS Inc., USA). The mean values of cell cycle percentages were compared by Dunnett *t*-test. A *p*-value less than 0.05 was considered significant.

Results

Effects of curcumin on proliferation and survival. To examine the effect of curcumin on cell proliferation and survival, HAG-1 cells were treated with different concentrations of curcumin for 72 h. Curcumin exhibited a dose-dependent antitumor activity against HAG-1 cells. The 50% inhibitory concentration (IC₅₀) was approximately 25 µM after 72 h of exposure.

Time-course analysis of the effect of curcumin on cell cycle progression and apoptosis. To examine whether the inhibitory effects observed in cytotoxicity assays reflect the arrest or delay of cell cycle or apoptotic cell death, cells were treated with 25 µM curcumin, and the cell-cycle progression and apoptosis were evaluated after PI staining by fluorescence-activated cell sorting analysis. When HAG-1 cells were treated with curcumin, the proportion of cells in the G₂/M phase increased as early as 24 h after treatment, with a corresponding decrease in cells in the G₀/G₁ phase, in a time-dependent manner (Figure 1A and 1B), becoming nearly 2-fold at 72 h post-treatment. The sub-G₀/G₁ cell population, which represents apoptotic cells, increased gradually at 24 h (3.1%) and 48 h (6.8%) post-treatment, and abruptly increased up to 18.4% after 72 h treatment (Figure 1A and 1B). Furthermore, cleavage of PARP, which serves as an early marker of apoptosis, was demonstrated from 24 h

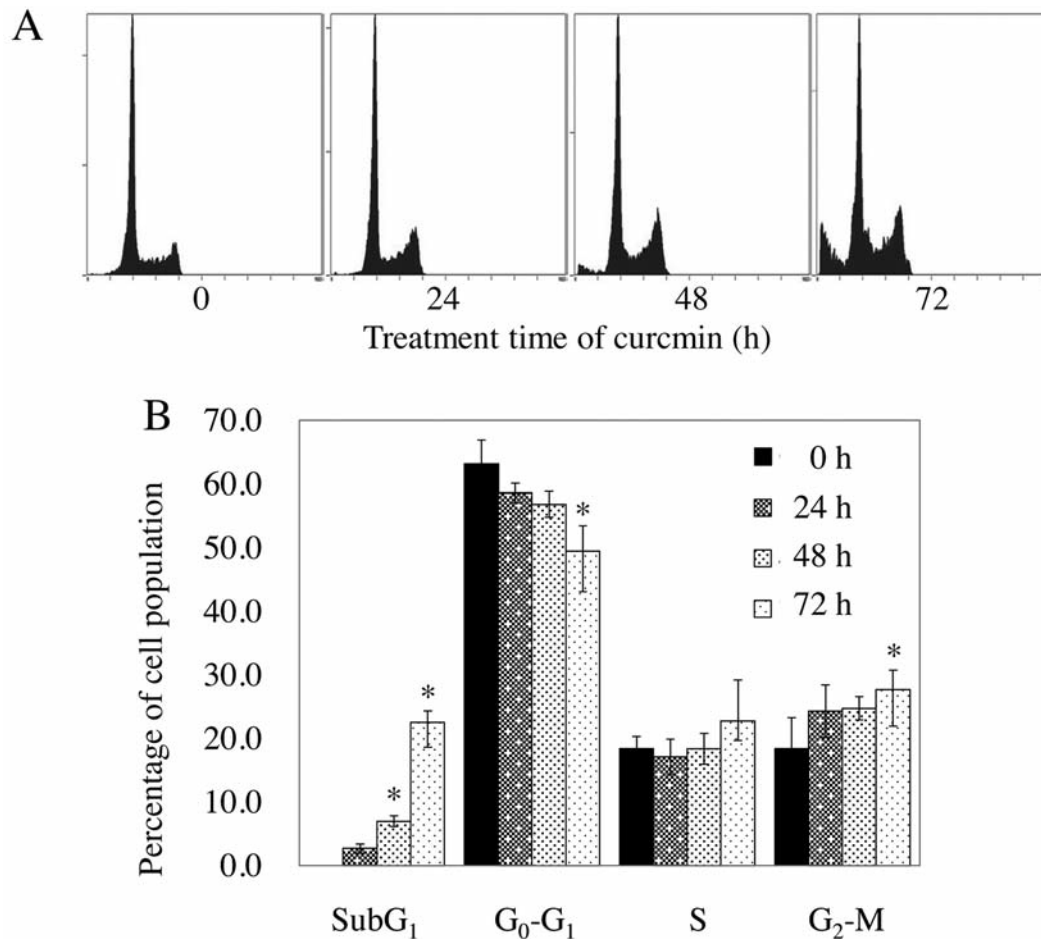


Figure 1. Time-course analysis of the effect of curcumin on cell-cycle progression and apoptosis determined by fluorescence-activated cell sorting. HAG-1 cells were stained with propidium iodide after exposure to curcumin (25 μ M) for 0, 24, 48, and 72 h, and analyzed by flow cytometry. A: Representative cell-cycle distribution determined by flow cytometry at indicated times of curcumin exposure. B: The percentage of the total cell population in the different phases of the cell cycle was determined with curve fitting using Kaluza ver.1.2 software. Data represent means of 3 independent experiments. Bars, SD of triplicates. * $p < 0.05$ relative to no treatment (0 h) for each stage of the cell cycle.

to 72 h post-treatment (Figure 2B). These data indicate that the observed curcumin-induced growth decline appeared to be due to combination effects of progressive expansion of the apoptotic cell population and G₂/M arrest of the cell cycle.

Effects of curcumin on activations of signaling molecules for cell proliferation and survival. Because activation of signaling molecules such as ERK1/2, AKT, and mTOR, have been considered as major factors contributing towards proliferation and survival, we examined the effects of curcumin on the expression and activation (phosphorylation) of these proteins. Upon treatment with 25 μ M curcumin, constitutive activity of ERK1/2 was enhanced as early as 2 h after treatment (Figure 2A), but the phosphorylation of AKT was substantially inhibited, in a time-dependent manner (Figure 2A). Phosphorylation of mTOR at Ser2448 was slightly reduced

by curcumin treatment, while the phosphorylation of S6K1 and 4E-BP1, both of which act downstream of mTOR, was clearly suppressed (Figure 2A). These data indicate that curcumin-induced reduction in cell proliferation and survival appears to be mediated by activation of the MAPK pathway as well as by inactivation of AKT, mTOR, and their downstream molecules (S6K1 and 4E-BP1).

Effects of curcumin on expression of pro- and anti-apoptotic proteins. To clarify the apoptotic mechanisms induced by curcumin, we examined the expression and activation of anti-apoptotic Bcl-2 and NF- κ B as well as pro-apoptotic Bax (Figure 2B). Upon treatment with 25 μ M curcumin, the expression and phosphorylation of Bcl-2 was inhibited in a time-dependent manner. In contrast, neither expression nor phosphorylation of NF- κ B was inhibited. The expression of

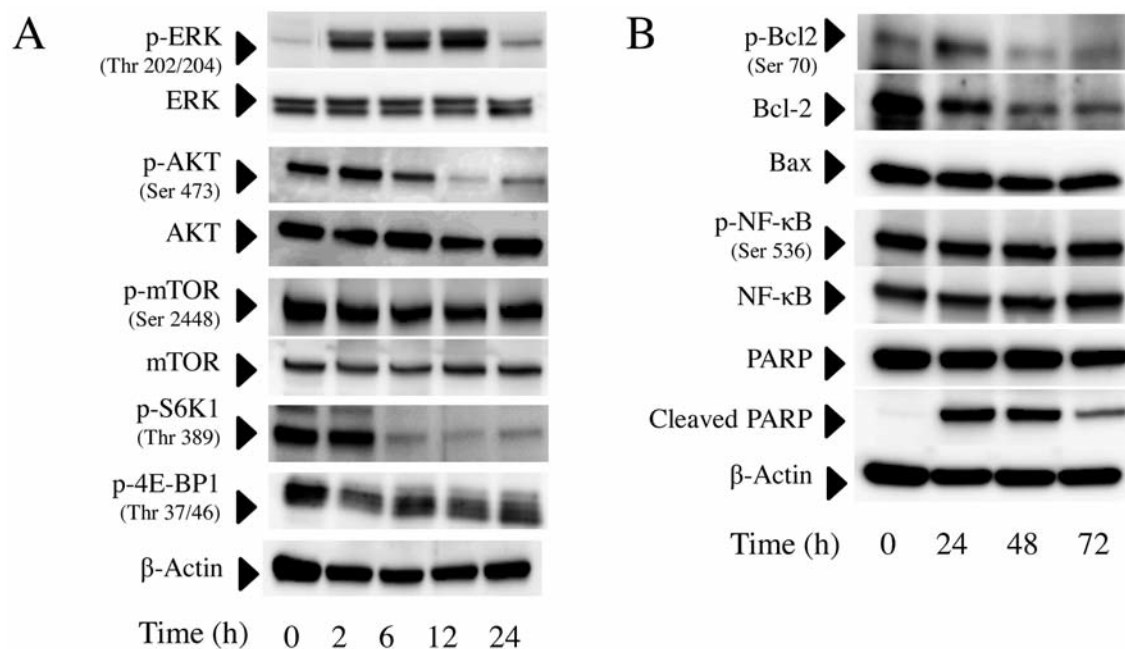


Figure 2. A: Effects of curcumin on the phosphorylation of ERK1/2, AKT/mTOR and downstream S6K1 and 4E-BP1. Western blots are shown for phospholylated and total ERK1/2, AKT and mTOR. Phospholylated S6K1 and 4E-BP1 are also shown. B: Effects of curcumin on the apoptotic and anti-apoptotic proteins including Bcl-2, Bax, and NF-κB. PARP and cleaved PARP are shown as an early marker of apoptosis.

Bax protein was unchanged relative to the incubation time with curcumin. These results suggest that reduced expression and de-phosphorylation of Bcl-2 provide a causative role for curcumin-induced apoptosis.

Discussion

In the present study, we found that the IC_{50} value of curcumin against HAG-1 gallbladder cancer cells is approximately 25 μ M for 72 h exposure, a comparable IC_{50} concentration observed for other human cancer cell lines. With this concentration, we showed that curcumin inhibited HAG-1 cell growth by arresting the cells in the G_2/M phase, accompanied by the increase in the sub- G_0/G_1 phase, with demonstrable cleavage of PARP. Cells in the sub- G_0/G_1 population represent apoptotic cells, and cleavage of PARP facilitates cellular disassembly and serves as a marker of cells undergoing apoptosis (13). Therefore, these data indicate that inhibitory effects of curcumin observed in cytotoxicity assays reflect the combination of curcumin-induced G_2/M phase cell-cycle arrest and apoptotic cell death.

Although curcumin has been shown to induce apoptosis in a variety of cancer cells, the mechanism whereby curcumin induces apoptosis varies with cell type (14, 15). Here we showed that curcumin down-regulates the expression and phosphorylation of Bcl-2 at Ser70 in HAG-1 gallbladder carcinoma cells. The antiapoptotic Bcl-2 protein resides in

the outer mitochondrial wall and inhibits cytochrome c release (16), and Bcl-2 phosphorylation at Ser70 has been shown to be required for its enhanced anti-apoptotic functions (17). Therefore, reduced expression and de-phosphorylation of Bcl-2 may play an important role for curcumin-induced apoptosis taking place in HAG-1 cells. We have also found that pro-apoptotic Bax protein resides in the cytosol and translocates to mitochondria following death signaling, thereby promoting the release of cytochrome c (16, 18). Although curcumin has been reported to induce the up-regulation of Bax in several cancer cell lines (19, 20), Bax was unchanged by curcumin in HAG-1 gallbladder carcinoma cells. Curcumin has been reported to prevent NF-κB activation induced by various agents through inhibition of p65 translocation to the nucleus and suppression of IκBα degradation in numerous cell types (21). By inhibiting NF-κB activation, curcumin has been shown to suppress the expression of various cell survival and proliferative genes, including Bcl-2, Bcl-xL, cyclin-D1, interleukin-6, cyclooxygenase-2 and matrix metalloproteinase-9, and subsequently arrest cell cycle, inhibit proliferation, and induce apoptosis (22). In HAG-1 cells, however, curcumin did not show any significant effects on NF-κB activation. Taken together, these results suggest that curcumin-induced apoptosis might be mediated mainly by inactivation of Bcl-2 through the mechanism independent of NF-κB signaling in HAG-1 gallbladder adenocarcinoma cells.

AKT plays a critical role in controlling survival and apoptosis by directly phosphorylating mTOR at Ser2448 (23, 24), leading to increased phosphorylation of S6K1 and 4E-BP1 (25). In many cancer cell lines, curcumin was found to inhibit phosphorylation of AKT/mTOR and its downstream targets, S6K1 and 4E-BP1, suggesting that curcumin may execute its apoptotic effect primarily through blocking mTOR-mediated signaling pathways (26, 27). Similarly, constitutive activity of AKT was inhibited upon treatment of HAG-1 cells with curcumin, with subsequent reduction of activities of mTOR and its downstream molecules S6K1 and 4E-BP1. Therefore, the phosphoinositol 3-kinase (PI3K)/AKT/mTOR signaling pathway is involved in the apoptosis of HAG-1 cells.

Interestingly, constitutive activity of ERK1/2 was enhanced upon treatment with curcumin. ERK1/2 is an important subfamily of MAPKs that control a broad range of cellular activities and physiological processes. Activation of ERK1/2 generally has mitogenic capacity and promotes cell survival, but under certain conditions, ERK1/2 has been shown to have pro-apoptotic functions (28). For example, DNA-damaging agents including etoposide, adriamycin, ionizing irradiation, and ultraviolet irradiation, activate ERK1/2 in various cell lines (29), and inhibition of ERK1/2 activation attenuates apoptosis or G₂-M arrest induced by etoposide, adriamycin, or UV. Conversely, enforced activation of ERK1/2 by overexpression of a constitutively activated MAP kinase kinase-1 (MEK1) Q56P mutant sensitizes cells to DNA damage-induced apoptosis (29). Since curcumin has been shown to induce DNA damage in colorectal carcinoma HCT116 cells (30) and HepG2 hepatoma cells (31), similar curcumin-induced apoptotic mechanisms involving activation of ERK may occur in HAG-1 gallbladder carcinoma cells. Therefore, our data suggest that the MAPK enhancement and inhibition of AKT-mTOR pathways might be important for the execution of curcumin-induced anti-proliferative effect and apoptosis in HAG-1 cells.

Cell-cycle checkpoints are important control mechanisms that ensure the proper execution of cell-cycle events. One of the checkpoints, the G₂/M checkpoint, blocks entry into mitosis. Our study demonstrated that curcumin inhibited the proliferation of HAG-1 gallbladder adenocarcinoma cells by inducing G₂/M cell-cycle arrest, causing a blockade of cell-cycle entry into mitosis. Curcumin has been shown to induce G₂/M arrest in many types of cancer cell such as colon, ovarian and glioma cell lines (32-34). In the present study, we found that the blockade of cell-cycle arrest at G₂/M was accompanied by an enhancement of constitutive activity of ERK1/2. There have been several reports suggesting a positive association between ERK activation and G₂/M cell-cycle arrest. Inhibition of ERK1/2 signaling resulted in more than 85% attenuation of irradiation-induced G₂/M arrest (35). Moreover, activation of ERK1/2 has been demonstrated to correlate with changes in the level and/or activity of several key regulators of the G₂/M checkpoint, including cyclin-dependent kinase-1 (CDK1) and Wee1 kinase

(35, 36). Previous studies have shown that concomitant decrease in the expressions of cyclin-B1 and CDK1 was demonstrated in curcumin-treated cells (37). Therefore, it is conceivable that curcumin-induced enhancement of ERK1/2 subsequently induces the change in these regulator molecules of the G₂/M checkpoint, leading to the arrest of the cell cycle at G₂/M.

Conclusion

Although the observations were obtained on a single human gallbladder cancer cell line, our findings suggest that antitumor activity of curcumin would be mediated by the induction of G₂/M arrest and apoptosis through multiple mechanisms involving enhanced MAPK activity, inhibition of AKT-mTOR pathway, and reduced Bcl-2 function, thus providing a mechanistic rationale for the potential use of curcumin in the treatment of patients with gallbladder cancer.

Conflicts of Interest

None.

Acknowledgements

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