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***Basic Study***

**Ursodeoxycholic acid induces apoptosis in hepatocellular carcinoma xenografts in mice**

Liu H *et al*. Ursodeoxycholic acid induces apoptosis *in vivo*

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**Abstract**

**AIM:** To evaluate the efficacy of ursodeoxycholic acid (UDCA) as a chemotherapeutic agent for the treatment of hepatocellular carcinoma (HCC).

**METHODS:** BALB/c nude mice were randomized into four groups 24 h before sc injection of hepatocarcinoma BEL7402 cells suspended in phosphate buffered saline (PBS) into the right flank. The control group (*n* = 10 animals) was fed a standard diet while treatment groups (*n* = 10 animals each) were fed a standard daily diet supplemented with different concentrations of UDCA (30, 50 and 70 mg/kg/d) for 21 d. Tumor growth was measured once each week, and tumor volume (V) was calculated with the following equation: V = (L × W2) × 0.52, where L was the length and W was the width of the xenograft. After 21 d, mice were killed under ether anesthesia, and tumors were excised and weighed. Apoptosis was evaluated through detection of DNA fragmentation with gel electrophoresis and the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay. Western blot analysis was performed to determine expression of apoptosis-related proteins BAX, BCL2, APAF1, cleaved-caspase-9, and cleaved-caspase-3.

**RESULTS:** UDCA suppressed tumor growth relative to controls. The mean tumor volumes were the following: control, 1090 ± 89 mm3; 30 mg/kg/d, 612 ± 46 mm3; 50 mg/kg/d, 563 ± 38 mm3; and 70 mg/kg/d, 221 ± 26 mm3. Decreased tumor volumes reached statistical significance relative to control xenografts (30 mg/kg/d, *P* < 0.05; 50 mg/kg/d, *P* < 0.05; 70 mg/kg/d, *P* < 0.01). Increasing concentrations of UDCA led to increased DNA fragmentation observed on gel electrophoresis and in the TUNEL assay (control, 1.6% ± 0.3%; 30 mg/kg/d, 2.9% ± 0.5%; 50 mg/kg/d, 3.15% ± 0.7%, and 70 mg/kg/d, 4.86 ± 0.9%). Western blots revealed increased expression of proteins which induce apoptosis, BAX, APAF1, cleaved-caspase-9 and cleaved-caspase-3, but decreased BCL2 protein, which is an inhibitor of apoptosis, following administration of UDCA.

**CONCLUSION:** UDCA suppresses growth of BEL7402 hepatocellular carcinoma cells *in vivo*,in part through apoptosis, and is thus a candidate for therapeutic treatment of HCC.

**Key words:** Hepatocellular carcinoma; Inhibitory effects;Mechanisms; Ursodeoxycholic acid; Xenografts

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**Core tip:** Hepatocellular carcinoma (HCC) ranks as the sixth most common cancer worldwide. Prognosis of HCC patients remains poor however due to the lack of effective therapies. In this study, ursodeoxycholic acid (UDCA) was investigated as a potential chemotherapeutic agent in a mouse model of HCC. Tumor growth was inhibited by increasing concentrations of UDCA over a 21-d period, and the effect was elicited through apoptosis. UDCA is thus a candidate chemopreventive and chemotherapeutic agent for hepatocellular carcinoma.

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**INTRODUCTION**

Worldwide, hepatocellular carcinoma (HCC) ranks as the sixth most common cancer, with over half a million new cases diagnosed each year[1]. HCC is particularly prevalent in Asia, where it is the third leading cause for cancer-related death[2,3]. The incidence is even higher in China alone[4], where HCC ranks as the second cause of cancer-related death[5-7]. Despite advances in surgical and chemo-radiotherapies, the prognosis of HCC patients remains poor. Thus it is crucial to develop new therapeutic options for treatment of this disease.

A potential chemotherapeutic agent is ursodeoxycholic acid (UDCA), a metabolite produced by intestinal bacterial, which is currently used in the treatment of liver disease. It has been reported to have a variety of therapeutic effects based upon its ability to reduce oxidative stress. UDCA has also been shown to lower biliary and serum concentrations of hydropholic bile acids, as well as tumor necrosis factor-α in chronic cholestasis[8,9]. Thus, UDCA appears to improve symptoms of nonalcoholic steatohepatosis (NASH)[10,11]. UDCA has been most successfully used for the treatment of primary biliary cirrhosis, which is thought to be mediated in part through immunosuppression[12]. In fact, the prognosis of primary biliary cirrhosis has improved not only due to earlier detection of the disease, but also to the increased use of UDCA for treatment[13]. Interestingly, while it has been reported to have anti-apoptotic properties, recent studies have demonstrated that UDCA reduced the frequency of colonic carcinogenesis by inhibiting interleukin-1 beta and deoxycholic acid-induced activation of NF-kappaB and AP-1[14].Furthermore, UDCA has been shown to induce apoptosis of HCC cells *in vitro*[15]. In our own studies, we have previously demonstrated that UDCA selectively inhibits proliferation and induces apoptosis in the HCC cell lines, HepG2 and BEL7402, *in vitro* by blocking the cell cycle and regulating the expression of genes involved in programmed cell death, such as *BAX*/*BCL2*[16].

Although a number of studies have demonstrated that UDCA inhibits cell proliferation and induces apoptosis in various cancer cells *in vitro*, little is known about the effect of UDCA on cancer cells *in vivo,* in particular with regard to HCC. Here, the efficacy of UDCA was investigated as a possible therapy in a mouse model of HCC. The results demonstrated that in BALB/c nude mice, UDCA suppressed the growth of sc injected BEL7402 cells, derived from a hepatocellular carcinoma, through the induction of apoptosis.

**MATERIALS AND METHODS**

***Ethics statement***

All animal experiments were carried out under an Institutional Animal Care and Use Committee-approved protocol.

***Cell culture***

BEL7402 cells (Shanghai Institute of Cell Biology, Chinese Academy of Sciences, Shanghai, China) were derived from a specimen obtained from a 53 year-old male patient with HCC in 1974. Cells were maintained in DMEM (Life Technologies, Grand Island, NY, United States) containing 10% fetal bovine serum supplemented with penicillin (100 units/mL) and streptomycin (100 µg/mL) and cultured at 37 ºC in a humidified chamber with 5% CO2.

***BEL7402 xenografts in immunodeficient mice***

Six-week-old male BALB/c immunodeficient mice (*n* = 40) were obtained from the experimental animal center of Shandong University (Shandong, China). The animals were housed in sterile filter-capped microisolator cages and provided with a sterilized diet and water. HCC BEL7402 cells (1 × 106/0.2 mL/mouse) were suspended in phosphate buffered saline (PBS) and injected sc into the right flank of mice. Mice were randomized into four groups one day before the injection of tumor cells. Group 1 (control, *n* = 10) was fed a standard diet; Group 2 (*n* = 10), a standard diet supplemented with UDCA (Sigma, St. Louis, MO, United States) at 30 mg/kg/d; Group 3 (*n* = 10), a standard diet supplemented with UDCA at 50 mg/kg/d; and Group 4 (*n* = 10), a standard diet supplemented with UDCA at 70 mg/kg/d. Body weights of animals in each group were measured before initiation of the experiment and after 21 d. Tumor growth was measured once each week over the 21 d, and tumor volume (*V*) was calculated as *V* = (*L* × *W*2) × 0.52, where *L* was the length and *W* was the width of a xenograft. After 21 days, mice were killed under ether anesthesia.The tumors were excised and weighed. A portion of the tumor was snap-frozen for protein analysis, and the remaining tissue was fixed in phosphate buffered formalin to obtain sections for histological analysis and immunohistochemistry.

***DNA isolation and evaluation***

DNAs were isolated from homogenized tissues or cells harvested and rinsed twice with ice-cold PBS. Samples were treated with proteinase K (0.1 g/L; Sigma) in 0.3 mL of buffer containing Tris-HCl (10 mmol/L, pH 7.4), EDTA (25 mmol/L), and SDS (0.5%) at 37 °C for 12 h. DNA was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated in NaOAc (3 mol/L) and 2 volumes of ice-cold absolute ethanol. The precipitated DNA was rinsed once with 70% ethanol, resolubilized in TE buffer (Tris-HCl 10 mmol/L and EDTA 1 mmol/L, pH 8.0), and incubated with RNase I (10 g/L) for 1 h at 37°C. Genomic DNAs (10 mg/well) and markers were run on 1.5% agarose gels containing ethidium bromide (0.1 g/L) for 2 h at 60 V and were visualized with ultraviolet light.

***Detection of apoptotic cells in situ***

Apoptotic cells were detected with terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) using the ApoTag Plus Peroxidase *in situ* Apoptosis Detection Kit (Chemicon, Temecula, CA, United States) according to the manufacturer’s instructions. In brief, tissue sections were de-paraffinized, rehydrated through a graded alcohol series, and rinsed in distilled water. The tissue sections were incubated with proteinase K for 20 min at room temperature and subsequently incubated with terminal deoxynucleotidyl transferase (TdT) buffer containing 0.3 U/L TdT (Life Technologies) and 0.04 nmol/L biotinylated dUTP (Boehringer Mannheim GmbH, Mannheim, Germany) in a humidified chamber for 1 h at 37 °C. Slides were rinsed with PBS, and signal was amplified with horseradish peroxidase-conjugated streptavidin. Sections were counterstained with hematoxylin for 30 s. Cells undergoing apoptosis contained dark brown staining nuclei, and the number of TUNEL-positive cells were determined by analyzing 1000 cells in randomly selected fields of three sections for each group. A section from rat mammary glands provided by the manufacturer was used as a positive control.

***Western blot analysis***

Xenograft tissue was lysed for 40 min on ice in buffer containing 50 mmol/L NaCl, 0.5% Triton X-100, 50 mmol/L Tris-HCl (pH 7.4), 25 mmol/L NaF, 20 mmol/L EDTA, 1 mmol/L DTT, 1 mmol/L Na3VO4, and protease inhibitors at a concentration of 10 mg/mL (Roche, Mannheim, Germany). Protein lysates were centrifuged at 14800 × *g* for 15 min to remove cellular debris. Supernatants were collected, and protein concentrations were measured with the bicinchoninic acid assay (BCA; Life Technologies). Protein (20 mg) was loaded onto a 4% to 12% NuPAGE gel (Life Technologies) and transferred onto a polyvinylidene difluoride membrane following electrophoresis. Membranes were blocked for 1 h with 5% nonfat dry milk in PBS with 0.1% Tween 20 (PBST), incubated with primary antibody (cleaved caspase-3 and cleaved caspase-9: Cell Signaling, Beverly, MA, United States; anti-mouse BAX, anti-human BCL2, anti-human APAF1, and anti-actin: Santa Cruz Biotechnology, Dallas, TX, United States) in 5% bovine serum albumin/0.1% PBST overnight at 4°C, rinsed three times for 5 min each with PBST, and then incubated with horseradish peroxidase-conjugated secondary antibody in 5% nonfat dry milk/PBST for 1 h at room temperature. Blots were rinsed with PBST three times, and transferred proteins were visualized with Super Signal chemiluminescent substrate (Life Technologies).

***Preparation of mitochondrial and cytosolic fractions and localization of cytochrome c***

Preparation of mitochondrial and cytosolic fractions was performed as previously described[11]. For isolation of mitochondria, the tumor tissue was minced on ice, resuspended in 10 mL of ice-cold Buffer A (200 mM mannitol, 50 mM sucrose, 10 mM KCl, 1 mM EDTA, 10 mM HEPES-KOH (pH 7.4), 0.1% bovine serum albumin, 10 μg/mL aprotinin, and 1 mM phenylmethylsulfonyl fluoride), and homogenized with a glass Dounce homogenizer and a tight Teflon pestle. Homogenates were centrifuged at 600 × *g* for 15 min at 4°C to pellet debris, and the supernatants collected and centrifuged at 3500 × *g* for 15 min at 4 °C to pellet mitochondria. Floating lipid layers were aspirated, and the mitochondrial pellets were resuspended in Buffer A. Suspensions were centrifuged at 1500 × *g* for 5 min at 4 °C, and the supernatants were recentrifuged at 5500 × *g* for 10 min. The last two steps were repeated twice, and used as mitochondrial protein lysates for western blot analysis. For preparation of cytosolic extracts, the tumor was homogenized in ice-cold buffer (20 mM HEPES-KOH (pH 7.0), 10 mM KCl, 1.5 mM MgCl2, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol, 250 mM sucrose, 10 mg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride) with a Dounce homogenizer. Supernatants were centrifuged at 14000 × *g* for 15 min in a microcentrifuge. The resulting supernatants were used for Western blot analysis. For detection of cytochrome *c*, cytosolic and mitochondrial proteins (20 mg) were separated on SDS-PAGE, transferred to polyvinylidene difluoride membrane, and incubated with anti-cytochrome *c* antibody (Santa Cruz Biotechnology). b-actin (AC-15; Sigma) was used as a cytoplasm-specific marker andoxidative complex 1 protein (20C11; Life Technologies) as a mitochondria-specific marker.

***Statistical analysis***

SPSS version 11.0 (SPSS, Inc.; Chicago, IL, United States) was used for data processing. All data were expressed as the mean ± standard deviation (SD). The student’s*t* test was used for the comparison between two groups. A *P* value < 0.05 was considered statistically significant.

**RESULTS**

***Increasing concentrations of UDCA inhibit growth of BEL7402 HCC in vivo***

BEL7402 cells were s.c. injected into mice and growth of xenografts treated with UDCA was monitored over 21 d. The effect of UDCA treatment at different doses was first investigated as a function of the weight of the animals and tumor volume. Animals were weighed at day 0 and a mean weight was calculated for each experimental group and the controls. The mean body weight for all groups was 17.8 ± 1.8 g. At day 21, body weights generally had decreased as xenografts developed with the most dramatic decrease in untreated controls. The mean body weight was 14.5 ± 1.5 g, 15.7 ± 1.6 g, 16.7 ± 1.7 g and 17.6 ± 1.8 g for controls and the UDCA groups at 30, 50 and 70 mg/kg/d, respectively. Statistical analysis demonstrated that body weight was significantly different between the treatment groups and controls (30 mg/kg/d, *P* < 0.05; 50 mg/kg/d, *P* < 0.05; 70 mg/kg/d, *P* < 0.01).

As expected, tumor volume (1090 ± 89 mm3)increased significantly in control animals over the 21 d. In the experimental groups, growth of tumors was inhibited with increasing doses of UDCA (Figure 1A). Differences between the mean volumes of treated tumors and controls were statistically significant at each dose: 30 mg/kg/d, 612 ± 46 mm3, *P* < 0.05; 50 mg/kg/d, 563 ± 38 mm3, *P* < 0.05; and 70 mg/kg/d, 221 ± 26 mm3,*P* < 0.01 (Figure 1B).

***UDCA treatment causes DNA fragmentation in BEL7402 xenografts***

Apoptosis is one of the mechanisms underlying UDCA growth inhibition *in vitro*. To determine whether UDCA elicits growth inhibition *in vivo* through apoptosis, DNA from treated xenografts was isolated and examined by agarose gel electrophoresis for the presence of the characteristic DNA ladder. DNA ladders were observed in genomic DNA isolated from tumors treated with UDCA after 21 d. Furthermore, the DNA fragmentation increased with increasing UDCA dose (Figure 2).

***Apoptosis detected in situ increases in UDCA treated BEL7402 xenografts***

Apoptosis induced by UDCA was further evaluated with the TUNEL assay which enables the detection of fragmented DNA *in situ*. The TUNEL assay revealed that the mean percentage of apoptotic cells increased with increasing UDCA dose: control, 1.6% ± 0.3%; 30 mg/kg/d, 2.9 ± 0.5%; 50 mg/kg/d, 3.15% ± 0.7%, and 70 mg/kg/d, 4.86% ± 0.9%. The increased percentage of apoptotic cells in treated *vs* control xenografts was significant (*P* = 0.041, 0.029, and 0.016 for 30 mg/kg/d UDCA 50mg/kg/day UDCA, and 70 mg/kg/d UDCA, respectively) (Figure 3).

***Protein expression of BAX, BCL2, APAF1, cleaved caspase-3 and cleaved caspase-9 is altered in UDCA induced apoptosis***

Several proteins involved in mediating and regulating apoptosis were examined by western blot in order to further elucidate the mechanism of UDCA-induced cell death in BEL7402 xenografts. With the administration of UDCA, the protein levels of BAX, apoptotic protease-activating factor-1 (APAF1), cleaved-caspase-9, and cleaved-caspase-3, proteins executing apoptosis, were up-regulated, whereas BCL2, a protein known to inhibit cell death, was down-regulated. The expression of these proteins furthermore increased with increasing doses of UDCA (Figures 4-6).

***Treatment with UDCA leads to release of cytochrome c into the cytosol***

Release of the mitochondrial protein cytochrome *c* into the cytosol signals the involvement of the mitochondria in programmed cell death. In order to localize cytochrome c during UDCA induced apoptosis, cytosolic and mitochondrial protein extracts were prepared from treated and control xenografts and analyzed by western blotting. UDCA treatment resulted in release of cytochrome *c* into the cytoplasm of BEL7402 relative to control xenografts (Figure 7).

**DISCUSSION**

The prognosis of HCC remains poor worldwide due to a high recurrence rate despite state of the art treatments. Based on previous work *in vitro*, UDCA was investigated here as a potential chemotherapeutic agent for the treatment of HCC *in vivo*. First, the results indicated that UDCA suppressed growth of HCC *in vivo*; continued UDCA administration, initiated one day ahead of implantation of the BEL7402 cells in nude mice, significantly inhibited the growth of xenografts compared to controls. Second, UDCA inhibition of tumor development was mediated in part through apoptosis. Finally, UDCA treatment at the doses used did not lead to the development of life threatening lesions or toxic side effects in the mice. Taken together, results from both *in vitro* and *in vivo* experiments support further investigation of UDCA as a chemotherapeutic agent for HCC.

UDCA, a hydrophilic bile acid, has been previously found to have therapeuticic effects. Firstly, UDCA was recognized as an effective agent in the treatment of primary biliary cirrhosis, from various biochemical and physiological aspects[12]. Secondly, UDCA is known as a cytoprotective agent. UDCA prevents apoptosis induced by a variety of stress stimuli including cytotoxic bile acids such as deoxycholic acid (DCA), and it has been shown to antagonize DCA-induced apoptosis in human colon cancer cells[17,18] and in it is a chemopreventive agent in the azoxymethane model of experimental colonic carcinogenesis[19]. Finally, it has been reported that UDCA and its derivatives induce apoptosis in several cancer cell lines, such as human HCC cells[20], human prostate cancer cells [21], human cervical carcinoma cells[22] and human breast carcinoma cells[23]. Thus, UDCA and its derivatives may inhibit carcinogenesis through different mechanisms in a variety of tissue types.

The appearance of a DNA ladder and the results of the TUNEL assay indicate that the inhibitory properties of UDCA *in vivo* are mediated in part through the induction of apoptosis. Additional experiments determined that molecular components of the apoptotic machinery associated with mitochondria were in fact altered under UDCA treatment. For example, UDCA induced the expression of BAX but down-regulated the expression of BCL2. BAX protein has a proapoptotic effect causing release of cytochrome c[24-26] and increasing outer membrane permeability[27]. In contrast, the BCL2 protein is anti-apoptotic and prevents both the loss of mitochondrial membrane potential and the efflux of cytochrome c[24-26]. Furthermore, BCL2 proteins are directly associated with the mitochondrial membrane and effectively regulate its integrity[28,29]. In addition, cytochrome c was localized in the cytosolic fraction indicating its release from the mitochondria. Cytochrome *c* is a crucial mediator of the pathway, as it leads to the activation of a complex (apoptosome) of caspase-9 and caspase-3 through the adaptor protein APAF1[30,31]. Once released, cytochrome *c* promotes the activation of pro-caspase-9 directly within the apoptosome complex[31,32]. The formation of the APAF1/caspase-9 apoptosome is a crucial event in the apoptotic cascade[33].

Based on these results, UDCA induced apoptosis appears to be mediated through a mitochondrial pathway. The mitochondrial apoptotic pathway has also been reported to play an important role in the apoptosis of other types of human carcinoma cells[34,35]. Thus, the identification of new drugs that stabilize the formation of an active apoptosome complex is a possible strategy for effective treatment of HCC as well as other cancers[36].

In conclusion, oral administration of UDCA was effective in suppressing the growth of BEL7402 xenografts in mice. Our results support further investigation of UDCA as a candidate for the treatment of liver cancer.

**COMMENTS**

***Background***

Hepatocellular carcinoma (HCC) is the seventh most common cancer worldwide, and the third leading cause of cancer-related deaths. Novel treatment strategies are necessary. A candidate therapy is administration of ursodeoxycholic acid (UDCA), which has been shown to be of therapeutic value in liver disease and to induce apoptosis in cancer cell lines. UDCA is effective in the treatment of primary biliary cirrhosis, mediated in part through immunosuppression, and nonalcoholic steatohepatosis. In addition, UDCA has been shown to inhibit the development of colonic carcinogenesis and induced apoptosis specifically in the HCC cells HepG2 and BEL7402, as opposed to a normal human hepatic line L-02 *in vitro*.

***Research frontiers***

HCC is a major cause of morbidity and mortality worldwide. Despite advances in surgical and chemo-radiotherapies, the prognosis remains poor for HCC patients. Therefore, it is crucial to develop novel therapies for the disease. While a number of studies have demonstrated efficacy of UDCA on cancer cells *in vitro*, little is known about the role of UDCA *in vivo*, especially with regard to HCC. This study is the first to evaluate the potential efficacy of UDCA in the treatment of HCC *in vivo*.

***Innovations and breakthroughs***

Previous work from our group has established that UDCA selectively inhibits proliferation and induces apoptosis of the human HCC cell lines HepG2 and BEL7402 in comparison to the normal human hepatic cell line L-02 *in vitro*. This study is the first to evaluate the potential efficacy of UDCA as a chemotherapeutic agent through oral administration in BALB/c nude mice bearing sc xenografts derived from BEL7402. Through several state of the art methods, such as DNA ladder detection, TUNEL, and western blot analysis, UDCA was found to suppress tumor growth and induce apoptosis through a mitochondrial pathway. Further delineation of the molecular basis of the effect might be exploited in the future to develop additional therapies for HCC.

***Applications***

UDCA has been previously established to elicit therapeutic effects in the treatment of liver diseases, such as primary biliary cirrhosis and nonalcoholic steatohepatosis. Recent studies have also indicated that UDCA reduced the frequency of colonic carcinogenesis and induced apoptosis of HCC cells *in vitro*. Our results further demonstrate that UDCA suppresses development of liver cancer *in vivo*. The results thus indicate that patients with HCC might benefit from treatment with UDCA as a chemotherapeutic agent.

***Terminology***

Ursodeoxycholic acid, a hydrophilic bile acid produced by intestinal bacteria, has been found to have several therapeutic effects.

***Peer-review***

The aim of the study is to evaluate the potential efficacy of UDCA as a chemotherapeutic agent for the treatment of HCC. Oral administration of UDCA suppressed the growth of xenografts derived from the HCC cell line BEL7402 in BALB/c nude mice. The research findings are of interest to the scientific and medical community as the prognosis of the large number of people affected worldwide by this disease remains poor. The data presented are clear and support the conclusions.

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 **A**



 **B**

**Figure 1 Ursodeoxycholic acid suppresses tumor growth *in vivo*.** A:Tumorvolume is plotted as a function of time in days. Tumor volumes plotted represent the median value of xenografts from 10 animals in each group; B: The median value of the tumor volume for each group after 21 d of treatment are graphically depicted. Differences between control and treated xenografts at 21 d reached statistical significance (*P* < 0.05)at all ursodeoxycholic acid (UDCA) doses (30 mg/kg/d, a*P* < 0.05; 50 mg/kg/d, b*P* < 0.05; 70 mg/kg/d, c*P* < 0.01).



**Figure 2 Ursodeoxycholic acid induces a DNA ladder in hepatocellular carcinoma xenografts in mice.** Genomic DNA was isolated from xenografts and electrophoresed on 1.5% agarose gels to determine integrity of the DNA. M: DNA markers; lane 0: control; lanes 1-3: ursodeoxycholic acid (UDCA) 30 mg/kg/d, UDCA 50 mg/kg/d, and UDCA 70 mg/kg/d.



**Figure 3 Systemic administration of ursodeoxycholic acid induces apoptosis in BEL7402 xenografts in immunodeficient mice.** A: TUNEL staining performed to detect apoptotic cells *in situ* in tissue sections prepared from control and treated xenografts as indicated (magnification × 400); B: Apoptotic cell numbers from control and treated xenografts represented graphically. Data are presented as the mean ± SD for 1000 cells in randomly selected fields on three sections for each group. Differences between control and treated xenografts reached statistical significance (30 mg/kg/d, a*P* < 0.05; 50 mg/kg/d, e*P* < 0.05; 70 mg/kg/d, f*P* < 0.01).



**Figure 4 Expression of cleaved-caspases 3 and 9 is induced in response to ursodeoxycholic acid in xenografts.** Western blot analysis performed with protein lysates prepared from control and treated tumors. Blots were incubated with antibody against cleaved-caspases 3 and 9, and b-actin was used as an internal control. 0: control; lanes 1-3: ursodeoxycholic acid (UDCA) 30 mg/kg/d, UDCA 50mg/kg/d, and UDCA 70mg/kg/d.



**Figure 5 Ursodeoxycholic acid induces expression of APAF1.** Western blot analysis for the expression of APAF1 was performed with protein lysates prepared from control and treated xenografts. 0: control; 1-3 lanes: ursodeoxycholic acid (UDCA) 30 mg/kg/d, 50 mg/kg/d, and UDCA 70 mg/kg/d. β-actin was used as an internal control.



**Figure 6 Ursodeoxycholic acid induces BAX but suppresses BCL2 protein levels.** Western blot analysis for the expression of BAX and BCL2 proteins was performed with protein lysates prepared from control and treated xenografts. 0: control; lanes 1-3: ursodeoxycholic acid (UDCA) 30 mg/kg/d, UDCA 50 mg/kg/d, and UDCA 70 mg/kg/d. β-actin was used as an internal control.



**Figure 7 Cytochrome *c* is localized to the cytosolic fraction in response to ursodeoxycholic acid.** Western blot analysis for the localization of cytochrome c was performed with protein lysates prepared from cytosolic and mitochondrial cellular fractions in control and treated xenografts. β-actin and oxidative complex I were used as marker and loading controls for cytosolic and mitochondrial fractions respectively.