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Thesis for the Degree of Master of Science

Hepatoprotective effect of ursodeoxycholic acid on tacrine-induced HepG2 cells

By

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Department of Food and Life Science

The Graduate School

Pukyong National University

February 2009

Hepatoprotective effect of ursodeoxycholic acid on tacrine-induced HepG2 cells

Tacrine에 의해 손상된 HepG2 cell에 대한 ursodoxycholic acid의 보호기전

Advisor: Prof. Hyeung-rak Kim

By

Ling Wu

A thesis submitted in partial fulfillment of requirements
For the degree of

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Pukyoung National University

February 2009

Hepatoprotective effect of ursodeoxycholic acid on tacrine-induced HepG2 cells

A dissertation

By

Ling Wu

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February 2009

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Hepatoprotective effect of ursodeoxycholic acid on tacrine-induced HepG2 cells

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Abstract

친수성 담즙산의 일종인 UDCA는 여러 가지 간의 질병, 말하자면 원발성 담즙성 간경변증 (PBC), 일차성 경화성 담관염 (PSC), 만성 C형 간염 등에 치료제로 널리 사용되고 있다. 콜린에스테라제의 길항적 저해제의 하나인 타크린은 치매치료제로써 간 세포독성을 지니고 있는 것으로 알려져 있다. UDCA를 간암세포 HepG2에 대한 독성확인을 위해 25μM, 50μM, 100μM의 농도로 UDCA를 처리한 결과 독성이 없음을 확인하였다. 타크린 0.2 mM을 UDCA 25μM, 50μM, 100μM와 동시에 처리한 결과 UDCA가 농도 의존적으로 간 세포보호 효과가 나타났음을 관찰할 수 있었다. 또 UDCA의 처리에 의하여 타크린이 일으킨 PARP, caspase 3와 caspase 9의 쪼개짐이 농도 의존적으로 억제 되였다는 것을 알 수 있었다. UDCA는 항암작용을 가지고 있는 PI3K의 발현을 증가시킴으로써 pAkt도 증가하는 것을 관찰할 수 있었고 또 다른 cell survival signal pathway인 NFκB를 활성화시킴으로써 apoptosis저해 단백질 가족인 IAP family의 발현을 증가시켰다. 나아가서는 Bcl-2 family인 Bad,

Bax의 발현을 효과적으로 억제하고 Bcl-x_L, Bcl-2의 발현양을 증가시킴으로써 미토콘드리아의 구멍의 뚫림에 의하여 생긴 Cytochrome c가 cytosol내로의 방출을 막아준다. 따라서 타크린이 HepG2에 일으킨 간 독성에 대한 UDCA의 간 세포보호 효과는 PI3K/pAkt의 신호전달 경로를 통한 전사인자의 발현과 이들 전사인자의 downstream에 존재하는 Bcl-2 family인 Bad, Bax의 발현억제와 Bcl-x_L, Bcl-2의 발현증가를 통하여 결과적으로 DNA repair작용을 하는PARP의 쪼개짐을 억제하는 것으로 판단된다.

1. Introduction

Ursodeoxycholic acid (UDCA, identical to ursodiol), a dihydroxy bile acid, is a major constituent of dried bile of the Chinese black bear (Dinesh & Rakesh, 2001). In black bear's bile, UDCA is the major bile acid (Lee *et al*., 1993), when it is only about 3% of total bile acids in human bile (Gustav *et al*., 2002). The chemical form of UDCA from the Chinese black bear's bile was first defined by Shoda in 1927. It has two hydroxy groups at 3α and 7β positions (Alvarez *et al*., 2007) and called 3α, 7β-dihydroxy-5β-cholanic acid. The chemical structure of UDCA is shown in Figure 1.

There are two primary bile acids (cholic acid, CA and chenodeoxycholic acid, CDCA) in humans, which are synthesized from cholesterol in liver and stored in the gall bladder (Paul *et al*., 2005). When excreted into the colon, primary bile acids can be converted to secondary bile acids by intestinal bacteria. That is, cholic acid is converted to deoxycholic acid (DCA), while chenodeoxycholic acid is converted to lithocholic acid (LCA) and UDCA (Kaoru *et al*., 2005) Furthermore, some of these endogenous bile acids are well known to be conjugated to glycine or taurine, which are water soluble, amphipathic, membrane-impermeable end products of cholesterol metabolism. (Alan *et al*., 1999)

It has been already reported in several studies that bile acids show either cytoprotective or cytotoxic effects, according to bile acid species. (Michihiro *et al*., 2007., Joohyun *et al*., 2003., Anabela *et al*., 2004) The hydrophilic properties of these bile acids depend on the number, position, and orientation of the steroid hydroxyl group(s). It has been shown that

cytoprotective or cytotoxic effect of bile acids depends on its hydrophilicity (Sagawa *et al*., 1993; Komichi *et al*., 2003). Hydrophilic bile acids, such as UDCA and its taurine conjugates TUDCA, appear to protect against cholestasis and the toxicity induced by the hydrophobic bile acid, such as deoxycholic acid.(Rodrigues *et al*., 1998; Marieke *et al*., 2004)

The liver plays a major role in metabolism, for example, converting nutrients derived from food into essential blood components, storing vitamins and minerals, regulating blood clotting, producing proteins and enzymes, maintaining hormone balances, and metabolizing and detoxifying substances which are harmful to the body. Some hepatocytotoxins such as carbon tetrachroride (CCl_4) (Teruo *et al*., 2005), ethanol (Wu *et al*., 2006), tacrine, acetaminophen (Priyanka *et al*., 2008; Milagros *et al*., 2000), along with lipopolysaccharide (Asuka *et al*., 1999) have been investigated for hepatotoxic research.

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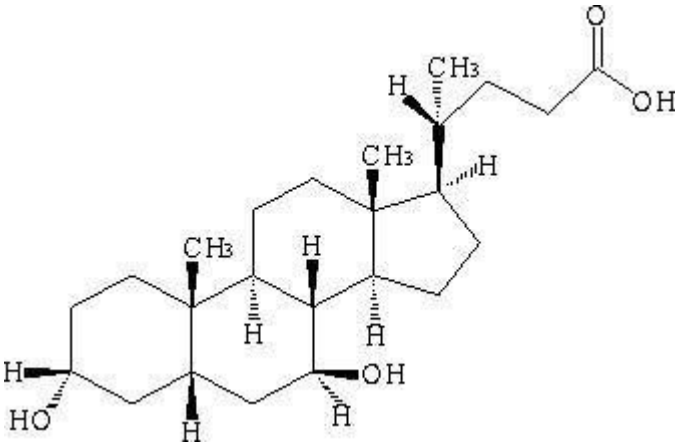

Fig.1. Chemical structure of ursodeoxycholic acid

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Tacrine (1, 2, 3, 4-tetrahydro-9-aminoacridine; THA) acts as a cholinesterase competitive inhibitor to block the degradation of acetylcholine in the neurons of cerebral cortex thereby increasing cholinergic transmission (Osseni et al. 1999; Wu and Yang *et al*., 1989 and Hunter *et al*., 1989). Although it is the first drug licensed in the USA and Europe for the treatment of Alzheimer's disease (Eagger *et al*., 1991), hepatotoxicity occurred in 30~50% of patients who took the medicine for along time in therapeutic doses (Watkins *et al*., 1994), the mechanism of which remains to be not fully established. Since intracellular glutathione represents an important protective factor against oxidative damage, the alteration of intracellular glutathione concentration by tacrine in cultured hepatocytes (Lagadic-Gossmann *et al*., 1998) might suggest the involvement of generation of reactive oxygen species (ROS). A mitochondrial dysfunction such as its membrane potential decrease and stimulation of its respiration elicited by THA has been reported by Berson *et al*. (1996) and Robertson *et al*. (1998). It has been always argued that tacrine induces hepatotoxicity with liver cell necrosis (Knapp *et al*., 1994), or with cell apoptosis (Debbasch *et al*., 1998; Fariss *et al*., 1996).

UDCA has been reported to protect hepatocytes, hepatoma cells, osteogenic sarcomas and Hela cells from apoptosis induced by several stimulant, for example, okadaic acid, hydrogen perosxide and more hydrophobic bile acids (Rodrigues *et al*., 1999; Mitsuyoshi *et al*., 1999) The mechanism of the beneficial effect of UDCA on tacrine-induced hepatotoxicity has not been clear. A major survival pathway, the activation of the transcription factor nuclear factor (NF)-κB was considered in this study. Besides inducing the expression of pro-survival

Bcl-2 members, NFκB additionally transactivates a number of other antiapoptotic genes, such as IAPs (inhibitors of apoptosis proteins) (Gewies, 2003). The IAP family including cIAP1/2, XIAP, Survivin and Livin was originally discovered in baculovirus and subsequently identified in human cells (Schoemaker *et al*., 2002). XIAP plays an important role in cell survival as a member of IAP family, and characterized by baculoviral IAP repeat domains, which can inhibit caspase 3 and caspase 9 acitivity by direct binding (Deveraux *et al*., 1997 and 1999; Riedl *et al*., 2001)

Another survival pathway is the phosphatidylinositol-3 kinase (PI3K) pathway (Fig. 2). This kinase cascade leads to activation of a number of cellular intermediates, of which a serine threonine protein kinase (Akt) is one of the most important survival factors (Datta *et al*., 1999). Akt activation protects against apoptosis via several mechanisms, including the phosphorylation of pro-apoptotic Bcl-2 family member Bad, which can inhibit heterodimerization of Bad with Bcl-x_L and Bcl-2 in BH3 domain to prove its anti-apoptotic function (Zha *et al*., 1997). The endogenous Bcl-2 family oncoproteins is one of the important apoptotic regulators, whose members have both pro- and anti-apoptotic effect. For instance, the previously mentioned Bcl-2 and Bcl-x_L are the two most important anti-apoptotic proteins while Bax, Bad, Bak, Bid, Bik and Bcl-x_s represent the principal pro-apoptotic ones (Reed *et al*., 1998). Bid had been shown to directly cause release of cytochrome c in cell free systems (Luo *et al*., 1998), either help chaperone Bax to the mitochondria, or by directly allosterically modifying Bax, promotes its insertion into the outer mitochondrial membrane, a requisite step for Bax-induced cytochrome c release (Hajime *et al*., 2001)

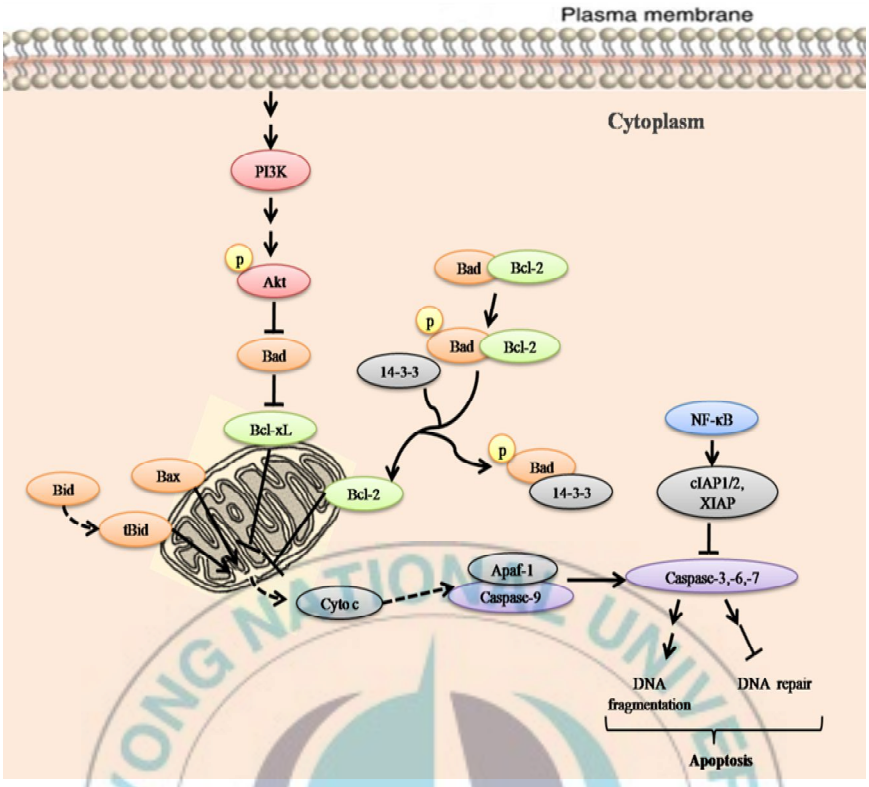

Fig. 2 Potential pathway

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In this study, an human hepatoma cell line, HepG2 was selected to investigation for UDCA that protect against tacrine-induced hepatotoxicity. HepG2, a well differentiated transformed cell line which retains many cellular functions often lost by primary hepatocytes, is a reliable model, easy to culture, well characterized, and widely used for biochemical and nutritional studies where many antioxidants and conditions can be assayed with minor interassay variations (Alía *et al*., 2005).

In the present report we want to investigate the protective effect of UDCA on tacrine-induced hepatotoxicity in HepG2 cells and the related cell signaling mechanism.

2. Materials and Methods

2-1. Materials

MEM (Minimum essential medium), penicillin-streptomycin mixture, 0.25% tripsin-EDTA, fetal bovine serum (FBS) were purchased from HyClone Laboratory Inc. (Utah, USA), sodium pyruvate, non-essential amino acid were purchased from Gibco BRL (Gaithersburg, MD), Celltiter 96 AQ one solution Cell Proliferation Assay kit was purchased from Promeaga Co. (WI, USA), and DMSO (dimethyl sulfoxide) was purchased from Sigma Chemical (St. Louis, MO). Culture dish, 6-well plate, 96-well plate, centrifuge tube and scrapper were purchased from Corning Incorporated (Corning, NY), Protein standard marker was purchased from Amersharm Pharmacia (Piscataway, NJ) and the enhanced chemiluminescence (ECL) detection kit was purchased from Perkin Elmer Life Science (Wellesley, MA, USA). Polyclonal antibodies against PI3K, PARP, pAkt, caspase-3, -9, Bad, Bcl-xL, Bcl-2, cytochrome c, Actin, and secondary antibodies, horse radish peroxidase conjugated anti-mouse IgG, anti-rabbit IgG, and anti-goat IgG were purchased from Santa Cruz Biotechnology (CA, USA). XIAP and cleaved caspase-3 were purchased from Cell Signaling Technology (Beverly, MA). Tacrine was provided by Professor JaeSue Choi in Pukyoung National University.

2-2. Methods

2-2-1. Cell culture and treatment

HepG2 cells (ATCC, MD, US) were maintained in MEM containing 2.0 mM L-glutamine, 0,1 mM non-essential amino acids, 1.0 mM sodium pyruvate, penicillin-streptomycin (100 U/ml penicillin and 100

μg/ml streptomycin in 0.85% saline) and 10% heat-inactivated FBS at 37℃ in 95% air-5% CO_2. The medium was replaced twice a week and cells were trypsinized and diluted at 1:3 ratio every 3 days. For experiments, confluented cells were cultured in medium without FBS for 24 hrs, and were coincubated with tacrine and UDCA (both dissolved in DMSO) at various concentrations and periods of time, with PBS and DMSO for control. The final concentration of DMSO did not exceed 0.5%. UDCA was reconstituted as 100 mM stock solutions in DMSO, while tacrine was prepared in 40 mM PBS, filter sterilized, and stored at -20℃.

2-2-2. Cell viability and hepatoprotection assay

HepG2 cells were seeded onto 96-well plates at a density of 10,000 cells/well in MEM plus 10% FBS. After 24 hrs, Vehicle or 10, 25, 50,100, and 200 μM UDCA were treated to cells in serum-free MEM medium. To confirm cytotoxicity of tacrine on HepG2 cells, cells were loaded with 0.1, 0.2, 0.4, 0.8, and 1.2 mM tacrine. Celltiter 96 AQ one solution Cell Proliferation Assay kit was diluted at 1:20 in serum free MEM medium, and 100 μl solution was added to each well of 96-well plates. Cells were incubated in a humidified incubator at 37 ℃ for 2 hrs. Absorbance of each well was measured with ELISA reader at 490 nm.

For assessment of hepatoprotective effect of UDCA on tacrine-induced HepG2 cells, cells were coincubated with 0.2 mM tacrine and UDCA (25-100 μM). The controls were treated with 0.5% DMSO. Celltiter 96 AQ one solution Cell Proliferation Assay kit was used again for the UDCA hepatoprotection.

2-2-3. Morphological analysis

HepG2 cells were plated onto 24-well plate and grown to 80% confluence. After 24 hrs' FBS starvation, cells were treated with 0.2 mM tacrine and 25, 50, and 100 μM UDCA for 24 hrs. Cells were washed with PBS twice and observed under light microscopy. Magnification, × 200.

2-2-4. Hoechst 33342 staining

To observe nuclear morphologic changes after tacrine and UDCA treatment, Hoechst 33342 staining was performed. Sample treatmented cells were fixed with 1ml fixing solution(formaldehyde:PBS = 1:9) at room temperature for 10 min and then washed once with PBS. Cells were covered with 500 μl of 0.2% Triton X–100 in PBS and incubated at room temperature for 10 min. The Triton X–100 was removed and cells were washed with 1ml of PBS to remove any excess. Two μg/ml solution of Hoechst 33342 was added to each well, incubated for 30 min and protected from light, then washed with PBS. 100% EtOH was added to each well for removing water. One drop of mounting medium was supplemented to cells. Nuclear morphology was photographed with a fluorescence using blue filter. Magnification, ×200

2-2-5. DNA isolation and documentation of DNA fragmentation

After tacrine (0.2 mM) plus UDCA (25-100 μM) treatment for 24 hrs, the agarose electrophoresis for DNA fragmentation from cells was performed as previously described (Anabela et al., 2004) and aliquots (3 μg) of isolated DNA were separated by 2% agarose gel, and visualized with an ultraviolet transluminator.

2-2-6. Western Blot analysis

2-2-6-1. Preparation of total cell lysates

HepG2 cells were washed twice with ice-cold PBS, scraped with lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet-40, 1% Tween-20, 0.1% SDS, 1mM Na_3VO_4, 10μg/ml leupeptin, 50 mM NaF, 1 mM PMSF) and stood on ice for 1 hr for lysis. After centrifuge at 18,000 g for 10 min, protein content of supernatant was measured, and aliquots (40 μg) of protein were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% non-fat dried milk in TBST buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.06% Tween-20) for 1 hr and incubated for 2 hrs with primary antibody in TBST buffer containing 5% non-fat dried milk. The blots were treated with horseradish peroxidase-conjugated secondary antibody in TBST buffer containing 5% non-fat dried milk for 1 hr, and immune complex was detected using ECL detection kit.

2-2-6-2. Purification of mitochondrial and cytosolic extracts

Cytosolic and mitochondrial extracts were prepared as described previously (Emanuele *et al.,* 2004). Briefly, HepG2 cells were washed twice with PBS and harvested by centrifugation (500 g for 5 min). Cell pellets were resuspended in lysis buffer A (20 mM Hepes, 1.5 mM $MgCl_2$, 10 mM KCl, 2 mM EDTA, 1 mM dithiothreitol and 250 mM sucrose) containing protease inhibitor cocktail by pipetting up and down gently with a pipette, and then centrifuged at 2000 g for 10 min at 4℃ Supernatant (S1) was collected and the pellet again resuspended in buffer A to obtain a new supernatant (S2). S1 and S2 were mixed and centrifuged at 14,000 rpm for 10 min at 4℃. The pellet and the

supernatant represent mitochondrial and cytosolic fractions, respectively.

2-2-6-3. Nuclear extraction

Nuclear extracts were prepared as described previously (Schoonbroodt *et al*., 1997). Cells were washed twice with PBS and harvested by centrifugation (1,200 rpm for 5 min). Cell pellets were resuspended in hypotonic buffer (10 mM HEPES/KOH, 10 mM KCl, 2 mM MgCl2, 0.1 mM EDTA, 1 mM DTT, and 0.5 mM PMSF, pH 7.9), leftonice for 15 min, vortexed, and centrifuged at 13,000 rpm for 1 min. The nuclearpellet were gently resuspended in 20 ml of ice cold saline buffer (50 mM Hepes/KOH, 50 mM KCl, 1 mM DTT, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, and 0.5 mM PMSF, pH 7.9), left ice for 30 min, vortexed, and centrifuged at 13,000 rpm for 20 min at 4 ℃.

2-2-7. Statistical analysis

All data are expressed as the mean ± SEM. Data were analyzed using one-way ANOVA or Duncan's multiple range test. Differences were considered significant of $P < 0.05$. All analyses were performed using SPSS (Statistical Package for Social Science) for Windows, version 10.07 (SPSS, Chicago, IL).

3. Results

3-1. Effect of ursodeoxycholic acid on HepG2 cells

3-1-1. Ursodeoxycholic acid increases HepG2 cell viability.

To examine the cell viability of UDCA on HepG2 cells, cells were cultured to 96-well plates at a density of 10,000 cells/ml. 24 hrs later, Vehicle or 10, 25, 50,100, and 200 μM UDCA were treated to cells in serum-free MEM medium. After another 24 hrs' incubation, cell viability was determined by MTS assay. Fig. 3 shows that there weren't any decreases in UDCA-treated HepG2 cell viability. This result suggests that there were no inhibitory effects of UDCA at any concentrations on HepG2 cell proliferation.

3-1-2. Tacrine induces cytotoxicity on HepG2 cells.

To determine the appropriate concentration of tacrine required to induce HepG2 cells, the cytotoxicity of tacrine to HepG2 cells was measured by MTS assay. As shown in Fig. 4, such a tacrine-induced reduction in cell viability was detected in a dose-dependent pattern. Compared with the control, the treatment of cells to tacrine at 0.2 mM led to a 50% decrease of cell viability in HepG2 cells at 24 hrs. According to this result, 0.2 mM tacrine was used in the following experiments.

3-1-3. Ursodeoxycholic acid protects HepG2 cells from on tacrine-induced cytotoxicity.

To ascertain whether UDCA has a hepatoprotective effect on tacrine-induced HepG2 cells, cells were cultured and exposed with 0.2

mM tacrine and various concentrations of UDCA, concurrently, for 24 hrs. The cell viability was also quantitated by MTS assay. As illustrated in Fig. 5, the more the concentration of UDCA increased, the more the viability of tacrine-induced HepG2 cells enhanced. It means that UDCA has a protective effect on tacrine-elicited HepG2 cells in a dose-response manner.

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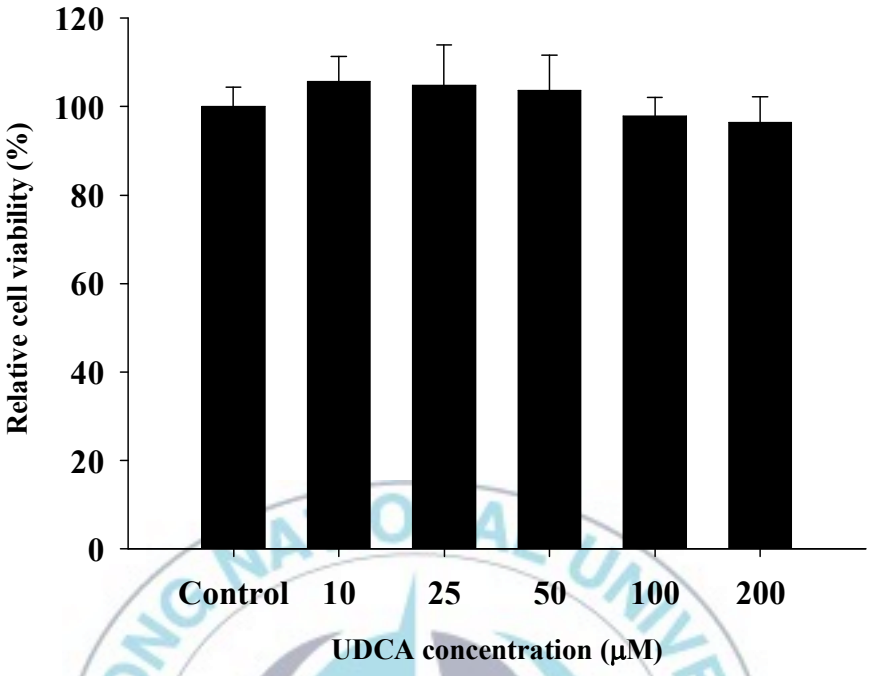

Fig. 3 Cell viability of ursodeoxycholic acid on HepG2 cells

HepG2 cells were preincubated without FBS for 24 hrs at 37℃, and treated with various concentrations of UDCA. Cell viability was assessed by measuring MTS reduction with ELISA reader at 490 nm.

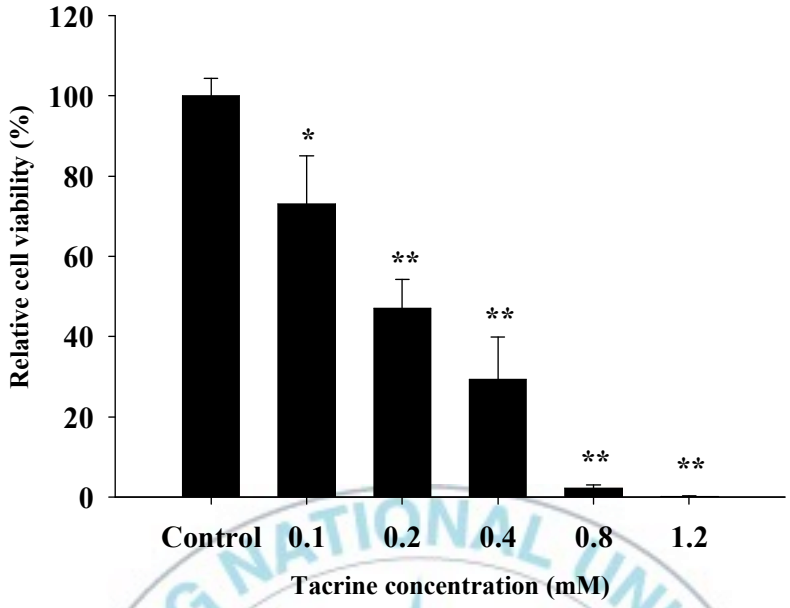

Fig. 4 Cytotoxicity of tacrine on HepG2 cells

HepG2 cells were preincubated without FBS at 37℃, and treated with various concentrations of tacrine for 24 hrs. Cell viability was assessed by measuring MTS reduction with ELISA reader at 490 nm. Values statically different are indicated by *($p<0.05$), **($p<0.01$).

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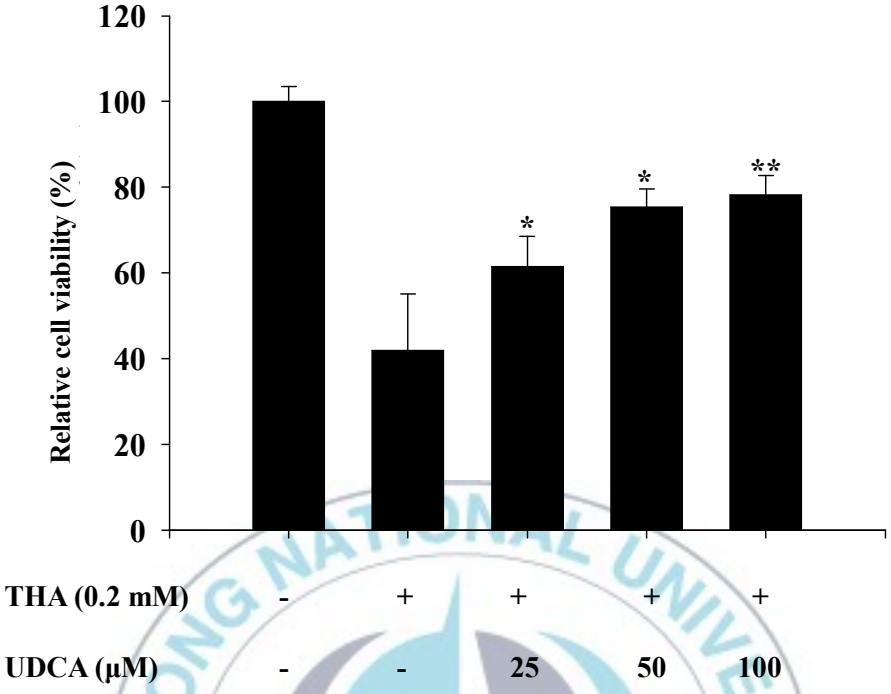

Fig. 5 The protective effect of UDCA on tacrine-induced HepG2 cells

HepG2 cells were starved with FBS at 37℃, and coincubated with 0.2 mM of tacrine and different concentrations of UDCA for 24 hrs. Cell viability was assessed by measuring MTS reduction with ELISA reader at 490 nm. Values statically different are indicated by *($p<0.05$), **($p<0.01$).

3-1-4. UDCA suppresses tacrine-induced HepG2 cell morphological change.

As described previously, when UDCA was supplemented to tacrine-induced HepG2 cells, the cell viability was increased. Morphological changes observed by phase-contrast microscopy (Fig. 6) shows that, when cells were treated with 0.2 mM tacrine, they rounded up and detached from the tissue culture dish. Conversely, UDCA suppressed these morphological changes as its concentration increased.

3-1-5. UDCA suppresses tacrine-induced HepG2 cell nuclear morphological change.

To explore the potential mechanisms by which UDCA inhibited tacrine-evoked cell death, we further determined whether UDCA prevented tacrine-induced nuclear fragmentation with chromatin condensation in HepG2 cells by Hoechst 33342 staining. Cells were treated as previously described, and tacrine caused cell nuclear morphological change was observed by Hoechst 33342 staining and quantified by using fluorescence microscopy. As shown in Fig. 7, HepG2 cells who were treated with tacrine exhibited numerous fragmented nuclei, and cells coincubated with tacrine and UDCA displayed that the numbers of fragmented nucleus decreased gradually in UDCA dose-dependent pattern.

3-1-6. UDCA inhibits tacrine-induced DNA fragmentation in HepG2 cells.

As a criterion to distinguish the hepatotoxicity induced by tacrine occurred through between apoptosis and necrosis, DNA fragmentation

was determined. Consistent with the previous nuclear changes, cells treated with 0.2 mM tacrine alone as a negative control or 0.2 mM tacrine plus 25, 50, and 100 μM UDCA for 24 hrs started to show DNA fragmentation by 2% gel electrophoresis (Fig. 8). In UDCA plus tacrine-treated cells, the level of tacrine-induced DNA fragmentation became weak at 50 μM of UDCA treatment. Taken together, these results suggest that tacrine inhibited the proliferation of HepG2 cells through apoptotic death induction, and UDCA protected HepG2 cells from these effects in dose-dependent manner.

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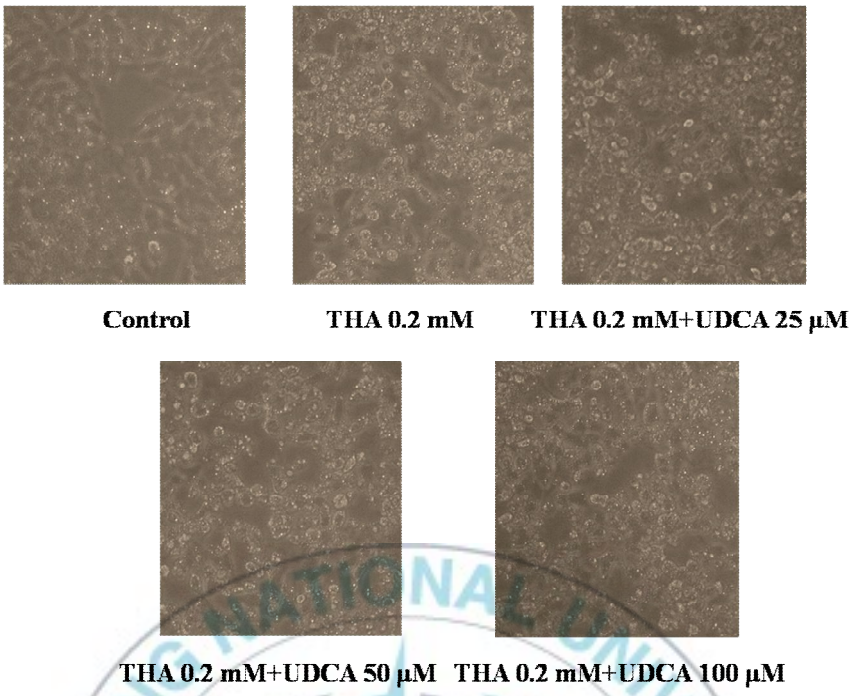

Fig. 6 Effect of UDCA on tacrine-induced HepG2 cell morphology

Morphological changes by treatment with 0.2 mM tacrine and 25,50, and 100 μM UDCA in HepG2. After 24 hrs incubation, cells were examined under light microscopy. Magnification, × 200.

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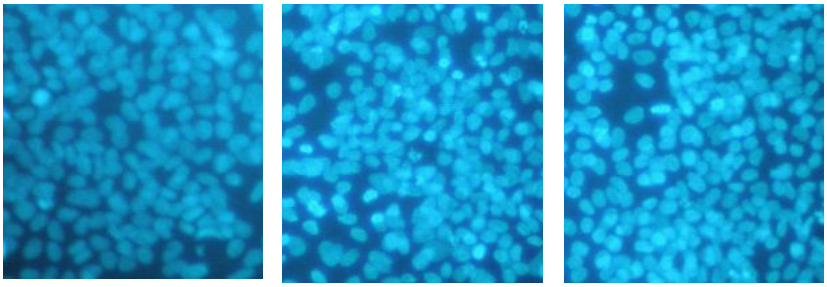
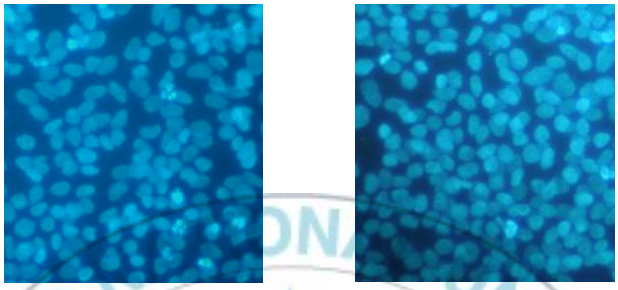


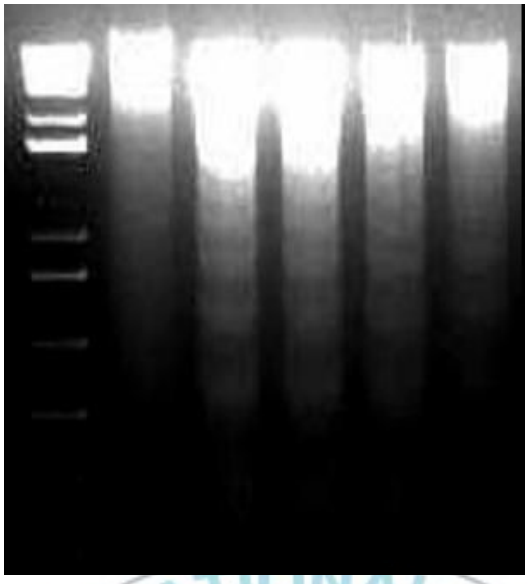
Control THA 0.2 mM THA 0.2 mM+UDCA 25 μM



THA 0.2 mM+UDCA 50 μM THA 0.2 mM+UDCA 100 μM

Fig. 7 Nuclear morphology of UDCA protected HepG2 cells

Morphological changes by treatment with 0.2 mM tacrine and 25,50, and 100 μM UDCA in HepG2. After 24 hrs incubation, cells were examined under light microscopy. Magnification, × 200.



THA (0.2 mM)	**-**	**+**	**+**	**+**	**+**
UDCA (μM)	**-**	**-**	**25**	**50**	**100**

Fig. 8 Effect of UDCA on tacrine caused DNA fragmentation in HepG2 cells

Cells were coincubated with 0.2 mM tacrine and various concentrations of UDCA. 3 μg of DNA fraction was seperated on 2% agarose gel. The first lane is standard DNA marker. UDCA inhibited tacrine-induced DNA fragmentation.

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3-2. Western blot analysis

3-2-1. UDCA protects HepG2 cells from tacrine caused PARP cleavage and caspase 3, caspase 9 activation.

We have previously shown that UDCA increased cell proliferation, inhibited DNA fragmentation and repressed chromatin condensation and the typical morphological changes in HepG2 cells which were induced by tacrine treatment. It has been reported that caspase 3 is the most efficient processing enzyme for PARP (Tewari *et al*., 1995). To further ensure that the variations of the levels of the related proteins expression, western blot analysis was then adopted in the experiment. The UDCA effect of inhibition of PARP cleavage caused by 0.2 mM tacrine was shown from Fig. 9. It also shows that compared the tacrine plus UDCA supplemented cells with the tacrine treated alone cells, the level of pro-caspase 3 was increased, while cleaved caspase 3 (p17 and p19) was decreased. Because caspase 9 activates caspase 3 via the cleavage of the procaspse 3, we examined whether UDCA affect the activity of caspase 9. As expected, the marker of caspase 9 activity-the expression of cleaved caspase 9 was decreased by UDCA in dose-response manner. At the same time, the protein of procaspase 9 was increased in the same pattern. Thus, the results are consistent with the view that the UDCA expression leads to attenuated activity of the cleaved caspase 3, caspase 9 and the cleavage of PARP induced by 0.2 mM tacrine.

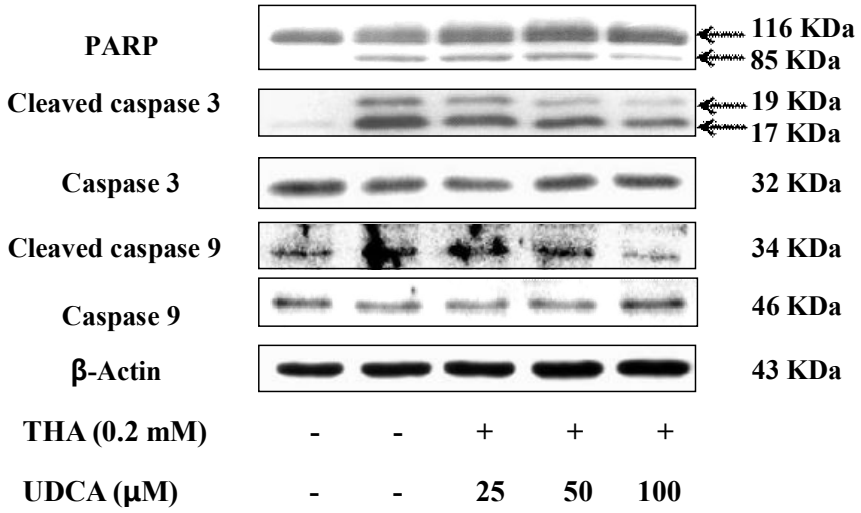

Fig. 9 Western blot analysis of PARP, caspase 3 and caspase 9 expression

HepG2 cells were starved with FBS at 37℃, and coincubated with 0.2 mM tacrine and various concentrations of UDCA. UDCA inhibited tacrine-induced cleavage of PARP, caspase 3 and caspase 9 in HepG2 cells. β-actin, the housekeeping gene, was used as an internal control.

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3-2-2. UDCA protects tacrine-induced hepatotoxicity in HepG2 cells through PI3K/Akt cell survival pathway.

For the phosphatidylinositol 3-kinase (PI3K)/Akt signal transduction pathway plays a central role in intracellular processes such as cell survival, proliferation, angiogenesis and motility (Mariana *et al*., 2008), we further determined the levels of PI3-kinase and phospho-Akt. Fig. 10 shows that comparing with the 0.2 mM tacrine treated cells, the levels of the cell survival gene-PI3K and its downstream protein pAkt were significantly enhanced by UDCA expression.

3-2-3. UDCA increases the expression of NFκB in nuclear and cytosol and XIAP in whole cell lysates in tacrine plus UDCA treated HepG2 cells

Next, we analyzed the expression of pAkt downstream protein, NFκB, which activates transcription of anti-apoptotic genes (Mariana *et al*., 2008), and one of the Bcl 2 family protein, Bid. As shown in Fig. 11, inclusion of UDCA in the tacrine-induced HepG2 cells caused a dose-dependent increase in the expression of NFκB in cytosolic fraction and decrease in nuclear extract and total Bid. As demonstrated before, XIAP is probably the most potent apoptosis inhibitor, so the UDCA-evoked stabilization of XIAP (Fig. 11) would render cells resistant to apoptosis that induced by 0.2 mM tacrine. Taken together, these data suggest that the IAP family protein dependent PI3K/Akt pathway is involved in the survival of hepatocarcinoma HepG2 cell lines.

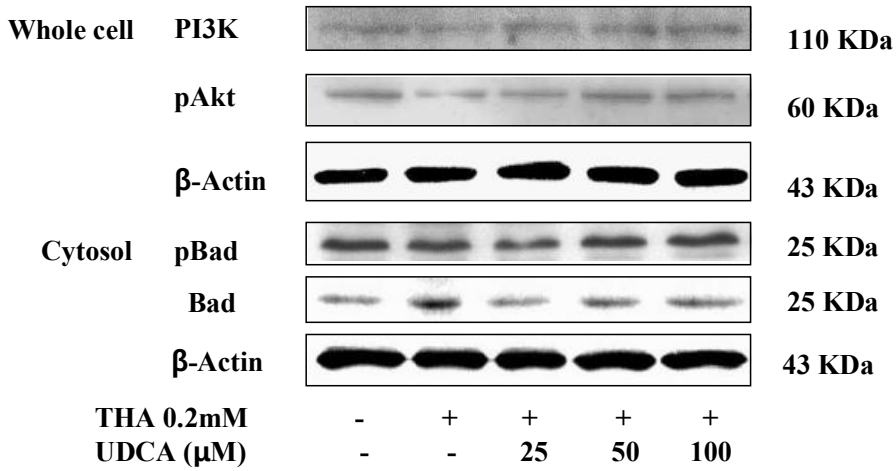

Fig. 10 UDCA protects HepG2 cells from tacrine treatment by activating PI3K/Akt signal pathway.

Cells were coincubated with 0.2 mM tacrine and various concentrations of UDCA. β-actin was used to control for loading differences.

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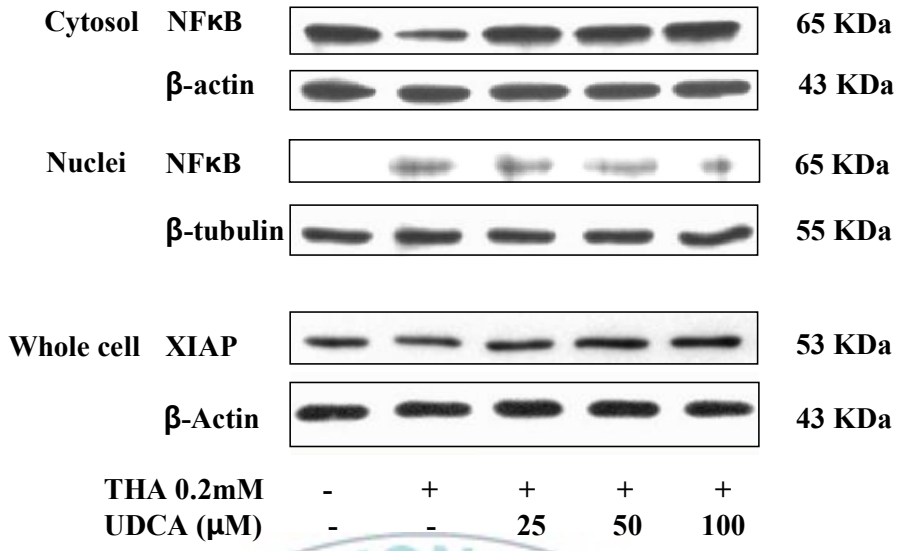

Fig. 11 Expression and regulation of NFκB and XIAP in response to UDCA on tacrine-treated HepG2 cells

Cells were coincubated with 0.2 mM tacrine and various concentrations of UDCA. β-actin was used to control for loading differences. Aliquots (40 μg) of protein were separated by SDS-PAGE and transferred onto a nitrocellulose membrane, and immune complex was detected using ECL detection kit. β-actin was shown for the control blot.

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3-2-4. UDCA regulates the expression of Bcl-2 family proteins and cytochrome c in cytosolic and mitochondrial level in tacrine plus UDCA treated HepG2 cells.

During apoptosis, cytochrome c is released from mitochondria into the cytoplasm (Li *et al*., 1997). Increased amount of cytochrome c was detected in the cytosolic fraction when 0.2 mM tacrine was treated to HepG2, and UDCA treatment inhibited its release in dose-dependent pattern (Fig. 12). We in turn assessed the affect of UDCA treatment on the cytosolic expression of Bcl-2 related proteins found in tacrine-induced HepG2 cells. Expression levels of Bcl-2, Bcl-x_L, Bax was measured by Western Blot. As the UDCA concentration was increased, a progressive loss of Bcl-2 and Bcl-x_L was observed in cytoplasm. These results indicate that tacrine-induced cytochrome c release from mitochondria into cytoplasm in HepG2 cells can be directly controlled by Bcl-2 family proteins regulated by UDCA.

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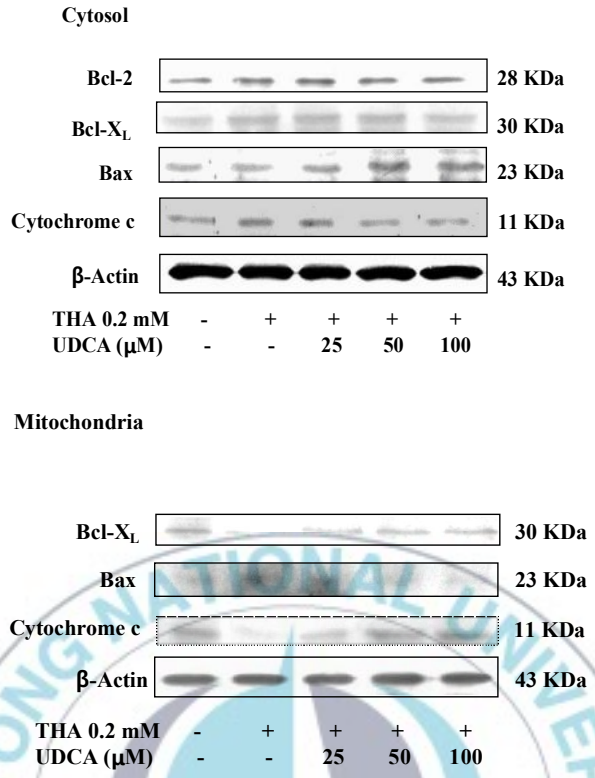


Fig. 12 Effect of UDCA treatment on the levels of Bcl 2 family proteins and cytochrome c in tacrine-induced HepG2 cells

Tacrine treated cells were fractionated by cytosol and mitochondrial lysis buffer. One hundred μg of cytosolic, 30 μg of mitochondrial proteins were seperated by SDS-PAGE. β-Actin was used as an internal control.

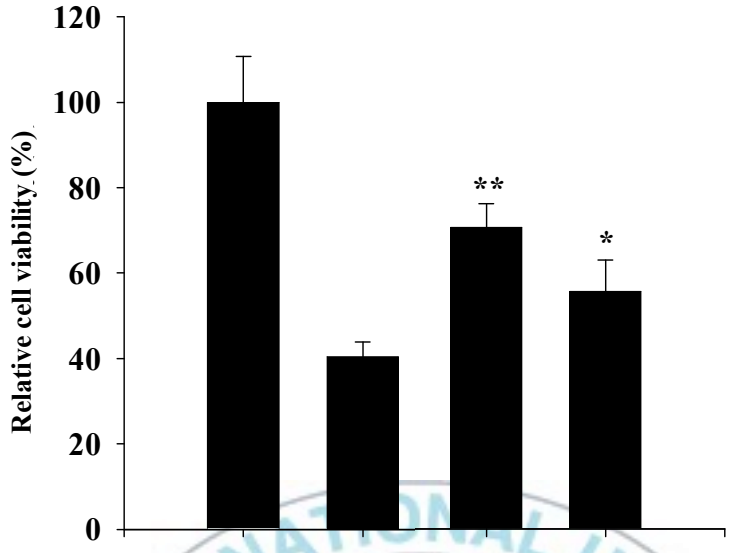
3-3. The PI3K inhibitor LY294002 prevents the protective effect of UDCA on tacrine-induced HepG2 cells.

3-3-1. The PI3K inhibitor LY294002 decreases HepG2 cell viability.

We have previously shown that UDCA protected HepG2 cells from tacrine treatment through PI3K/Akt pathway. Here, to further determine the mechanisms, we assessed whether the protective effect of UDCA was inhibited by PI3K inhibitor, LY294002. LY294002 (20 μM) was treated to cells 1 hr before tacrine (0.2 mM) and UDCA (100 μM) were inserted. After another 24 hrs, cell viability was measured by MTS assay. Combined exposure to tacrine and UDCA with LY294002, the cell proliferation was decreased, in contrast to tacrine and UDCA treated cells (Fig. 13).

3-3-2. Western blot analysis of PI3K inhibitor LY294002 on HepG2 cells

To further verify the role of the PI3K/Akt pathway of UDCA on tacrine-induced HepG2 cells, cells were treated as described before and the expression of the related proteins were measured by Western blot analysis. Fig. 14 shows that cell treatment of UDCA and tacrine combined with LY294002 significantly inhibited the expression of PI3K, pAkt, and therefore increased activation of caspase 3. In line with these results, the cleavage of PARP, the substrate for the activated caspase 3, was increased by LY294002 (Fig. 14). LY294002 also increased the release of cytochrome c from mitochondria to cytoplasm. These results reveal that the PI3K inhibitor, LY294002 can significantly inhibit PI3K/Akt pathway dependent cell survival effective gene expression which operated by UDCA to tacrine-treated HepG2 cells.



THA 0.2 mM	-	+	+	+
UDCA 100 μM	-	-	+	+
LY294002 20 μM	-	-	-	+

Fig. 13 Effect of PI3K inhibitor (LY294002) on cell viability of tacrine and UDCA treated HepG2 cells

Cells were seeded on 96-well plate. After 24 hrs' FBS starvation, 20 μM LY294002 was supplemented to HepG2 cells for 1 hr, then 0.2 mM tacrine and 100 μM UDCA was added. Cell viability was assessed by measuring MTS reduction with ELISA reader at 490 nm. Values statically different are indicated by *($p<0.05$), **($p<0.01$).

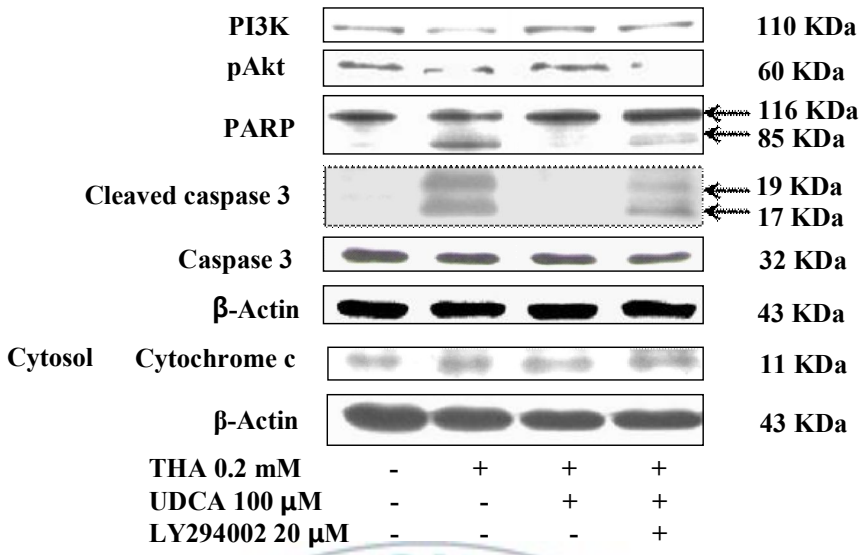


Fig. 14 PI3K inhibitor, LY294002, inhibits the expression of PI3K and the downstream proteins in UDCA protected HepG2 cells.

HepG2 cells were seeded in 6-well plate. After 24 hrs' FBS starvation, 20 μM LY294002 was supplemented to HepG2 cells for 1 hr, then 0.2 mM tacrine and 100 μM UDCA was added. Cell extracts (40 μg) were separated by SDS-PAGE.

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4. Discussion

Several earlier studies reported that UDCA could protect cells from the pro-apototic effects of a variety of reagents including more hydrophobic bile acids such as DCA (Botla *et al*., 1995; Cecilia *et al*., 1998). For the Alzheimer's disease drug, acetylcholinesterase inhibitor-tacrine has a side effect to induce hepatotoxicity, this study shows that the hepatoprotective effect of UDCA on tacrine-induced HepG2 cells. Better understanding of the mechanism underlying UDCA's protection on tacrine-induced hepatotoxicity may provide new concepts for the medical treatment of hepatotoxicity.

In this study, we have shown that the hydrophobic bile acid UDCA has no toxic effect on HepG2 cells even up to 200 μM, so 25, 50, and 100 μM were selected to use in the experiment. Tacrine induces apoptotic cell death in HepG2 cells, and the appropriate concentration of THA treated to cells, 0.2 mM, which corresponded with Galisteo *et al*. (2000). A significantly increase of the proliferation of tacrine-treated HepG2 cells was performed by UDCA supplement. Based on the results, we expect that UDCA is a potential agent in the treatment of tacrine caused human hepatocyte injury.

Here, we observed a DNA fragmentation occurred in tacrine-treated HepG2 cells. The cleavage produces ladders of DNA fragments that are the size of integer multiples of a nucleasome length (180-200 bp) (Huang *et al*., 1997) Their characteristic patterns revealed by agarose gel electrophoresis are widely used as one of the biomarkers of apoptosis. The other biochemical markers, a characteristic ultrastructural appearance and numerous fragmented nuclei observed under an electron

microscope were displayed in tacrine-evoked HepG2 cells, but UDCA prevented these phenomena in dose-response manner. With these results we predict that exposure of HepG2 to tacrine induces apoptotic cell death.

During apoptosis, PARP is selectively cleaved to 24 KDa and 85 KDa fragments representing the N-terminal DNA-binding domain and the C-terminal catalytic subunit, respectively, by several caspases, especially by caspase 3 (Zhao *et al*., 2006). In this experiment, following the degradation of procaspase 3, the caspase 3 substrate PARP minor 85 KDa fragment was detected after tacrine administration. Again, UDCA inhibited the cleavage of these proteins. Generally, once activated caspase 9 goes on to cleavage of caspase 3 and caspase 7 which cleave several cellular targets, including poly (ADP-ribose) polymerase. Therefore, consistent with these data, the expression of caspase 9 appeared the same tendency with caspase 3. Taken together, these results suggest that suppression of tacrine-induced hepatotoxicity by UDCA treatment in HepG2 cells was mainly caused by regulation of caspase activation through down-expression of cleaved caspase 3 and caspse 9.

UDCA has been described as decreasing apoptosis via modulation of mitochondrial function by inhibiting mitochondrial membrane depolarization and cytochrome c release in hepatic and nonhepatic cells (Rodrigues *et al*., 1999, 1998). Since the activation of pro-caspase 9 associated with the release of cytochrome c from mitochondria to the cytosol-an indicator of apoptosis, we determined the expression of cytochrome c release into cytosolic fraction. Following the down-expression of cytochrome c release in cytoplasm, we obtained down-regulated cytosolic Bcl-2, Bcl-x_L in UDCA plus tacrine treated HepG2

cells. The cell death regulator molecules, Bcl-2 family regulates mitochondrial integrity and apoptosis, which include the pro-apoptotic Bax and the anti-apoptotic Bcl-2, Bcl-x_L. Mechanistically, our data support the suppression of tacrine-induced apoptosis by UDCA through an inhibition of the mitochondria pathway: increased mitochondrial Bcl-2 and Bcl-x_L, decreased mitochondrial Bax, release of cytochrome c, augmented pro-caspase 9 and pro-caspase 3 (through inhibition of cleavage) and suppressed PARP cleavage and DNA fragmentation.

The detail mechanisms for how UDCA affects the mitochondria to inhibit apoptosis signaling as well as a possible involvement of the MAPK (ERK, JNK, p38MAPK) or the PI3K/AKT survival pathway is quite necessary. Our experiment revealed that UDCA treatment didn't lead to any alteration on the expression of phospho-ERK (data not shown). Conversely, we found that the magnitude of the PI3K activity was significantly increased by UDCA supplement in tacrine-induced HepG2 cells. In line with previous result, the level of the PI3K downstream effector, phospho-Akt was enhanced by UDCA in dose-dependent pattern. Next, we determined the Akt downstream protein, Bad. When another pro-apoptotic protein of Bcl-2 family Bad active, Akt phosphorylates Bad on Ser136, causing Bad to dissociate from the Bcl-2/Bcl-x_L complex and lose its proapoptotic function (Marlene *et al*., 2008). In agreement with previously published data, in our study, marked activation of Akt clearly activated Bad by UDCA in HepG2 cells. From these observations, we found that UDCA protect HepG2 from tacrine-induced hepatotoxicity by activation of Bad through the PI3K/Akt cell survival signal pathway.

In the result of Akt activation, certain proteins are phosphorylated and

lead to cell survival. For example, phosphorylation of IκB by Akt leads to activation of NFκB that promotes IAP family proteins. This study revealed that UDCA up-regulated NFκB-dependent gene activation and induced XIAP expression, possibly suggesting that UDCA protective effect on tacrine-induced apoptosis in HepG2 cells occurred partially via suppressing NFκB translocation from cytosol to nuclei and induction of XIAP expression.

We further reported that the PI3K inhibitor, LY294002, can efficiently block the UDCA-induced activation of Akt, Bad, NFκB and XIAP. Furthermore, in contrast to UDCA effect, it increased the activation caspase 3, the cleavage of PARP and release of cytochrome c into cysolic fraction. This strongly suggests that PI3K/Akt dependent pathway displays an important role in UDCA protective effect on tacrine-induced apoptosis in HepG2 cells.

Our observations presented in this study recommended that UDCA carried out its protective work on tacrine-treated HepG2 cells by activating a PI3K/Akt dependent survival signal that is mediated by NFκB and IAP family. This concept indicated that the net effect of UDCA in mediating hepatoprotection reflects a balance between pro- and anti-apoptotic processes. Therefore, the investigation of this experiment is novel in demonstrating alteration of mitochondrial function and inhibition of apoptosis by UDCA at physiologically relevant concentrations in tacrine-induced human hepatoma cell line (HepG2).

5. Reference

Alan F. Hofmann. (1999) The continuing importance of bile acids in liver and intestinal disease. *Arch Intern Med*. 159: 2647-2658

Alía, M., Ramos, S., Mateos, R., Bravo, L., Goya, L. (2005) Response of the antioxidant degense system to t-Butyl hydroperoxide and hydrogen peroxide in a human hepatoma cell line (HepG2). *J. Biochem. Mol. Toxicol*. 19: 119-128

Alvarez, M., Jover, A., Carrazana, J., Meijide, F., Soto, V.H., Vázquez, Tato. J. (2007) Crystal structure of chenodeoxycholic acid, ursodeoxycholic acid and their two 3β,7α- and 3β,7β-dihydroxy epimers. *J. Steroids*. 72: 535-544

Anabela P. Rolo, Carlos M. Palmeira, Jon M. Holy, and Kendall B. Wallace. (2004) Role of mitochondrial dysfunction in combined bile acid-induced cytotoxicity: the switch between apoptosis and necrosis. *Toxicol. Sci*. 79: 196-204

Asuka Nanbo, Hitoshi Nishimura, Tatsushi Muta and Shigehar Nagassawa. (1999) Lipopolysacchride stimulates HepG2 human hepatoma cells in the presence of lipopolysaccharide-binding protein via CD14. *Eur. J. Biochem*. 260: 183-191

Berson A., Renault S., Letteron P., Robin MA>, Fromenty B., Fau D., Le Bot MA., Riche C., Durand-Schneider AM., Feldmann G. and Pessayre

D (1996) Uncoupling of rat and human mitochondria: a possible explanation for tacrine-induced liver dysfunction. *Gastroenterology* 110:1878-1890

Botla R., Spivey JR., Aguilar H., Bronk SF. and Gores GJ. (1995) Ursodeoxycholate (UDCA) inhibits the mitochondrial membrane permeability transition induced by glycochenodeoxycholate: a mechanism of UDCA cytoprotection. *J. Pharmacol. Exp. Ther.* 272(2): 930-938

Datta S. R., Brunet A. and Greenberg M. E. (1999) Cellular survival: a play in three Akts. *Genes Dev.* 13:2905-2927

Debbasch C., Osseni R.A., Rat P., Christen M.O., Thevenin M., Adolphe M. and Warnet J.-M. (1998) Evidence of apoptosis in tacrine cell death. *Toxicol. Lett.* 95: 106-106

Deveraux QL., Takahashi R., Salvesen GS. and Reed JC. (1997) X-linked IAP is a direct inhibitor of cell-death proteases. *Nature* 388: 300-304

Deveraux QL. and Reed JC. (1999) IAP family proteins-suppressors of apoptosis. *Genes. Dev.* 13: 239-252

Dinesh Kumar and Rakesh K Tandon. (2001) Use of ursodeoxycholic acid in liver disease. *J. Gastroenterol. Hepatol*. 16: 3-14

Eagger S.A., Levy R. and Sahakian B.J. (1991) Tacrine in Alzheimer's disease. *Lancet* 337: 989-992

Emanuele S., Anneo A.D., Bellavia G., Vassallo B.,. Lauricella M., De Blasio A., Vento R. and Tesoriere G. (2004) Sodium butyrate induces apoptosis in human hepatoma cells by a mitochondria/caspase pathway, associated with degradation of β-catenin, pRb and Bcl-X_L. *Eur. J. Cancer* 40: 1441-1452

Fariss M. W., Mumaw V. R. and Walton L. P. (1996) Tetrahydroaminoacridine-induced apoptosis in rat hepatocytes. *Toxicol. in vitro.* 10: 383-393

Galisteo M, Rissel M, Sergent O, Chevanne M, Cillard J, Guillouzo A, Lagadic-Gossmann D. (2000) Hepatotoxicity of tacrine: occurrence of membrane fluidity alterations without involvement of lipid peroxidation. *J Pharmacol Exp Ther.* 294(1):160-167

Gewies, Andreas. (2003) Introduction to apoptosis. Aporeview: 1-26

Gustav Paumgartner and Ulrich Beuers. (2002) Ursodeoxycholic acid in cholestatic liver disease: mechanisms of action and therapeutic use revisited. *Hepatology.* 36: 525-531

Hajime Higuchi, Hideyuku Miyoshi, Steven F. Bronk, Hong Zhang, Nicholas Dean and Gregory J. Gores (2001) Bid antisense attenuates bile acid-induced apoptosis and cholestatic liver injury. *J. Pharmacol. Exp.*

Therapeut. 299(3): 866-873

Huang P., Ballal K. and Plunkett W. (1997) Biochemical characterization of the protein activity responsible for high molecular weight DNA fragmentation during drug-induced apoptosis. *Cancer Res.* 57: 3407-3414

Hunter, A. J., Murray, T. K., Jones, J. A., Cross, A. J. and Green, A. R. (1989) The cholinergic pharmacology of tetrahydroaminoacridine in vivo and in vitro. *Br J Pharmacol.* 98(1):79-86

Hyekyung Yang, Mi Kyeong Lee and Young Choong Kim. (2005) Protective activities of stilbene glycosides from Acer mono leaves against H_2O_2-induced oxidative damage in primary cultured rat hepatocytes. *J. Agric. Food Chem*. 53: 4182-4186

Joohyun Sohn, Vladimir I. Khaoustov, Qing Xie, Charles C. Chung, Bhuvaneswari Krishnan and Boris Yoffe. (2003) The effect of ursodeoxycholic acid on the surviving in thapsigargin-induced apoptosis. *Cancer Lett.* 191: 83-92

Kaoru Ishizaki, Teruaki Imada, Makoto Tsurufuji. (2005) Hepatoprotective bile acid 'ursodeoxycholic acid (UDCA)' property and difference as bile acids. *Hepatology Research*. 33: 174-177

Knapp M. J., Knopman D. S., Solomon P. R., Pendlebury W., Davis C.S. and Gracon S. I. (1994) High doses of tacrine used in Alzheimer disease:

a randomized study during 30 weeks. *Journal of the American Medical Association* 271: 985-991

Komichi D, Tazuma S, Nishioka T, Hyogo H, Une M, Chayama K. (2003) Unique inhibition of bile salt-induced apoptosis by lecithins and cytoprotective bile salts in immortalized mouse cholangiocytes. *Dig. Dis. Sci.*48: 2315-22

Lagadic-Gossmann D., Rissel M., Le Bot MA. and Guillouzo A. (1998) Toxic effects of tacrine on primary hepatocytes and liver epithelial cells in culture. *Cell Biol. Toicol.* 14: 361-373

Lee R. Hagey, Diane L. Crombie, Edgard Espinosa, Martin C. Carey, Hirotsune Igimi, and Alan F. Hofmann. (1993) Ursodeoxycholic acid in the Ursidae: biliary bile acids of bears, pandas, and related carnivores. *J. :Lipid Research*. 34: 1911-1917

Li P., Nijhawan D., Budihardjo I. Srinivasula S. M., Ahmad M., Alnemri E. S. and Wang XD. (1997) Cytochrome *c* and dATP-dependent formation of apaf-1/caspase-9 complex initiates an apoptic protease cascade. *Cell* 91: 479-489

Luo X., Budihardjo I., Zou H., Slaughter C. and Wang X. (1998) Bid, a Bcl-2 interacting protein, mediates cytochrome *c* release from mitochondria in response to activation of cell surface death receptors. *Cell*. 94: 481-490

Marieke H. Schoemaker, Laura Conde de la Rosa, Manon Buist-Homan, Titia E. Vrenken, Rick Havinga, Klaas Poelstra, Hidde J. Haisma, Peter L. M. Jansen, and Han Moshange. (2004) Tauroursodeoxycholic acid protects rat hepatocytes from bile acid-induced apoptosis via activation of survival pathways. *Hepatology*. 39: 1563-1573

Mariana G. García , Laura D. Alaniz, Rosalía I. Cordo Russo,
Elida Alvarez and Silvia E. Hajos. (2008) PI3K/Akt inhibition modulates multidrug resistance and activates NF-kappaB in murine lymphoma cell lines. *Leuk. Res*.

Marlene T. Mathews and Bradford C. Berk (2008) PARP-1 inhibition prevents oxidative and nitrosative stress-induced endothelial cell death via transactivation of the VEGF receptor 2. *Arterioscler. Thromb. Vasc. Biol.* 28:000-000

Michihiro Nonaka, Susumu Tazuma, Hideyuki Hyogo, Keishi Kanno and Kazuaki Chayama. (2007) Cytoprotective effect of tauroursodeoxycholate on hepatocyte apoptosis induced by peroxisome proliferator-activated receptor gamma ligand. *J. Gastroenterol. Hepatol.*

Milagros Gtalisteo, Mary Rissel, Odile Sergent, Martine Chevanne, Josiane Cillard, Andre Guillouzo, and Dominique Lagadic-Gossmann. (2000) Hepatotoxicity of tacrine: occurrence of membrane fluidity alterations without involvement of lipid peroxidation. *J. Phamacol. Exp. Ther.* 294: 160-167

Mitsuyoshi H., Nakashima T., Sumida Y., Yoh T., Nakajima Y., Ishikawa H., Inaba K., Sakamoto Y., Okanoue T. and Kashima K. (1999) Ursodeoxycholic acid protects hepatocytes against oxidative injury via induction of antioxidants. *Biochem. Biophys. Res. Commun.* 263:537-542

Osseni, R.A., Debbasch, C., Christen, M.-O., Rat, P. and Warnet, J.-M. (1999) Tacrine-induced reactive oxygen species in a human liver cell line: the role of anethole dithiolethione as a scavenger. *Toxicology in Vitro* 13: 683-688

Paul Dent, Youwen Fang, Seema Gupta, Elaine Studer, Clint Mitcell, Sarah Spiegel, and Philip B. Hylemon. (2005) *Hepatology*. 42: 1291-1299

Priyanka Priyadarsiny, Sunil K. Khattar, Renu Malik, Venkatesha Udupa, Arigila Seshaiah, Shamsur Rahman, Vyas Madhavrao Shingatgeri, Roop Singh Bora and Kulvinder Singh Saini. (2008) Differential gene expression analysis of a known hepatotoxin, N-acetyl-p-amino-phenol (APAP) as compared to its non-toxic analog, N-acetyl-m-amino-phenol (AMAP) in mouse liver. *J. Toxicol. Sci.* 33(2): 163-173

Reed JC. (1998) Bcl-2 family proteins. *Oncogene* 17: 3225-3236

Riedl SJ., Renaus M. and Schwarzenbacher R. (2001) Structural basis for the inhibition of caspase 3 by XIAP. *Cell* 104: 791-800

Robertson DG., Braden TK., Urda ER., Lalwani ND. and de la Iglesia FA. (1998) Elucidation of mitochondrial effects by tetrahydroaminoacridine (tacrine) in rat dog, monkey and human hepatic parenchymal cells. *Arch. Toxicol.* 72: 362-371.

Rodrigues, Cecilia M. P., Fan Guangsheng, Wong, Philliip Y., Kren, Betsy T. and Steer, Clifford J. (1998) Ursodeoxycholic acid may inhibit deoxycholic acid-induced apoptosis by modulating mitochondrial transmembrane potential and reactive oxygen species production. *Mol. Med.* 4(3): 165-178

Rodrigues, Cecilia M. P., Fan Guangsheng, Ma Xiaoming, Kren, Betsy T. and Steer, Clifford J. (1998) A novel role for ursodeoxycholic acid in inhibiting apoptosis by modulating mitochondrial membrane perturbation. *J. Clin. Invest*. 101(12): 2790-2799

Rodrigues, Cecilia M. P., Ma Xiaoming, Cheryle Linehan-Stieers, Fan Guangsheng, Kren, Betsy T. and Steer, Clifford J. (1999) Ursodeoxycholic acid prevents cytochrome c release in apoptosis by inhibiting mitochondrial membrane depolarization and channel formation. *Cell Death. Differ*. 6: 842-854

Sagawa H, Tazuma S, Kajiyama G. (1993) Protection against hydrophobic bile salt-induced cell membrane damage by liposomes and hydrophilic bile salts. *Am. J. Physiol.* 264: G835-9

Schoonbroodt S, Legrand-Poels S, Best-Belpomme M, Piette J. (1997) Activation of the NF-kappa B transcription factor in a T-lymphocytic cell line by hypochlorous acid. *J. Biochem.* 321: 777-785.

Schoemaker, M. H., Ros, J. E., Homan, M., Trautwein, C., Liston, P., Poelstra, K., H. van Goor, Jansen, P. L.M. and Moshage, H. (2002) Cytokine regulation of pro- and anti-apoptotic genes in rat hepatocytes: NF-κB-regulated inhibitor of apoptosis proteins(cIAP2) prevents apoptosis. *J. Hepatol.* 36: 742-750

Teruo Miyazaki, Masaaki Karube, Yasushi Matsuzaki, Tadashi Ikegami, Mikio Doy, Naomi Tanaka and Bernard Bouscarel. (2005) Taurine inhibits oxidative damage and prevents fibrosis in carbon tetrachloride-induced hepatic fibrosis. *J. Hepatol.* 43: 117-125

Tewari, M. and Dixit, V.M. (1995) Yama/CPP32b, a mammalian homolog of CED-3 is a CrmA-inhibitable protease that cleaves the death substrate poly (ADP-ribose) polymerase. *Cell* **81**: 801–809

Watkins P. B., Zimmerman H. J., Knapp M. J., Gracon S. I. and Lewis K. W. (1994) Hepatotoxic effects of tacrine administration in patients with Alzheimer's disease. *Journal of the American Medical Association* 13: 992-998

Wu H., Cai P., Dahn L. Clemens, Thomas R. Jerrells, G.A. Shakeel Ansari and Bhupendra S. Kaphalia. (2006) Metabolic basis of ethanol-induced cytotoxicity in recombinant HepG2 cells: role of nonoxidative

metabolism. *Toxicol. Appl. Pharmacol.* 216: 238-247

Wu CS. and Yang JT.(1989) Tacrine protection of acetylcholinesterase from inactivation by diisopropylfluorophosphate: a circular dichroism study. Mol Pharmacol. 35(1):85-92

Zha Jiping, Hisashi Harada, Konstantin Osipov, Jennifer Jockel, Gabriel Waksman, and Stanley J. Korsmeyer (1997) BH3 domain of Bad is required for heterodimerization with Bcl-xL and pro-apoptotic activity. *J Biol Chem.* 272(39):24101-24104

Zhao, M. H., Guo, T., Wang, M. W., Zhao, Q. C., Liu, Y. X. and Sun, X. H. (2006) The course of uncarinic acid E-induced apoptosis of HepG2 cells from damage to DNA and p53 activation to mitochondrial release of cytochrome *c*. *Biol. Pharm. Bull.* 29 (8): 1639-1644

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