Pharmacological evaluation of several major ingredients of Chinese herbal medicines in human hepatoma Hep3B cells

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Abstract

Long-dan-tan (Chinese name) is one of the most common herbal medicines used by Chinese people with chronic liver disease. Accumulated anecdotal evidence suggests that Long-dan-tan may show a beneficial effect in patients with hepatocellular carcinoma. Long-dan-tan is made from five plants: Gentiana root, Scutellaria root, Gardenia fruit, Alisma rhizome, and Bupleurum root. In this study, we have examined the cytotoxic effects of the five major ingredients isolated from the above plants, i.e. gentiopicroside, baicalein, geniposide, alisol B acetate and saikosaponin-d, respectively, on human hepatoma Hep3B cells. Annexin V immunofluorescence detection, DNA fragmentation assays and FACScan analysis of propidium iodide-staining cells showed that gentiopicroside, baicalein, and geniposide had little effect, whereas alisol B acetate and saikosaponin-d profoundly induced apoptosis in Hep3B cells. Alisol B acetate, but not saikosaponin-d, induced G2/M arrest of the cell cycle as well as a significant increase in caspase-3 activity. Interestingly, baicalein by itself induced an increase in H2O2 generation and the subsequent NF-κB activation; furthermore, it effectively inhibited the transforming growth factor-β (TGF-β)-induced caspase-3 activation and cell apoptosis. We suggest that alisol B acetate and saikosaponin-d induced cell apoptosis through the caspase-3-dependent and -independent pathways, respectively. Instead of inducing apoptosis, baicalein inhibits TGF-β-induced apoptosis via increase in cellular H2O2 formation and NF-κB activation in human hepatoma Hep3B cells.

Keywords: Alisol-B-monoacetate; Saikosaponin-d; Baicalein; Apoptosis; Hep3B

1. Introduction

Herbal medicines which have been used by Chinese people for thousands of years are now being manufactured in many countries as quality-controlled drugs with standardized quantities of ingredients. Long-dan-tan (Chinese name) is one of the most common herbal medicines used by Chinese people suffering from chronic liver disease. Accumulated anecdotal evidence suggests that Long-dan-tan may show a beneficial effect in patients with hepatocellular carcinoma. However, to date it has not been scientifically evaluated in human hepatoma.

Long-dan-tan is made from five important plants, i.e. Gentiana rhizome, Scutellaria root, Gardenia fruit, Alisma rhizome, and Bupleurum root. In this study we have examined the cytotoxic effects of the five major ingredients isolated from the above herbs, i.e. gentiopicroside, baicalein, geniposide, alisol B acetate and saikosaponin-d, respectively, on human hepatoma Hep3B cells. It has been suggested that gentiopicroside exhibits a moderate hepatoprotective effect on D-galactosamine/lipopolysaccharide-induced liver injury in mice (Hase et al., 1997). Baicalein is a flavonoid, and shows anti-inflammatory and antioxidant activities and inhibits hepatic fibrosis (Chang et al., 1993; Lin and Shieh, 1996; Shimizu, 2000). Furthermore, it also plays a role in growth regulation, influencing apoptosis and anti-proliferation, in several types of cancer cells including human prostate cancers (DU145 and PC-3), pancreatic cancers (PANC-1, MiaPaca2, Capan2 and HPAF), colon cancers (Caco-2) and breast cancers (MDA-MB-435 and MCF-7) (So et al., 1997; Ding et al., 1999; Kuntz et al., 1999; Chan et al., 2000). In addition to these anticancer effects, baicalein also profoundly inhibited ascorbic acid-induced lipid peroxidation in rat liver microsomes (Gao et al., 1995). Therefore, herbal medicines whose major ingredient is baicalein are frequently used in...
the treatment of liver disease. Geniposide, an iridoid glucoside of the Gardenia fruit, has been suggested to show a chemo-preventive effect on early acute hepatic damage induced by aflatoxin B1 (Wang et al. 1991). It is also biologically active as an anti-tumor agent against C-6 glioma cells (Wang et al. 1992). Alisol B acetate, a triterpene from Alisma plantago-aquatica, has a glucocorticoid-like structure, and induces apoptosis in human acute lymphoblastic leukemia CEM cells and human fibrosarcoma HT1080 cells (Chen et al. 2001; Lee et al. 2001). Furthermore, it also inhibits antibody-mediated allergic reactions in rat models, and is may have potential for the treatment of allergic reactions (Kubo et al. 1997). The anti-cancer effect of saikosaponin-d was investigated on human hepatoma cell lines (PLC/PRF/5, HepG2) and human pancreatic cancer cell lines (BxPC-3). It was suggested that saikosaponin-d inhibited the cell growth and DNA synthesis of all the tested cell lines (Motoo and Sawabu 1994). Most of these major ingredients of Longdan-tan exhibit these anti-cancer activities in numerous types of cancers. However, to date there has been little study to elucidate the anti-cancer effect in Hep3B cells, which contain copies of the hepatitis B virus (HBV) genomes in their chromosomes (Twist et al. 1981), actively secrete HBsAg (Aden et al. 1979), and are considered to be representative cell models for developing anti-cancer agents against HBV-related hepatocellular carcinoma.

In our laboratory, we examine many purified compounds and crude extracts from herbs used in Chinese medicinal prescriptions to elucidate their active components and elucidate their mechanisms of action. Based on anecdotal evidence and scattered reports of beneficial outcome, we were interested in the pharmacological evaluation of Longdan-tan and its main ingredients regarding its anti-cancer effect on human hepatocellular carcinoma. Our objectives were: (1) to carry out a scientific assessment of these major ingredients, and to evaluate the therapeutic potential of this prescription, and (2) to determine the active components and explore their mechanisms of action. And hopefully, based on our examination, the beneficial and harmful components could be determined, and the dosage in this prescription could be reevaluated for optimal therapy.

2. Materials and methods

2.1. Cell culture and growth factor

Hep3B cells (American Type Culture Collection) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS; Gibco BRL), 100 U/ml penicillin G and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of air/CO2 (19:1). Human recombinant lyophilized transforming growth factor-β1 (TGF-β1, 2 mg) (Sigma) was dissolved in 1 ml of 4 mM HCl containing 0.1% bovine serum albumin, and divided into 50-μl aliquots before storage at −70 °C.

2.2. Annexin V-labeling of apoptotic cells

In situ detection of apoptotic cells was carried out using fluorescein-labeled annexin V, and fluorescence microscopy. Briefly, cells were cultured in chamber slides for 24 h and then treated with vehicle, gentiopicroside, baicalein, geniposide, alisol B acetate, or saikosaponin-d for 30 min in a serum-free medium. Then, TGF-β1 or vehicle was added to the cells. After the incubation period, cells were washed twice with phosphate-buffered saline (PBS) and then 100 μl of annexin V reaction mixture (annexin V-FITC and propidium iodide in binding buffer from R&D Systems) was added for a further 15-min incubation. After the above treatment, cells were washed twice with a binding buffer. Finally, photomicrographs were obtained with a fluorescence microscope (Nikon).

2.3. DNA fragmentation assay

Hep3B cells were cultured in 100-mm dishes in 10 ml medium at plating densities of 2×10⁶ cells/dish, and incubated at 37 °C in a humidified atmosphere of air/CO2 (19:1) for 24 h prior to addition of experimental agents. Cells were then exposed to alisol B acetate (50 μM) or saikosaponin-d (5 μM) for another 24 h. After the treatment, cells were washed twice with PBS and harvested with incubation buffer (10 mM EDTA, 50 mM Tris–HCl, pH 8.0, 0.5% (v/v) sarkosyl and 1 μg/ml proteinase K). The cells were incubated at 56 °C for 3 h, and then RNase (final concentration of 50 μg/ml) was added to the incubation buffer for a further 1-h incubation. The DNA was extracted by phenol/chloroform/isooamyl alcohol (25:24:1, v/v) and then precipitated with ethanol. The extracted DNA was separated and stained by electrophoresis in 2% agarose gel with ethidium bromide.

2.4. FACScan flow cytometric assay

Cells were seeded onto six-well plates, serum starved, and treated with or without the indicated agent for 24 h. Cells were then washed twice with ice-cold PBS, and collected by centrifugation at 200×g for 5 min at 4 °C. The cells were fixed in 70% (v/v) ethanol at 4 °C for 30 min. After fixation, cells were treated with 0.2 ml DNA extraction buffer (0.2 M Na2HPO4 and 0.1 M citric acid buffer, pH 7.8) for 30 min and then centrifuged and resuspended in 1 ml propidium iodide staining buffer (0.1% Triton X-100, 100 μg/ml RNase A, 80 μg/ml propidium iodide in PBS) at 37 °C for 30 min. Cells were detected using a cytofluorometer, and analyzed by FACScan and CellQuest program (Becton Dickinson).
2.5. Preparation of cytosolic extracts and measurement of caspase-3 activity

Cells were seeded onto six-well plates for 24 h and then treated with vehicle, alisol B acetate, saikosaponin-d or baicalein for 30 min. Then, vehicle or TGF-β1 was added to the cells for 16 h. After the incubation period, cells were washed twice with ice-cold PBS, and then collected by centrifugation at 2000×g for 5 min at 4 °C. The cell pellet was resuspended in lysis buffer (25 μl/10^6 cells). After a 10-min incubation on ice, the cell homogenates were centrifuged at 10,000×g for 1 min and supernatants were removed for determination of caspase-3 activity. Proteolytic reactions were performed in a total volume of 100 μl reaction buffer containing 5 μl of cytosolic extracts and 5 μl DEVD-pNA, obtained from a commercial assay kit (Caspase-3 Colorimetric Assay Kit, R&D Systems). The reaction mixture was incubated at 37 °C for 1–2 h, and then the formation of p-nitroanilide was measured at 405 nm by an ELISA reader.

2.6. Measurement of H_2O_2 generation

Intracellular H_2O_2 levels were measured by the fluorescent probe DCFH-DA. This cell-permeable dye, once inside the cells, is cleaved by endogenous esterase into DCF. The intracellular non-fluorescent form of DCFH is oxidized, commonly by H_2O_2, into the fluorescent form, DCF. Hep3B cells were seeded onto six-well plates for 24 h and then incubated in the absence or presence of TGF-β1 or baicalein for 4 h. Then 30 min before termination of the incubation, DCFH-DA (10 μM) was added to the cells, and incubated for the last 30 min at 37 °C. Cells were then harvested for the detection of H_2O_2 accumulation using FACScan flow cytometric analysis.

2.7. Assay for nuclear translocation of NF-κB

DNA binding activities of NF-κB were determined using electrophoretic mobility shift assay (EMSA). Hep3B cells were incubated in the absence or presence of baicalein, baicalein plus catalase or NF-κB competitor for 1 h, and then cells were washed twice with ice-cold PBS and collected in buffer A, containing 10 mM HEPES (pH 7.9), 1.5 mM MgCl_2, 10 mM KCl, 0.5 mM dithiothreitol and 0.2 mM phenylmethane sulphonyl fluoride for 10 min on ice and centrifuged at 2000 rpm for 3 min. The pellet was re-suspended in buffer B containing 20 mM HEPES (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM MgCl_2, 0.2 mM EDTA, 0.5 mM dithiothreitol and 0.2 mM phenylmethane sulphonyl fluoride, vortexed vigorously, and allowed to stand on ice for 20 min. The supernatants with soluble nuclear proteins were collected by centrifugation at 14,000 rpm for 5 min, and stored at −70 °C. For the NF-κB–DNA binding assay, each nuclear extract (2 μg) was incubated with a 35-base pair double-stranded 32P-labeled probe encoding the κB consensus sequence (5'-AGT TGA GGG GAT CCC CCC AGG C-3') in binding buffer containing 10 mM Tris–HCl, 40 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 1% Nonidet P-40, 1% deoxycholate, 3 μg/ml polydeoxyinosinic-deoxy-rytidylic acid at room temperature for 30 min. Then samples were applied to native 5% polyacrylamide gels and analyzed on autoradiography. For competition assay, 20-fold molar excess unlabeled consensus oligonucleotide was added at 30 min prior to the addition of the labeled probe. Components of NF-κB proteins were identified by supershift assay using antibodies against p50 or p65 antibodies.

2.8. Materials and data analysis

DMEM, 0.1% trypsin–0.04% EDTA solution, FBS, and antibiotics (penicillin G and streptomycin) were obtained from Gibco (Grand Island, NY, USA). Propidium iodide, baicalein, RO-318220, and human recombinant lyophilized TGF-β1 were obtained from Sigma (St. Louis, MO, USA). Gentioficuside, geniposide, alisol B acetate, and saikosaponin-d were obtained from Nacalai Tesque (Kyoto, Japan). The Caspase-3 Activity Assay Kit and Annexin V-FITC Apoptosis Detection Kit were from R&D Systems (Minneapolis, MN, USA).

Data are presented as the mean and S.E.M. for the indicated number of separate experiments. Statistical analysis of data was performed with one-way analysis of variance (ANOVA) followed by a t-test, and P-values less than 0.05 were considered significant.

3. Results

In this study, we have examined the effects of gentiopicroside, baicalein, geniposide, alisol B acetate and saikosaponin-d on cytotoxicity in human hepatoma Hep3B cells, by means of cell morphology identification and in situ labeling of annexin V-reaction techniques. The results demonstrated intact cell morphology and negative staining of annexin V in control cells (Fig. 1A) as well as in gentiopicroside (100 μM, Fig. 1B), baicalein (100 μM, Fig. 1C) and geniposide (100 μM, Fig. 1D)-treated cells. However, it showed an apoptotic character and a positive annexin V reaction of the green fluorescence staining in alisol B acetate (50 μM)-treated cells (Fig. 1E). The cells exposed to saikosaponin-d (5 μM) showed a cytotoxic morphology and a positive annexin V and propidium iodide reaction of the green and orange fluorescence staining, respectively (Fig. 1F). The cytotoxic effect, induced by alisol B acetate (50 μM) and saikosaponin-d (5 μM), was further examined using DNA ladder detection assays. The data showed that both agents profoundly induced DNA fragmentation in Hep3B cells, indicating the occurrence of an apoptotic reaction (Fig. 1G). The effects of these
Fig. 1. Effect of the major ingredients of Long-dan-tan on apoptosis in Hep3B cells. Cells were exposed to vehicle (A), gentiopicroside (300 μM, B), baicalein (100 μM, C), geniposide (300 μM, D), alisol B acetate (50 μM, E), or saikosaponin-d (5 μM, F) for 24 h. Then, the cell morphology or double immunostaining (inset) with annexin V (green fluorescence) and propidium iodide (orange fluorescence) was detected as described in the Materials and methods section. Furthermore, cells were treated with vehicle (lane 1), alisol B acetate (50 μM, lane 2) or saikosaponin-d (5 μM, lane 3) for 24 h, and then the cells were harvested for the detection of DNA ladder using DNA fragmentation assays as described in the Materials and methods section.

Fig. 2. Effect of the major ingredients of Long-dan-tan on cell cycle progression in Hep3B cells. Cells were exposed to vehicle, gentiopicroside, baicalein, geniposide, alisol B acetate or saikosaponin-d for 24 h. Then, the cells were harvested for the detection of cell cycle progression using FACScan flow cytometric analysis as described in the Materials and methods section.

Ingredients on cytotoxicity were also examined using FACScan flow cytometric analysis. As demonstrated in Fig. 2, gentiopicroside, baicalein, and geniposide had little influence on the phase distribution of the cell cycle progression. However, alisol B acetate and saikosaponin-d significantly induced an increase in sub-G1 phase, indicating the induction of apoptosis in Hep3B cells. The concentration-dependent responses of alisol B acetate- and saikosaponin-d-induced effects were analysed. The data showed that both agents induced cell apoptosis in a
Fig. 3. Effect of alisol B acetate and saikosaponin-d on cell cycle progression in Hep3B cells. Cells were exposed to vehicle, alisol B acetate or saikosaponin-d of the indicated concentration for 24 h. Then, the cells were harvested for the detection of cell cycle progression using FACScan flow cytometric analysis as described in the Materials and methods section. Data are expressed as the mean and S.E.M. of four determinations. *P<0.05, **P<0.01 and ***P<0.001 compared with control.

concentration-dependent manner with IC_{50} values of 42.4 and 2.95 μM, respectively (Fig. 3A,C). Interestingly, alisol B acetate induced-apoptosis was associated with a significant but modest increase in G2/M phase arrest of the cell cycle (Fig. 3B), while in contrast, saikosaponin-d had little effect on the G2/M phase distribution (Fig. 3D), although it was more potent (~14 times) than alisol B acetate in eliciting cell apoptosis. Accordingly, we examined the role of cdc2 kinase on alisol B acetate-induced G2/M phase arrest of the cell cycle, since it has been suggested that the regulation of cdc2 kinase activity is relevant to cell cycle progression as well as G2/M phase arrest of the cell cycle (Doree and Galas, 1994). The data showed that olomoucine (60 μM), a cdc2 kinase inhibitor, showed little effect on G2/M phase by itself (olomoucine 11.3±0.8 vs. control 9.6±0.8; P>0.05, n=3); it also had no influence on the alisol B acetate-induced effect (olomoucine 20.4±2.1% vs. alisol B acetate alone 23.1±1.9%; P>0.05, n=3) indicating the involvement of a cdc2 kinase-independent pathway. Furthermore, to investigate the apoptotic mechanism, several pharmacological agents were used in this study. As demonstrated in Fig. 4, both the activation and down-regulation of protein kinase C by long-term exposure of low (30 nM) and high concentration (1 μM) of phorbol 12-myristate 13-acetate (PMA) did not induce any apoptosis revealing that the regulation of protein kinase C activity was not involved in the alisol B acetate- and saikosaponin-d-mediated function (Fig. 4A).

Additionally, RO-318220 (3 μM, a protein kinase C inhibitor), olomoucine (60 μM) and SB203580 (10 μM, a p38 mitogen-activated protein kinase (MAPK) inhibitor) did not modify, while N-benzyloxy carbonyl-Asp-Glu-Val-Asp-fluoromethylketone (zNAD-fmk; 50 μM) significantly inhibited alisol B acetate-induced apoptosis, suggesting the involvement of caspase activation in this reaction (Fig. 4B). In contrast, all of these inhibitors had little effect on saikosaponin-d action, although zNAD-fmk showed an inhibitory tendency (Fig. 4C). The caspase activation plays a pivotal role in the execution of apoptosis. Recent studies have demonstrated that caspase-3 is the major caspase activated in response to distinct apoptotic stimuli (Wilson, 1998). Furthermore, there are several lines of evidence suggesting that the activation of caspase-3 is involved in anti-tumor agent-induced apoptosis in Hep3B cells (Chen et al., 2002). We determined caspase-3 activity in this study, and the data showed that the alisol B acetate induced a significant increase, whereas saikosaponin-d showed little effect, on the caspase-3 activity. This suggests that caspase-3 activity plays a role in alisol B acetate other than the saikosaponin-d-mediated mechanism (Fig. 5).

The data showed that gentiopicroside, baicalein and geniposide did not induce any apoptosis in Hep3B cells. Interestingly enough, baicalein inhibited the transforming growth factor β_{1} (TGF-β_{1})-induced apoptosis in a concentration-dependent manner (Fig. 6). The data demonstrated that TGF-β_{1} induced a significant increase in caspase-3 activity (Fig. 5); however, it had little effect on H_{2}O_{2} generation in Hep3B cells (Fig. 7). Baicalein completely abolished the caspase-3 activation (Fig. 5) induced by TGF-β_{1}. It is worth noting that baicalein by itself induced the increase in H_{2}O_{2} generation in a
Fig. 5. Effect of the major ingredients of Long-dan-tan on caspase-3 activity. Cells were treated without (control) or with the indicated agent (alisol B acetate 30 μM, saikosaponin-d 3 μM, TGF-β 10 ng/ml, baicalein 100 μM) for 16 h, and then cells were lysed for the detection of caspase-3 activity as described in the Materials and methods section. Data are expressed as mean ± S.E.M. of four determinations. *P < 0.05 and **P < 0.01 compared with the control, and #P < 0.01 compared with TGF-β alone.

4. Discussion

In recent years Long-dan-tan has been one of the herbal medicines most commonly administered to Chinese people
addition, the cells become rounder and apoptotic bodies are identified. According to these criteria, the present study revealed apoptosis in Hep3B cells in response to alisol B acetate and saikosaponin-d, but not to gentiopicroside, baicalein or geniposide. Apoptosis was also detected and quantified using FACScan flow cytometric analysis. The data were consistent with those of annexin V-reaction techniques. Furthermore, it also revealed that saikosaponin-d was the most potent ingredient to cause apoptosis in Hep3B cells. However, the double staining of red fluorescence with propidium iodide showed that saikosaponin-d also induced marked necrosis in these cells (Fig. 1F). This might be explained by the severe apoptosis induced by the high concentration of saikosaponin-d (5 μM), since low concentrations (less than 3 μM) of this ingredient only induced the apoptotic reaction (data not shown). Two fundamental forms of cell death, that is to say apoptosis and necrosis, occurred after the apoptotic stimuli were also observed in a variety of cell types. Filipovic and colleagues have suggested that oxidant-induced apoptosis activates poly(ADP-ribose) polymerase (PARP), and that the subsequent ATP and NAD depletions contribute to necrotic cell death in renal epithelial cells (Filipovic et al., 1999). Li and colleagues observed that β-lapachone induced cell death in a spectrum of human carcinoma cells; it induced apoptosis in human ovary, colon, and lung cancer cells, and necrotic cell death in four human breast cancer cell lines (Li et al., 1999). Therefore, it has been suggested that the necrosis might be secondary to the apoptosis in response to several apoptotic stimuli, or that these two different types of cell death share the same signaling events in their mechanisms of action.

In this study, it was evident by FACS analysis that alisol B acetate induced a modest but significant increase in G2/M arrest (Fig. 3B), which is characterized by abnormal metaphase morphology, of the cell cycle. It has been suggested that disturbance in cdc2 kinase activity and tubulin assembly/disassembly contribute to the increase in...
G2/M arrest in numerous types of cells. It has been demonstrated that taxol stabilizes microtubules and induces G2/M arrest as well as cell apoptosis coincident with cdc2 activation (Donaldson et al., 1994). Furthermore, TGF-β1 induced an increase in the cell population in the G2/M phase, and a transient increase in cdc2 expression at an early phase of apoptosis in hepatoma FaO cells. Treatment with olomoucine blocked TGF-β1-induced apoptosis in these cells (Choi et al., 1999). On the other hand, Nehme et al. (2001) suggest that docetaxel causes phosphorylation and hence inactivation of cdc2 kinase resulting in G2/M arrest in DU145 and LNCaP prostate cancer cells. In this study, olomoucine did not affect the basal or alisol B acetate-mediated G2/M arrest, revealing a mechanism beyond regulation by cdc2 kinase. However, further studies are required to define whether alisol B acetate disturbs tubulin assembly/disassembly in these cells.

Several molecular signals have been suggested to be involved in the regulation of apoptosis in various types of cells. Protein kinase C, a family of serine/threonine protein kinases, is involved in signal transduction pathways that regulate cell proliferation, apoptosis, differentiation and numerous cellular responses (Swannie and Kaye, 2002). Its central role in these processes, such as tumor initiation, progression, invasion, angiogenesis and response to antitumor agents, makes it a potential therapeutic target in cancer treatment. We treated Hep3B cells with long-term exposure to low or high concentration of PMA to activate or inactivate protein kinase C; however, cells demonstrated a nearly unaltered cell cycle distribution. Furthermore, RO-318220, a non-selective protein kinase C inhibitor, was employed to examine the functional involvement of this enzyme. However, cells pre-treated with or without RO-318220 did not behave distinctly differently in their viability against alisol B acetate or saikosaponin-d treatment, suggesting that protein kinase C was not responsible for Hep3B cell death incurred by alisol B acetate- and saikosaponin-d, p38 MAPK, another serine/threonine protein kinase, belongs to the MAPK superfamily and appears to play a crucial role in cell apoptosis, cytokine production and transcriptional regulation in response to diverse extracellular stimuli, including irradiation, heat shock, cytokines and chemotherapeutic agents (Obata et al., 2000). In this work, SB203580, a selective inhibitor of p38 MAPK, did not influence the alisol B acetate- and saikosaponin-d-induced effect. In a parallel experiment using Western blot detection, both alisol B acetate and saikosaponin-d showed little effect on the basal level of phosphorylated p38 MAPK expression (data not shown). These data indicate that neither alisol B acetate- nor saikosaponin-d-induced apoptosis is through the activation of p38 MAPK.

Recent studies have suggested that apoptosis requires the participation of endogenous cellular enzymes. Central to the apoptotic program is a family of cysteine proteases, termed caspases (Stennicke and Salvesen, 1998; Budihardjo et al., 1999). It appears that apoptotic processes promoted by a variety of stimuli converge on the activation of a member of the caspase family. In living cells caspases are present as inactive zymogens and become activated following the apoptotic stimuli. To date, more than ten distinct human caspase genes have been identified (Villa et al., 1997). Among these caspases, the activation of caspase-3 is the crucial event in a variety of cells leading to the execution of apoptosis (Stennicke and Salvesen, 1998; Budihardjo et al., 1999). In the present study, we found that caspase-3 participates in the apoptosis induced by alisol B acetate, suggesting a central role of caspase-3. On the other hand, we did not detect any elevation of caspase-3 activity in response to saikosaponin-d, revealing the involvement of a caspase-3-independent mechanism. There are also increasing lines of evidence suggesting the existence of caspase-3-independent cell apoptosis (Cahir-McFarland and Kieff, 2002). It has been demonstrated that the epidermal growth factor enhances cisplatin-induced cell death in human ovarian cancer cells by activating an apoptotic pathway that is independent of caspase-3 (Cenni et al., 2001). Additionally, a low-molecular-weight fraction of human seminal plasma has been suggested to induce caspase-3-independent apoptosis in prostatic epithelial cells (Untergasser et al., 2001). However, the detail mechanism regarding saikosaponin-d action needs to be further studied and identified.

It has been recognized that TGF-β1 induces an apoptotic reaction when added to cultures of hepatoma cell lines, or primary cultures (Gressner et al., 1997). Therefore, TGF-β1 is thought to be a very important factor in growth regulation in hepatocytes and hepatoma cells. In this study, we also examined the effect of gentiopicroside, baicalein and geniposide on TGF-β1-induced apoptosis in Hep3B cells. We found that baicalein could efficiently prevent the TGF-β1-induced apoptosis. Baicalein is a well-known 12-lipoxygenase inhibitor. However, in our data (not shown), the predominant 12-lipoxygenase metabolite, 12-HETE, did not reverse the baicalein-induced anti-apoptotic effect, suggesting that the baicalein-mediated effect in the present study is not through the inhibition of 12-lipoxygenase. Furthermore, it has been suggested that baicalein exhibits antioxidant activity in both in vitro and in vivo studies (Hara et al., 1992). One possible route of baicalein-mediated anti-apoptosis is via its antioxidant activity. However, in this study TGF-β1 did not markedly generate reactive oxygen species (ROS), demonstrating that the antioxidant effect could not reasonably explain baicalein-mediated mechanisms. As a point of interest though, baicalein by itself profoundly induced the formation of H2O2; this effect was also detected in other flavonoids (Miura et al., 1998). There is accumulating evidence suggesting that H2O2 appears to serve as a messenger mediating the activation of NF-κB, which plays a crucial role in the apoptotic, anti-apoptotic, immune and inflammatory responses in a many cells (Boland, 2000; Karin and Lin, 2002). Furthermore, it has been suggested that diverse agents, such as calcium ionophore, tumor necrosis factor-α, interleukin-1 and lipopolysaccharide, thought to activate
NF-κB by distinct intracellular pathways, might all act through a common mechanism involving the synthesis of ROS (Schreck et al., 1991). In the present study, baicalein alone markedly induced the activation of NF-κB. This baicalein-induced effect on the NF-κB level was significantly attenuated by catalase, a H₂O₂ degrading enzyme, indicating that H₂O₂ serves as the upstream activator of NF-κB activation of baicalein action. Furthermore, we also tried to identify the effect of alsisol B acetate and saikosaponin-d on NF-κB nuclear binding activity in Hep3B cells. We found that these two agents had little effect on this transcription factor. However, it is worth noting that baikalin (100 μM) significantly inhibited the alsisol B acetate-induced apoptosis in Hep3B cells as seen by FACSscan flow cytometric analysis (data not shown). This has considerable implications for the efficacy of Long-dan-tan when comparing individual components. In order to increase the anti-cancer action against Hep3B cells, it seems that the dose of baikalin in Long-dan-tan should be decreased.

The anti-apoptotic effect of baikalin seems to contradict the report by Chang and colleagues, where flavonoids such as baikalin, baicalin and wogonin, decreased cell viability in human hepatoma cells (HepG2, Hep3B and SK-Hep1) using MTT assay methods (Chang et al., 2002). However, the cell lines showed different drug sensitivities, with SK-Hep1 being the most sensitive and Hep3B the most resistant in their study. These results are consistent with our unpublished data that baikalin (up to 50 μM) induced cytotoxicity in both human hepatoma HepG2 and HA-22T cells, but not Hep3B cells. However, the data also suggest that different hepatoma cells respond in different ways to baikalin. Whether the same effects occur on hepatoma cells in vivo needs further investigation.

In summary, after general examination of the five ingredients in the present study, three groups of distinct actions are categorized. Alsisol B acetate and saikosaponin-d, by themselves, cause a profound cytotoxic effect on human hepatoma Hep3B cells, baikalin is the effective anti-apoptotic constituent against TGF-β1-induced apoptosis, and gentiopicroside and geniposide exhibit little effect on apoptotic regulation in these cells.

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