Effect of wilfortrine on human hepatic cancer HepG2 cell proliferation potential *in vitro*

M. Yue[1]*, X.J. Shen[1]*, Y.X. Liu[2], X.Y. Lin[3], S.Q. Zhou[1] and Y.H. Song[4]

[1]Department of Gastroenterology,
Jinan Central Hospital Affiliated to Shandong University, Jinan,
Shandong, China
[2]Department of Oncology, Shengli Oil Central Hospital, Dongying,
Shandong, China
[3]Department of Pathology,
Shandong Provincial Hospital Affiliated to Shandong University, Jinan,
Shandong, China
[4]Department of Respiratory Medicine, Shandong Qianfoshan Hospital,
Shandong University, Jinan, Shandong, China

*These authors contributed equally to this study.
Corresponding author: X.J. Shen
E-mail: XingJieMM@126.com

Genet. Mol. Res. 14 (4): 15349-15355 (2015)
Received July 18, 2015
Accepted October 9, 2015
Published November 30, 2015
DOI http://dx.doi.org/10.4238/2015.November.30.12

ABSTRACT. Liver cancers are characterized by high morbidity and mortality owing to few effective drugs for its treatment. Wilfortrine has several pharmacological effects, including an inhibitory effect on liver cancer cell proliferation. However, whether wilfortrine can induce liver cancer cell apoptosis has not been elucidated. We investigated the role of wilfortrine on liver cancer cell HepG2 apoptosis and analyzed its possible mechanisms to provide a theoretical basis for clinical analysis of liver cancer pathogenesis. The liver cancer cell line HepG2 was treated with 40 mM wilfortrine for 48 h. Flow cytometry was applied to

detect HepG2 cell apoptosis and cell cycle changes. Western blot was used to analyze Bcl-2 and Bax expression. The HepG2 cell apoptosis rate increased significantly after treatment with wilfortrine, especially the early apoptosis rate ($P < 0.05$). However, wilfortrine did not change the cell cycle of HepG2 cells. After wilfortrine treatment, Bcl-2 expression decreased significantly ($P < 0.05$); on the contrary, Bax expression increased noticeably compared with the control group ($P < 0.05$). Wilfortrine can induce liver cancer cell HepG2 apoptosis, but with no effect on the cell cycle, mainly by promoting Bax expression and inhibiting anti-apoptotic protein Bcl-2 expression.

Key words: Wilfortrine; HepG2 cell; Liver cancer; Apoptosis

INTRODUCTION

Tripterygium wilfordii, also known as daemonorops margaritae root, xanthate, graceful jessamine herb, or daemonorops margaritae grass, is bitter. *T. wilfordii* plants contain a variety of alkaloids, of which wilfortrine is a small bioactive molecule. Wilfortrine is extracted from the wooden lianas of the *T. wilfordii* genus belonging to the Celastraceae family (Caspi and Polak., 2013; Wu et al., 2014). Wilfortrine has many pharmacological functions, including immunosuppression, that have significant inhibitory effects on hemolysin formation in mice. Intraperitoneal injection wilfortrine can inhibit graft-versus-host reaction. It can also inhibit delayed-type hypersensitivity and inhibit peripheral blood mononuclear cell proliferation reaction induced by phytohemagglutinin. Thus, wilfortrine can be used in the treatment of autoimmune diseases such as nephritis and rheumatoid arthritis (Cai et al., 2011; Xie et al., 2012; Ma et al., 2014). Several studies have suggested that wilfortrine has anti-inflammatory effects, such as inhibiting interleukin and tumor necrosis factor expression, and can play a role in the treatment of inflammatory diseases (Zheng et al., 1989; Ouyang et al., 2007).

Liver cancer is one of the common malignant tumors with the fifth highest rate of morbidity. It is a leading cause of death in patients with malignant tumors, ranking second after lung cancer (Cheong et al., 2014). China accounts for 55% of the global liver cancer morbidity (Takada et al., 2014). There are many treatment methods for liver cancer including surgery, radiotherapy, chemotherapy, immunotherapy, and interventional therapy. However, the efficacy of liver cancer treatment is poor with easy metastasis, high recurrence, and poor prognosis, resulting in short survival time and decreased quality of life. Thus, medical problems in liver carcinogenesis, development, diagnosis, treatment, and research need to be resolved urgently (Alsaied et al., 2014; Ling et al., 2014). Since the current anti-liver cancer drugs are less effective, the search for effective anti-liver cancer drugs has increased significance. Some researchers have confirmed that drugs from *T. wilfordii*, such as triptolide, have inhibitory effects on liver cancer cell proliferation. As a *T. wilfordii* extract, wilfortrine also has an inhibitory effect on liver cancer cell proliferation, but whether it can induce liver cancer cell apoptosis has not been elucidated (Chan et al., 2001; Huang et al., 2012). This study investigated the role of wilfortrine on liver cancer cell HepG2 apoptosis and analyzed its possible mechanisms to provide a theoretical basis for clinical analysis of liver cancer pathogenesis.

MATERIAL AND METHODS

Main instruments and reagents

HepG2 cell line was purchased from American Type Culture Collection (ATCC Cell Bank, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), EDTA, and penicillin-streptomycin were purchased from Hyclone (USA). Pancreatic enzyme EDTA digestive juice was purchased from Sigma (USA). Annexin-propidium iodide (PI) and cell cycle detection kits were purchased from Becton, Dickinson and Company (BD Company, USA). Polyvinylidene difluoride membrane was purchased from Pall Life Sciences (USA). Western blot-related chemical reagents were purchased from Beyotime Biotechnology Co., Ltd. (Shanghai, China). Enhanced chemiluminescence reagent was obtained from Amersham Biosciences (USA). Bcl-2 and Bax primary antibodies and horseradish peroxidase-tagged IgG secondary antibody were purchased from Cell Signaling (USA). Other common reagents were obtained from Sangon Biotechnology Co., Ltd. (Shanghai, China). A FACSCalibur flow cytometry instrument was purchased from Becton, Dickinson and Company. A cell incubator was purchased from Thermo Electric Company (Pennsylvania, USA). Wilfortrine was purchased from Tongwei Industrial Co., Ltd. (Shanghai, China).

HepG2 cell culture and grouping

The HepG2 liver cancer cell line was maintained in DMEM supplemented with 10% FBS and 100 U/mL penicillin-streptomycin in a humid atmosphere containing 5% CO_2 at 37°C. Wilfortrine (40 mM) was used to treat HepG2 liver cancer cells for 48 h.

Flow cytometry

After treatment with wilfortrine (48 h), cells were digested by trypsinization and washed with PBS. Flow cytometry was used to detect the apoptosis rate of HepG2 cells by determining the relative amount of annexin V-fluorescein isothiocyanate-positive/PI-negative cells.

Cell cycle analysis

All cells in the culture dish were harvested by trypsinization, washed in ice-cold PBS, and fixed in ice-cold 70% ethanol in PBS. Before staining, the cells from single-cell suspension were pelleted in a cooled centrifuge and resuspended at 4°C. Bovine pancreatic RNAase was added at a final concentration of 100 µg/mL, and cells were incubated at 37°C for 30 min, followed by incubation with 50 µg/mL PI for 1 h at 4°C. Cells (1 x 10^5 cells) were analyzed by flow cytometry.

Western blot

After treatment with wilfortrine (48 h), cells were harvested and homogenized with lysis buffer. Total protein was separated by denaturing 10% SDS-polyacrylamide gel electrophoresis. Detection was performed using a Bio-Rad Image Processing System (USA), and

analysis was performed with the Quantity One software (Bio-Rad). Antibody dilutions were 1:500 for Bcl-2 and 1:5000 for Bax. Protein levels were normalized to actin.

Statistical analysis

Numerical data are reported as means and standard deviation (means ± SD). Differences between means and rates were analyzed using the Student *t*-test and the chi-square test, respectively. All statistical analyses were performed using the SPSS16.0 software (Chicago, IL, USA). P values <0.05 were considered to be statistically significant.

RESULTS

Effect of wilfortrine on HepG2 cell apoptosis

Flow cytometry was applied to detect the effect of wilfortrine on HepG2 cell apoptosis. The HepG2 cell apoptosis rate increased significantly after treatment with wilfortrine ($P < 0.05$). The difference mainly appeared in the early apoptosis stage ($P < 0.05$), while the difference in the late apoptosis stage was not significant ($P > 0.05$; Figure 1). These results indicate that wilfortrine can inhibit liver cancer cell proliferation by inducing cell apoptosis.

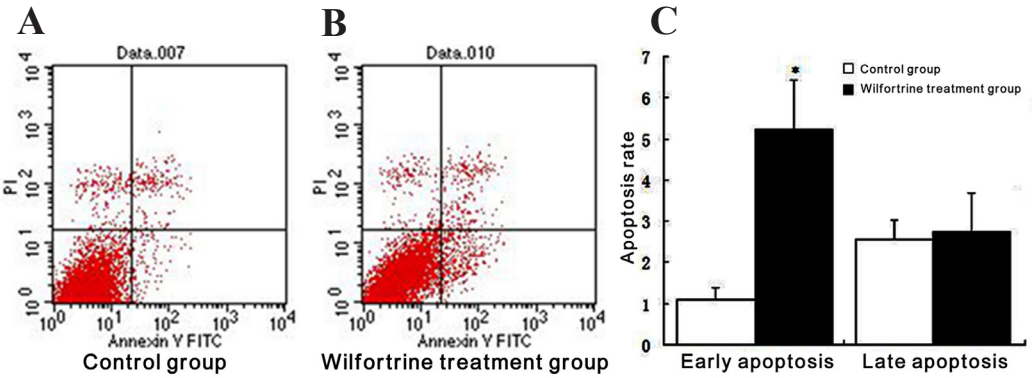

Figure 1. Effect of wilfortrine on HepG2 cell apoptosis. **A.** Flow cytometry analysis of control cells. **B.** Flow cytometry analysis of cells treated with 40 mM wilfortrine for 48 h. **C.** Rate of apoptosis [annexin V-fluorescein isothiocyanate (FITC)-positive/propidium iodide (PI)-negative cells] in early and late state apoptosis. *P < 0.05 compared with the control group.

Effect of wilfortrine on HepG2 cell cycle

Flow cytometry was applied to detect the effect of wilfortrine on the HepG2 cell cycle. After treatment with wilfortrine for 48 h, the HepG2 cell cycle was retarded, but this was not significant ($P > 0.05$; Figure 2). This result indicates that wilfortrine fails to affect the HepG2 cell cycle.

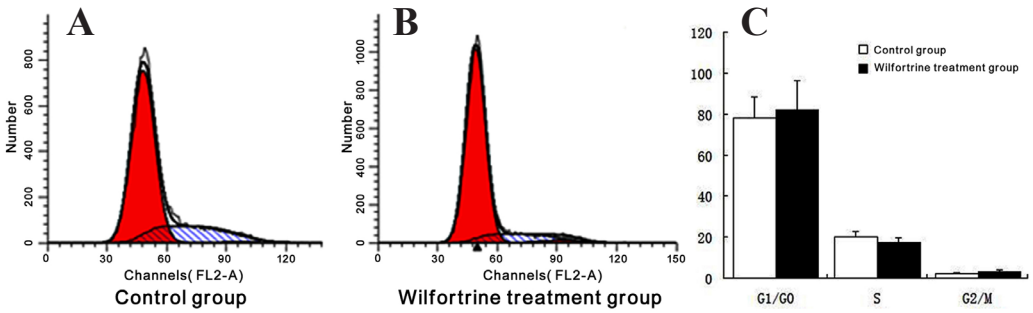

Figure 2. Effect of wilfortrine on HepG2 cell cycle. **A.** Flow cytometry analysis of control cells. **B.** Flow cytometry analysis of cells treated with 40 mM wilfortrine for 48 h. **C.** Rate of cell cycle.

Effect of wilfortrine on apoptosis-related protein expression in HepG2 cells

Western blot was applied to analyze further the effect of wilfortrine on the expression of the apoptosis-related proteins Bcl-2 and Bax in HepG2 cells. It was found that, after treatment with wilfortrine, Bcl-2 expression decreased significantly compared with the control ($P < 0.05$; Figure 3); on the contrary, Bax expression increased significantly ($P < 0.05$; Figure 4). These results suggest that wilfortrine could induce HepG2 cell apoptosis by inhibiting Bcl-2 and promoting Bax expression.

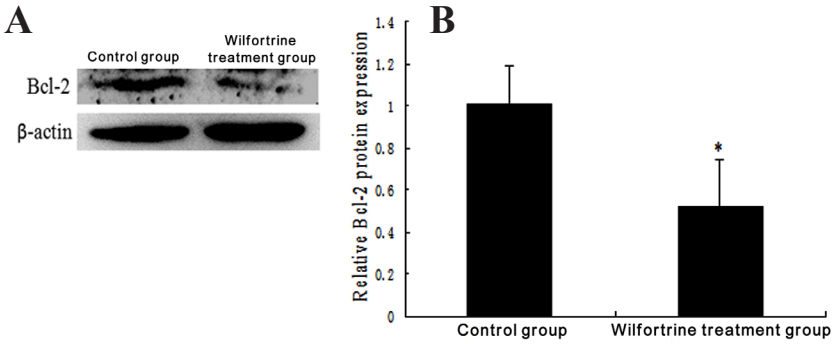

Figure 3. Effect of wilfortrine on Bcl-2 expression. **A.** Control cells and cells treated with 40 mM wilfortrine for 48 h. **B.** Relative Bcl-2 protein expression. *$P < 0.05$ compared with the control groups.

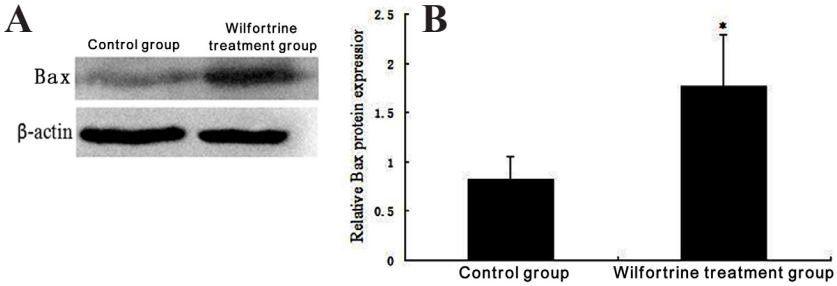

Figure 4. Effect of wilfortrine on Bax expression. **A.** Control cells and cells treated with 40 mM wilfortrine for 48 h. **B.** Relative Bax protein expression. *$P < 0.05$ compared with the control group.

DISCUSSION

Apoptosis plays an important role in the regulation of liver carcinogenesis and development and, thus, draws large attention in liver cancer investigation (Song et al., 2014). The anti-apoptotic mechanism of liver cancer cells is closely related to Bcl-2 overexpression and Bax inhibition (Fu et al., 2011). Apoptosis is a regulatory process of self-stabilization of the body that can inhibit tumor overgrowth. Overexpression of Bcl-2 can prevent damaged cells from undergoing apoptosis and lead to liver cancer progression, which is associated with the related proliferative and growth inhibition genes. For the inhibition effect of overexpressed Bcl-2 on apoptosis, it is considered that Bcl-2 can block the nuclear transportation, leading to reduced intracellular calcium ion concentration and antioxidant damage. As a result, the balance between cell proliferation and apoptosis is disturbed, resulting in carcinogenesis and liver cancer (Ke et al., 2014; Sabokrouh et al., 2014). Bax overexpression may promote cell apoptosis. Overexpression of Bax can initiate an apoptosis signal or inhibit Bcl-2 expression. Bax either initiated apoptosis signal by increasing expression or inhibited the expression of Bcl-2 protein as important inhibitors. The function depends on which one was the dominant, if it were Bax protein or Bax protein Dimer, inducing cell will end up in apoptosis; if it were Bcl-2 protein or Bcl-2 protein Dimer, apoptosis is inhibited. An imbalance in Bax and Bcl-2 protein expression results in abnormal cell expression (Leuenroth and Crews, 2008; Rich et al., 2014).

Previous studies have confirmed that wilfortrine, an extract from *T. wilfordii*, can inhibit liver cancer cell proliferation. However, its specific mechanism is still not clarified (Ji et al., 2002; Xiao et al., 2002; Leuenroth et al., 2007). Our study confirmed that wilfortrine could induce HepG2 cell apoptosis, especially in the early apoptosis stage. This suggested that wilfortrine could inhibit liver cancer cell proliferation through inducing cell apoptosis in the early stage. Although wilfortrine showed slight inhibition of the cell cycle, this was not significant compared with the control. Therefore, wilfortrine does not have an effect on HepG2 cell cycle. Further investigation confirmed that wilfortrine could induce HepG2 cell apoptosis by inhibiting Bcl-2 expression and promoting Bax expression.

In conclusion, wilfortrine can induce liver cancer HepG2 cell apoptosis, mainly by promoting expression of the apoptotic protein Bax and inhibiting expression of the anti-apoptotic protein Bcl-2, but not by regulating the cell cycle. Since wilfortrine is a commonly used Chinese herbal medicine with an easy extraction method, the in-depth study of its mechanism is helpful for its clinical application. On the other hand, since effective chemotherapy drugs for liver cancer are still lacking, investigation of wilfortrine function may provide new alternatives for clinical treatment of liver cancer.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

We thank the anonymous reviewers for reviewing this manuscript.

REFERENCES

Alsaied OA, Sangwan V, Banerjee S, Krosch TC, et al. (2014). Sorafenib and triptolide as combination therapy for hepatocellular carcinoma. *Surgery* 156: 270-279.

Cai MQ, Chen XH, He SW, OuYang XK, et al. (2011). Determination of four pyridine alkaloids from *Tripterygium wilfordii* Hook. f. in human plasma by high-performance liquid chromatography coupled with mass spectrometry. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci*. 879: 3516-3522.

Caspi O and Polak A (2013). Traditional immunosuppression - Lei Gong Teng in modern medicine. *Harefuah* 152: 404-409, 433.

Chan EW, Cheng SC, Sin FW and Xie Y (2001). Triptolide induced cytotoxic effects on human promyelocytic leukemia, T cell lymphoma and human hepatocellular carcinoma cell lines. *Toxicol. Lett*. 122: 81-87.

Cheong JY, Shin HD, Cho SW and Kim YJ (2014). Association of Polymorphism in MicroRNA 604 with Susceptibility to Persistent Hepatitis B Virus Infection and Development of Hepatocellular Carcinoma. *J. Korean Med. Sci*. 29: 1523-1527.

Fu Q, Huang X, Shu B, Xue M, et al. (2011). Inhibition of mitochondrial respiratory chain is involved in triptolide-induced liver injury. *Fitoterapia* 82: 1241-1248.

Huang W, He T, Chai C, Yang Y, et al. (2012). Triptolide inhibits the proliferation of prostate cancer cells and down-regulates SUMO-specific protease 1 expression. *PLoS One* 7: e37693.

Ji H, Yao S, Luo Q and Cao X (2002). TCM treatment of rheumatoid arthritis by supplementing the kidney and invigorating the blood circulation. *J. Tradit. Chin. Med*. 22: 252-255.

Ke Z, Li J, Zhe J, Liang LB, et al. (2014). Flow cytometric analysis of asialoglycoprotein receptor expression predicts hepatic functional reserve after hepatectomy. *J. Coll. Physicians Surg. Pak*. 24: 820-824.

Leuenroth SJ and Crews CM (2008). Triptolide-induced transcriptional arrest is associated with changes in nuclear substructure. *Cancer Res*. 68: 5257-5266.

Leuenroth SJ, Okuhara D, Shotwell JD, Markowitz GS, et al. (2007). Triptolide is a traditional Chinese medicine-derived inhibitor of polycystic kidney disease. *Proc. Natl. Acad. Sci. U. S. A*. 104: 4389-4394.

Ling D, Xia H, Park W, Hackett MJ, et al. (2014). pH-Sensitive Nanoformulated Triptolide as a Targeted Therapeutic Strategy for Hepatocellular Carcinoma. *ACS Nano*. 8: 8027-8039.

Ma L, Liu B, Jiang Z and Jiang Y (2014). Reduced numbers of regulatory B cells are negatively correlated with disease activity in patients with new-onset rheumatoid arthritis. *Clin. Rheumatol*. 33: 187-195.

Ouyang XK, Jin MC and He CH (2007). Simultaneous determination of four sesquiterpene alkaloids in *Tripterygium wilfordii* Hook. F. extracts by high-performance liquid chromatography. *Phytochem. Anal*. 18: 320-325.

Rich G, Williams D and Roche E (2014). Gastrointestinal: Hepatocellular carcinoma in an esophageal varix. *J. Gastroenterol. Hepatol*. 29: 1950.

Sabokrouh A, Goodarzi MT, Vaisi-Raygani A, Khatami S, et al. (2014). Effects of Treatment with Platinum Azidothymidine and Azidothymidine on Telomerase Activity and Bcl-2 Concentration in Hepatocellular Carcinoma-Induced Rats. *Avicenna J. Med. Biotechnol*. 6: 200-209.

Song S, Luo M, Song Y, Liu T, et al. (2014). Prognostic Role of SIRT1 in Hepatocellular Carcinoma. *J. Coll. Physicians Surg. Pak*. 24: 849-54.

Takada Y, Tohyama T and Watanabe J (2014). Biological markers of hepatocellular carcinoma for use as selection criteria in liver transplantation. *J. Hepatobiliary Pancreat. Sci*. doi: 10.1002/jhbp.195.

Wu J, Liu X, Chan CO, Mok DK et al. (2014). Petroleum ether extractive of the hips of *Rosa multiflora* ameliorates collagen-induced arthritis in rats. *J. Ethnopharmacol*. 157: 45-54.

Xiao YH, Chen DP, Yan JH and Yokoyama Y (2002). Mechanism of action of *Tripterygium wilfordii* polyglycoside on experimental endometriosis. *Eur. J. Gynaecol. Oncol*. 23: 63-67.

Xie FG, Li CJ, Yang JZ, Luo YM, et al. (2012). Study on chemical constituents from the root bark of *Tripterygium hypoglaucum*. *Zhong Yao Cai* 35: 1083-1087.

Zheng YL, Xu Y and Lin JF (1989). Immunosuppresive effects of wilfortrine and euonine. *Yao Xue Xue Bao*. 24: 568-572.