Tetrandrine inhibits hepatocellular carcinoma cell growth through the caspase pathway and G_2/M phase

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Received November 20, 2012; Accepted January 18, 2013

DOI: 10.3892/or.2013.2352

Abstract. Activation of p53-independent pathways plays an important role in phytochemical-induced apoptosis and is considered to be a crucial factor in the invasion and metastasis of cancer. Previous studies have shown that combined effects of *Stephania tetrandra* with medicinal herbs exhibit beneficial effects in cancer patients. Tetrandrine, an active component of *Stephania tetrandra* has been reported to have anticancer properties in cancer cells. However, the mechanism(s) of action of tetrandrine in liver cancer have yet to be fully elucidated. In this study, we investigated the effects of tetrandrine in hepatocellular carcinoma (HCC) cells. The results showed that tetrandrine inhibited HCC cell proliferation by suppression of cell cycle progression at the G_2/M phase. Changes in the expression levels of Bax, Bcl, p53, survivin, PCNA, PARP and p21 were observed. In addition, tetrandrine increased caspase-3 expression and induced DNA fragmentation in Huh-7 cells. The results suggest that the anti-cancer effect of tetrandrine in Huh-7 cells may be mediated by p53-independent pathways.

Introduction

Hepatocellular carcinoma (HCC) is the most common form of liver cancer and is one of the leading causes of cancer-related mortality worldwide (1). Invasion and metastasis of liver cancer contribute to treatment failure in the majority of cancer patients. Liver cancer is generally diagnosed at a later stage of cancer development. Liver metastasis and treatment are related to the characteristics of the tumor and the immune system of the host. Understanding the apoptotic pathways and their corresponding inhibitors enables us to formulate strategies for cancer therapy. The overexpression of genes that are associated with cell growth and programed cell death depend on the activity of the p53-associated pathways. During malignant progression, the human papillomaviruses integrate into the liver cell genome resulting in a loss of expression of oncogenes. The gene proteins may lead to interference with the tumor suppression protein p53, which is activated in response to stress and plays an important role in the regulation of cell cycle, DNA repair and apoptosis (2). Genomic alterations of p53 can be found in cancer and deletion of p53 in cancer cells could lead to their resistance to apoptosis (2). Tumor suppressor p53 is involved in transcriptional activation of the human Bax gene (3,4). Apoptosis-inducing factor plays a role in the regulation of caspase-independent cell death (5,6). The most common risk factor for HCC is the hepatitis B (HBV) or hepatitis C viral (HCV) infection, of which there is a high incidence rate in China (1). Approximately one fourth of the carriers could develop liver cancer. An antiviral approach against human transcriptional inactivation of viral infection is used for HCV infection. Complementary medicine using herbal ingredients against transcriptional inactivation of cancer cells showed minimal system toxicity and could be a promising agent for liver cancer therapy particularly at an early stage of liver carcinogenesis. Cancer patients can have a higher survival rate with the complementary treatment. Tetrandrine is a bisbenzylisoquinoline alkaloid, a naturally occurring compound isolated from the root of *Stephania tetrandra*, which was reported to exhibit a variety of pharmacological properties including anti-inflammatory, anti-rheumatic and anti-hypertensive effects (7). It can inhibit the proliferation of HeLa and HepG2 cells *in vitro* and suppress ascites tumors in mice (8). Tetrandrine was reported to suppress Wnt/β-catenin signaling and tumor growth of human colorectal cancer (9). A previous study showed that tetrandrine induced apoptosis by activating reactive oxygen species and repressing Akt activity in human liver cancer cells (10). The results suggest that mediation of the ROS/Akt pathway by tetrandrine can enhance the beneficial effects of tetrandrine in cells. Zhang *et al* showed that tetrandrine was used together with cisplatin to enhance growth suppression of ovarian cells and apoptosis (11). *In vivo* study of the combined effects of tetrandrine and cisplatin exhibited anticancer effects in the rat (11). The effect of tetrandrine with radiation on human esophageal cancer cell line TE1 showed that the expression of cyclin B1 protein increased while radiation-induced G_2 arrest was abrogated (12). The study suggests that the enhanced cytotoxicity and activation of ROS-dependent caspase-3 activity could induce programmed cell death in cancer (13).

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Key words: tetrandrine, Huh-7, cancer, cell cycle, inhibition

Another study indicated that two distinct pathways could lead to the apoptosis of cancer cells (14). Apoptosis of cancer cells was considered to be associated with mitochondrial release of inducing factors that occurs downstream of cytochrome *c* release in response to oxidative stress (15). A recent study showed that tetrandrine induced apoptosis via caspase cascade in human bladder cancer cells (16). Although tetrandrine was reported to have multiple biologic activities, the details of its anticancer properties are lacking. Therefore, we investigated the effect of tetrandrine on Huh-7 cancer cells.

Materials and methods

Cell culture and reagents. The human liver cell line Huh-7 was obtained from the American Type Culture Collection (USA) and cultured in DMEM (Gibco, USA) supplemented with RPMI and 0.25% trypsin-EDTA at 37˚C in an atmosphere of 5% CO_2-95% air. Tetrandrine was obtained from Sigma Chemicals (St. Louis, MO, USA).

In vitro cell viability assay. Cell proliferation was measured by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay (MTT) according to the manufacturer's protocols. Huh-7 cells ($1x10^5$ cells/well) were placed in 96-well microtiter plates (Corning) and incubated overnight. Cells were treated with either 1% serum DMEM as a control or with various concentrations of tetrandrine in 1% DMEM for 24, 48 or 72 h, respectively. At the end of the incubation period, 20 μl of a 5-mg/ml solution of MTT prepared in PBS was placed into each well and the cells were incubated for an additional 4 h. Cells were lysed in 200 μl DMSO and absorbance was measured at 570 nm. Six replicate wells were used for each analysis. The data represent the means ± SD of three independent experiments with 95% confidence intervals.

Western blot analysis of gene expression. Western blotting assay was used to analyze the expressions of apoptotic proteins in Huh-7 cells. Cells ($1x10^6$) seeded in 6-well plates were exposed to various concentrations of tetrandrine for 24, 48 and 72 h. The cells were harvested and lysated (40 μg of protein per lane) and fractionated by 10% SDS-PAGE as described below. The protein content was determined according to the Bradford method (17). Cells were collected in the 15-ml falcon and were re-suspended in 400 μl of cell DNA lysis buffer in a 1.5-ml microtube with vigorous mixing. Twenty microliters of 10 mg/ml proteinase K were added to each microtube. The mixtures were incubated at 37˚C for 3 h. Saturated NaCl solution (50 μl) was added to each sample tube prior to centrifugation at 7,000 x g for 15 min at room temperature. The supernatant containing DNA was collected. Ice cold absolute ethanol (1 ml) was added to the tubes with mixing followed by centrifugation at 14,000 x g at 4˚C for 20 min. The pellets were washed with 70% ethanol once. They were spun again at 14,000 x g at 4˚C for 20 min. The DNA pellet was allowed to air dry. TE buffer (50 μl) containing 0.2 mg/ml of RNase A was added to the DNA pellets for RNA digestion prior to incubation at 37˚C for 90 min. DNA solution (2 μl) from each sample was added to 998 μl TE buffer. The concentrations of the DNA in the diluted solutions were measured by UV spectrophotometry (Beckman, DU 650) at 260 nm.

Table I. The annealing and extension temperature used for different target genes.

Target gene	Annealing and extension temperature (˚C)
Bax	60
Bid	60
Bcl-2	55
GAPDH	60
p21	65
Survivin	60

Gel electrophoresis of DNA. Agarose (0.3 g) was added to 20 ml TBE buffer. Ethidium bromide (2 μl) was added to the agarose solution followed by the addition of 10 μl of the dissolved DNA samples and 2 μl 6X DNA loading dye. The mixtures were loaded on the 1.5% agarose gel and it was run at 75 V for 1 h. The DNA bands were visualized under UV illuminator. The gel was photographed for documentation.

Measurement of gene expression in tetrandrine-induced cancer cells. Huh-7 cells ($1x10^6$) were seeded on 6-well plates with different concentrations of tetrandrine (0, 7.5, 15 and 20 μM). After 24 h of incubation, cells were harvested by trypsinization and washed with 1X PBS. The cell platelets were collected by centrifugation at 600 g for 2 min followed by the addition of 1 ml TRIzol reagent. The mixtures were then incubated at room temperature for 5 min prior to the addition of 266 μl chloroform. Subsequently, the samples were centrifuged at 14,000 rpm for 15 min. The supernatant was mixed with 70% ethanol in 1:1 volume ratio. RNeasy Mini kit™ from Qiagen was used for the total RNA extraction. The mRNA extraction was conducted according to the manufacturer's method. The total RNA concentration of each sample assay was measured. The same amount of total RNA was used in each sample for cDNA synthesis. Transcriptor First Strand cDNA Synthesis kit from Roche was used for cDNA synthesis.

Reverse-transcription-PCR (RT-PCR) and detection of gene products. Each RT-PCR reaction mixture contained 11.2 μl water, 4 μl 5X Green GoTaq Flexi Buffer, 1.2 μl $MgCl_2$ (25 mM), 1 μl primer, 0.4 μl dNTP, 0.2 μl GoTaq® Hot Start Polymerase 5 U/μl, 2 μl cDNA template. The reaction was conducted at 95˚C for 5 min and the denaturation was at 95˚C for 30 sec. Annealing and extension were performed in one step over 1 min (Table I). The PCR products were detected by running a 2% agarose gel. Agarose (0.4 g) was dissolved in 20 ml 1X TBE buffer. The 2% agarose gel was run at 100 V for 30 min to separate the PCR products.

Measurement of protein expression in tetrandrine-induced apoptotic cells. Huh-7 cells ($1x10^6$) were seeded into a 100-mm dish with different concentrations of tetrandrine for 24 h before being trypsinized and collected by centrifugation. The number of cells collected in each sample was counted using a hemocytometer. Whole cells lysis buffer (200 μl) was

added per 1x10^6 cells and incubated at 37˚C for 30 min. The samples were then placed in boiling water for 10 min prior to centrifugation at 14,000 x g for 15 min. The supernatant of the samples was collected in a new micro-centrifuge tube for the protein concentration determination (17).

Protein gel electrophoresis by SDS-PAGE. Mini-PROTEAN® III cell with a 10-tooth comb from Bio-Rad was used for SDS-PAGE according to a previous method with modifications (17). A small quantity of isopropanol was layered on the top of the running gel solution before the excess isopropanol was discarded and dH_2O was used to wash the gel. Stacking gel solution (4%) was added onto the top of the running gel. The gel was allowed to stand until it polymerized. Sample loading dye (2X) was added to the protein samples in a 1:1 ratio prior to boiling at 100˚C for 10 min. Samples were loaded onto the wells and 1.5 μl of PageRuler™ Prestained Protein Ladder was added as a protein marker. The gel was allowed to run at constant voltage. Fifty V were used for running the stacking gel whereas 80, 100 and 120 V were used for running 10, 12 and 15% running gel, respectively. The SDS-PAGE was stopped after the dye front containing bromophenol blue reached the bottom of the gel.

Western blot analysis. Bio-Rad Semi-dry Trans-Blot electroblotter was used to transfer the protein onto the membrane according to the manufacturer's protocols. The gel was immersed into transfer buffer for 15 min for equilibration. The PVDF membrane was activated using 100% methanol and was washed twice using dH_2O to remove methanol prior to equilibration in the transfer buffer for 15 min. The running gel was placed above the membrane with another 3 pieces of paper. Constant current (0.1A) was used to transfer proteins for 2 h. The PVDF membrane was rinsed with TBST buffer twice. The desired dilution of antibody was added to the suitable percentage of non-fat milk solution which was the same as that used in blocking. The membrane was finally blotted with different antibodies overnight at 4˚C. The membrane was washed with 1X TBST containing 0.2% Tween-20 three times, 15 min each. After washing, the membrane was blotted with the corresponding secondary antibody. The dilution for anti-mouse and anti-rabbit were 1:10,000 and 1:6,667, respectively. The secondary antibodies were diluted in non-fat milk solution. The blocking solution contained the same concentration of non-fat milk as that for the primary antibody. The membrane was agitated for 1 h at room temperature. After probing with secondary antibody, the membrane was washed with 1X TBST containing 0.1% Tween-20 solution three times, each with 10 min.

Rodeo™ ECL Western Blotting Reagents from USB Biochemicals was used for the signal development. The two reagents in the kit were mixed in a 1:1 ratio. The mixture was equilibrated at room temperature for 5 min prior to use. Excess TBST solution on the membrane was removed by blotting it onto the M-fold tissue paper. The western blotting reagent mixture was added slowly onto the membrane with the size mobilized with proteins faced upward. Protein bands on the membrane were visualized after exposing to Fuji Super RX film. The intensities of the bands were analyzed using ImageJ program.

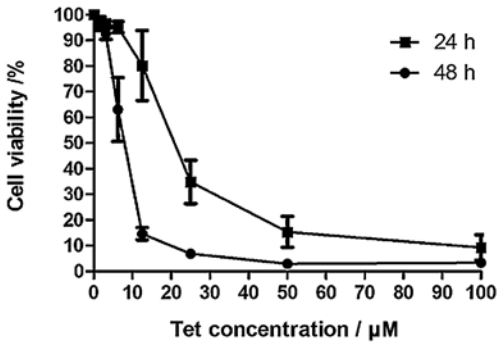

Figure 1. Huh-7 cells were seeded in 96-well microplates and were incubated with different concentrations of tetrandrine for 24 and 48 h. The viability of the cells was measured by MTT assay using DMSO as blank. The values are the means of three individual experiments. The data represent the means ± SD (n=3).

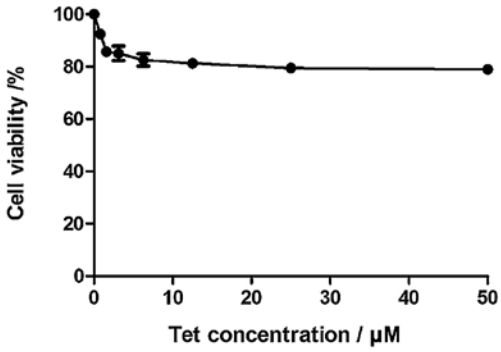

Figure 2. WRL68 cells were seeded in 96-well microplates and were incubated with different concentrations of tetrandrine for 24 h. The viability of the cells was measured by MTT assay using DMSO as blank. The values are the means of three individual experiments. The data represent the means ± SD (n=3).

Cell cycle analysis of tetrandrine-treated cells. Huh-7 cells (1x10^6) were seed into a 100-mm dish with different concentrations of tetrandrine for 24, 24 and 72 h. The tetrandrine-treated Huh-7 cells were trypsinized and were collected in a 15-ml falcon prior to centrifugation at 3,000 x g for 3 min. The cells were re-suspended in 1 ml 1X PBS prior to centrifugation at 3,000 x g for 3 min. The cells were collected by centrifugation in sequence after washing in 2 ml of 70% ethanol, RNase containing PBS solution, 1 ml propidium iodide solution. The cells in 1 ml propidium iodide solution were centrifuged at 3,000 x g for 3 min prior to flow cytometry analysis. The DNA content of the treated cells was recorded and analyzed by FACSCanto flow cytometer.

Statistical analysis. Statistical analysis was conducted using ANOVA. All experiments were performed three times independently. Data are expressed as the means ± SD.

Results

Fig. 1 shows the cell viability of Huh-7 cells following treatment with different concentrations of tetrandrine for 24 and 48 h. The viability of cells significantly decreased when tetrandrine concentration was increased. The IC_{50} of cells after 24 and 48 h of incubation were found to be 20.8 and 8.0 μM,

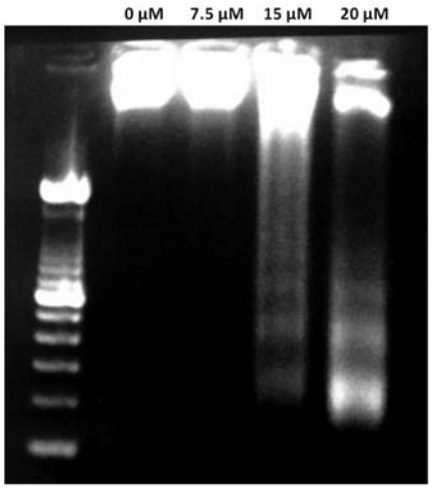

Figure 3. Huh-7 cells were incubated with 0, 7.5, 15 and 20 μM tetrandrine for 24 h. DNA contents extracted were analyzed by agarose gel electrophoresis with 2% agarose gel. The DNA ladders were visualized using UV illuminator after staining with ethidium bromide.

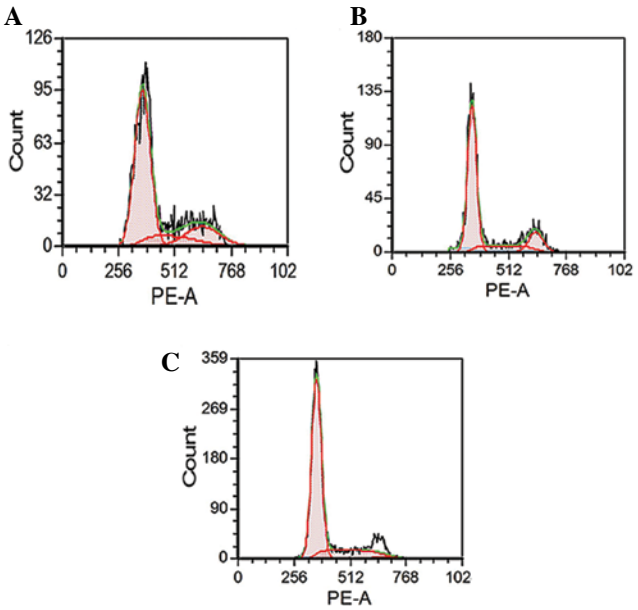

Figure 4. Flow cytometry analysis of Huh-7 cells treated with tetrandrine for 24 h. Huh-7 cells were incubated with (A) 0 μM, (B) 5 μM and (C) 10 μM of tetrandrine for 24 h. The DNA contents of the cells were evaluated by propidium iodide staining. Data represent the means of three individual sets of experiments.

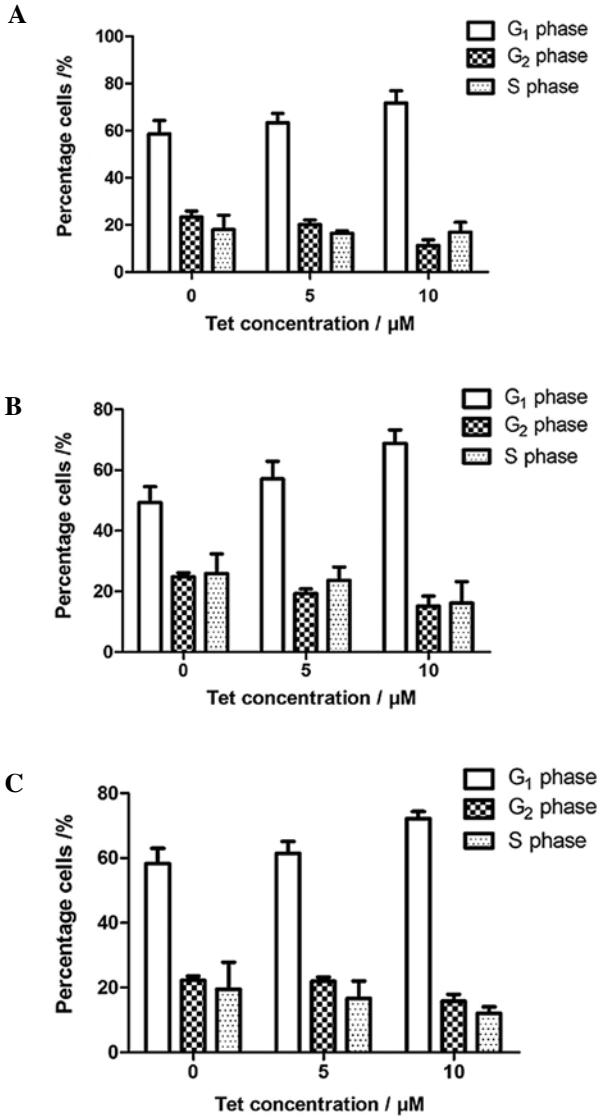

Figure 5. The graphs show the cell cycle analysis (%) of Huh-7 cells after (A) 24, (B) 48 and (C) 72 h of treatment with 0, 5 and 10 μM of tetrandrine. The data represent the means ± SD (n=3).

respectively. After 48 h of incubation, the cell viability was reduced. Fig. 2 shows the effect the tetrandrine on WRL68 cells. The cell viability of WRL68 cells decreased when tetrandrine concentration increased, but leveled off when its concentration reached 3.125 μM. No IC_{50} for WRL68 could be determined. Fig. 3 shows the DNA fragmentation of tetrandrine-treated Huh-7 cells. DNA ladders were observed in the tetrandrine-treated samples but not in the control. Fig. 4 shows the flow cytometry analysis of Huh-7 cells after treatment with tetrandrine for 24 h. The similar pattern of results of flow cytometry after 48 and 72 h of incubation of cells was recorded. The results are summarized in Fig. 5 and indicate the distribution of the cell populations in G_1, S and G_2 phase following treatment with tetrandrine. The cell percentage in each phase of the cell cycle was analyzed by FACS Express. The cell population in the G_1 phase increased from 58.63 to 71.72% after 24 h, from 49.30 to 68.74% after 48 h and from 58.30 to 72.15% after 72 h of treatment. For the cell population within the S phase, the percentages decreased slightly from 18.07 to 16.95% for 24 h, from 25.88 to 16.14% for 48 h and from 19.47 to 12.09% for 72 h of incubation. The cell population within the G_2 phase decreased at all the time points. It decreased from 2.30 to 11.33%, from 24.83 to 15.14% and from 22.24 to 15.76% for 24, 48 and 72 h of incubation, respectively. Fig. 6 shows the mRNA expression of survivin, Bax, Bid and p21 in tetrandrine-treated cancer cells. The expression levels of these genes were evaluated by the RT-PCR. Fig. 7 shows the western blots of different apoptotic proteins after Huh-7 cells were treated with tetrandrine. When the tetrandrine concentration was increased from 0 to 20 μM, the band intensities for the full-length PARP, pro-caspase-9, PCNA and Bcl-2 were found to be decreased while the intensities for bands of cleaved PARP,

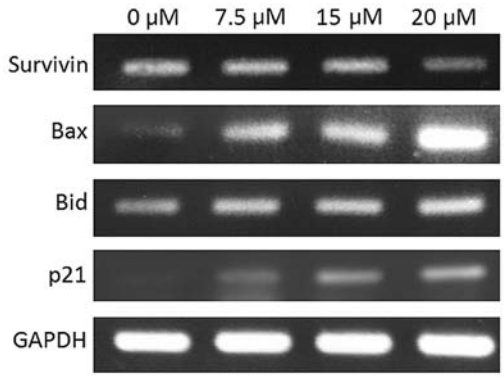

Figure 6. Huh-7 cells were treated with 0, 7.5, 15 and 20 μM tetrandrine for 24 h. The mRNA of lysed cells was extracted and was converted to cDNA. The gene expression levels of Bax, Bid, p21 and survivin were evaluated by reverse-transcription PCR. GAPDH was used as the control. Data represent the means of three individual sets of experiments.

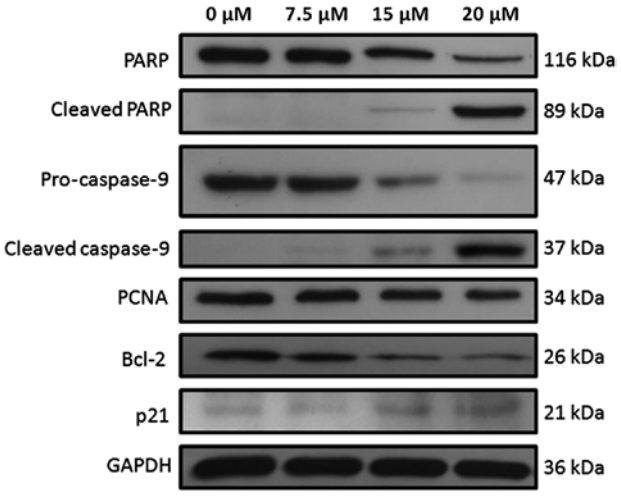

Figure 7. Huh-7 cells were treated with 0, 7.5, 15 and 20 μM of tetrandrine for 24 h. Equal amounts of cell lysates (60 μg) were subjected to electrophoresis. The levels of PARP, cleaved PARP, pro-caspase-9, cleaved caspase-9, PCNA, Bcl-2 and p21 were analyzed by western blotting. To ensure equal loading, the same blot was stripped and re-probed with anti-GAPDH which was used as the loading control. The results show the representative western blots from three individual experiments.

cleaved caspase-9 and p21 were found to be increased with the increased tetrandrine concentration. To ensure even loading of the total proteins, the blots were stripped and re-probed with anti-GAPDH. GAPDH was used as the control.

Discussion

The cell viability of Huh-7 decreased in a dose-dependent manner but remained almost unchanged in WRL68, the normal liver cells. The IC_{50} decreased from 20.8 to 8.0 μM for Huh-7 cells with time. The low value of IC_{50} suggests that tetrandrine is a considerable candidate as an anticancer agent. The viability of WRL68 cells dropped and leveled off approximately 80.0% at 50 μM. No IC_{50} for WRL68 could be determined from the MTT assay. The results suggest tetrandrine is one of the few phytochemicals that can differentially act against cancer cell viability.

Cell death of Huh-7 cells induced by tetrandrine is mediated through apoptosis. The results from the DNA fragmentation assay clearly indicated that DNA ladders were detected in the tetrandrine-treated Huh-7 cells. The results provide supporting evidence that tetrandrine induces apoptosis of Huh-7 cells with increased concentration of tetrandrine. It suggests that more genomic DNA molecules were cleaved to form smaller DNA fragments at a higher tetrandrine concentration. The Huh-7 cell line is a p53 gene mutated cell line. In the absence of the functional p53 protein, apoptosis can still occur in Huh-7 cells suggesting that apoptosis does not require the activation of the p53 gene. The results suggest that tetrandrine could induce apoptosis through the p53-independent pathway.

Tetrandrine induces G_1 phase cell cycle arrest. Studies show that there is a link between cell proliferation and apoptosis of Huh-7. The results of the cell cycle analysis by flow cytometry showed that the percentage of cells in the S and G_2 phases decreased whereas G_1 phase increased with increasing concentrations of tetrandrine at all the time points. It suggests that tetrandrine inhibits cell proliferation at a very early stage within the cell cycle. Tetrandrine induced programmed cell death via mediation of p21 and PCNA gene expression possibly through binding of p21 to cyclin-CDK 2 or -CDK 4 complexes and subsequently inhibits their activities. p21 plays an important role in cell cycle regulation by controlling the cell cycle progression from G_1 to S phase. PCNA is synthesized in the early G_1 and S phases during the cell cycle. It acts as an auxiliary factor for DNA polymerase δ in DNA synthesis during the S phase of the cell cycle. It is an important protein responsible for the regulation of DNA synthesis. The binding of p21 to PCNA inhibits the role of PCNA during DNA replication. Therefore, the decreased protein expression for PCNA indicates that there were fewer cells entering S phase after treatment with tetrandrine. The results showed that a higher inhibitory effect on Huh-7 cells was observed in proceeding from G_1 to S phase with higher concentrations of tetrandrine. Therefore, there were fewer proliferating cells and a larger population of cells was retained in G_1 phase after treatment. The data from RT-PCR and western blot analysis also support the notion that tetrandrine induces G_1 phase cell cycle arrest.

Tetrandrine-induced apoptosis involves the intrinsic, caspase-dependent pathway. Caspases are involved in both the initiation and execution of the programmed cell death. Western blot analysis showed that tetrandrine induced apoptosis of Huh-7 through caspase activity. The result showed that the protein expression for pro-caspase-9 decreased in a concentration-dependent manner. Furthermore, the expression for the cleaved, active caspase-9 was found to be similarly increased. As caspase-9 is the initiator for the intrinsic apoptotic pathway, the cleavage of pro-caspase-9 to form the active caspase-9 is essential for inducing cell death in tetrandrine-treated Huh-7 cells and the intrinsic apoptotic pathway is involved.

In the intrinsic apoptotic pathway, the caspase cascade involves active caspase-9 and pro-caspase-3. Following treatment of Huh-7 cells with tetrandrine, the protein expression for full-length PARP decreased in Huh-7 cells. The amount of cleaved PARP increased. PARP, an important protein for DNA repair, is the molecular substrate of active caspase-3.

The occurrence of PARP cleavage is associated with DNA fragmentation in cells resulting in cell death. The result is concordant with the DNA integrity analysis for Huh-7 cells shown in Fig. 3. In the gene expression analysis in Huh-7 cells (Fig. 6), survivin expression was found to be decreased in a concentration-dependent manner. It is reported that survivin is an anti-apoptotic protein that exerts its function by binding to caspase-3 and hence the caspase-3 activity is suppressed. The decreased gene expression suggests that tetrandrine could promote apoptosis by suppressing the expression of survivin.

Tetrandrine induces expression of proteins in Bcl-2. The proteins in the Bcl-2 family can be classified into two categories, the pro-apoptotic and anti-apoptotic proteins. These proteins are involved in the apoptotic pathway associated with mitochondrial control. The gene expressions of Bax and Bid increased. Both the Bax and Bid are pro-apoptotic proteins whereas Bcl-2 is an anti-apoptotic protein. The pro- and anti-apoptotic proteins exert their function in opposite ways. It is reported that Bcl-2 was overexpressed whereas the expression of Bax was downregulated in HCC (4). The elevated gene expressions of Bax and Bid, and the suppressed gene expression of Bcl-2, suggest that these proteins were involved in the apoptosis of Huh-7 cells.

Acknowledgements

This study was supported by grants from the Luck Tissue Mfy Ltd. We thank Matt Cheung for his technical assistance.

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