Antiproliferative and Apoptotic Effects of Tetrandrine on Different Human Hepatoma Cell Lines

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Abstract: Tetrandrine (TET), a bis-benzylisoquinoline alkaloid isolated from the dried root of Hang-Fang-Chi (*Stephania tetrandra* S. Moore), is well known to possess activities including antioxidant, anti-inflammation, anti-fibrotic and anticancer. It is used clinically to treat hypertension and silicosis. In the present study, the anti-proliferative and apoptotic effects of TET were evaluated on three different hepatoma cell lines, namely Hep G2, PLC/PRF/5 and Hep 3B. Using XTT assay, results showed that the IC₅₀ values of TET were 4.35 μ M for Hep G2, 9.44 μ M for PLC/PRF/5 and 10.41 μ M for Hep 3B cells. The CC₅₀ of TET against BNL-CL.2 mouse normal liver cells was 31.12 μ M. Interestingly, TET exhibited a lower IC₅₀ value and better selectivity against Hep G2 and PLC/PRF/5 cells than cisplatin. Microscopic observation study, DNA fragmentation assay and flow cytometric analysis further supported apoptotic effect of TET on both PLC/PRF/5 and Hep 3B cells. The cell cycle of PLC/PRF/5 treated with TET appeared to arrest at G2/M phase in a dose-dependent manner, whereas no effect was noted on the cell cycle of Hep 3B cells. The present study concludes that TET exhibited anti-proliferative effect on Hep G2, PLC/PRF/5 and Hep 3B cells in a dose-dependent manner. TET also possesses a lower IC₅₀ and better SI value than cisplatin against Hep G2 and PLC/PRF/5 cells. The effect of TET on cell cycle progression was found to vary with the type of hepatoma cells, suggesting the genetic make-up of the cells play an important role in the response to drug treatment.

Keywords: Tetrandrine; Hepatoma; Apoptosis; *Stephania tetrandra*.

Introduction

Tetrandrine (TET) (Fig. 1) is a bis-benzylisoquinoline alkaloid isolated from the root of *Stephania tetrandra* S. Moore (Menispermaceae), which is known as Hang-Fang-Chi in Chinese. Its plant material has been used in traditional Chinese medicine as an anti-rheumatic, anti-inflammatory and anti-hypertensive agent (Shen *et al.*, 2001). TET was reported to possess a very broad spectrum of pharmacological actions; for example plasma glucose lowering (Chen *et al.*, 2004), anti-inflammatory (Li *et al.*, 1989), immunosuppressive (Li *et al.*, 1989) and free radical scavenging activities (Cao, 1996). It also exhibited cytotoxic effect on various types of cancer cells, including A549 (Lee *et al.*, 2002), Hep G2 (Yoo *et al.*, 2002), U937 (Lai *et al.*, 1998; Jang *et al.*, 2004), MCF-7 (Wang *et al.*, 2002), HL-60 (Dong *et al.*, 1997), HeLa (The *et al.*, 1991) and T-HSC/Cl-6 (Zhao *et al.*, 2004) cells. However, it has never been tested on hepatoma PLC/PRF/5 and Hep 3B cells.

In rat model of silicosis, TET was found to possess anti-fibrotic activity. It effectively blocked the ability of quartz to stimulate oxidant release from pulmonary phagocytes, and protected hepatocytes against CCl₄ injury (Chen *et al.*, 1996). Clinically, TET has been used as a drug for treating hypertension and silicosis (Qian, 2002; Xie *et al.*, 2002).

Hepatocellular carcinoma (HCC) is one of the most prevalent malignant diseases in China, Taiwan, Korea and Sub-Africa (Chen *et al.*, 1996; He *et al.*, 1996). The major cause of HCC widespread in Taiwan is known to result from hepatitis B virus (HBV) infection (Harris, 1990). Most patients diagnosed with HCC have low recovery rates, and conventional and modified therapies currently available are rarely beneficial (Harris, 1990; Okuda, 1992). To evaluate the potential benefit of TET on treatment of hepatoma, three different HCC cell lines (Table 1), namely Hep G2, PLC/PRF5 and Hep 3B were tested in this study.

Hep G2 and Hep 3B cells are highly differentiated cells (Simon *et al.*, 1982) whereas PLC/PRF/5 is less divided but highly migrated hepatoma cells (Zvibel *et al.*, 1991). They behave functionally as highly differentiated liver parenchymal cells and are karyologically distinguishable from PLC/PRF/5, both by the presence of trisomy 6 (pter leads to q14) and with one of the long arm of chromosome 15 is 15q+ (Simon *et al.*, 1982). Hep G2 and Hep 3B cells, not PLC/PRF/5 cells, secrete IGF carrier protein (Moses *et al.*, 1983) and produce α -2-plasmin inhibitor (α -PI), a physiological inhibitor (Saito *et al.*, 1982).

Figure 1. The structure of tetrandrine.

Table 1. Characteristics of Hep G2, Hep 3B and PLC/PRF/5 Cell Lines

Cell Lines	HBV-DNA [*]	p53	Fas Receptor	RB [†]	Histology
Hep G2	–	Normal	Normal	+	Hepatoblastoma
PLC/PRF/5	+	Mutation	No function	+	Hepatocellular carcinoma
Hep 3B	+	Deletion	–	–	Hepatocellular carcinoma

^{*}Hepatitis B virus-DNA. [†]Retinoblastoma.

In this study, our objective was to examine the effects of TET on cell growth and apoptosis in different human hepatoma Hep G2, Hep 3B and PLC/PRF/5 cells.

Materials and Methods

Chemicals

Tetrandrine, with purity greater than 98%, was purchased from Fluka (St. Gallen, Switzerland). It was dissolved in dimethylsulfoxide (DMSO) to prepare a stock solution of 3.2 mM. For each experiment, it was diluted further with distilled water to desired concentrations (4, 8, 16 and 32 μ M). The final DMSO concentration was about 0.1%. Cisplatin (a commercial anticancer drug), propidium iodide, bovine pancreatic proteinase A, Histo-Paque[®]-1077 solution and sodium salt of 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT) kits were obtained from Sigma Aldrich Co. (St. Louis, MO, USA). Proteinase K was purchased from Roche Diagnostics (GmbH, Mannheim, Germany). The Bio-100[™] DNA Ladder marker was obtained from PROtech Technology Enterprise Co. (Taipei, Taiwan). All other chemicals used were of analytical grade.

Cell Culture

The human hepatoma PLC/PRF/5 (ATCC CRL8024), Hep 3B (ATCC HB8064) and Hep G2 (ATCC HB 8065) cells and BNL-CL.2 mouse normal liver (ATCC TIB-73) cells were obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA), supplemented with 10% fetal calf serum (FCS), 100 units penicillin G, 100 μ g/ml streptomycin and 0.25 μ g/ml amphotericin B (Gibco, Grand Island, NY, USA) in an incubator with humidified air at 37°C with 5% CO₂.

Antiproliferative Effect Assay

The effect of TET on cell proliferation was determined by XTT colorimetric assay (Roehm *et al.*, 1991). The cells (1×10^4 /well) were plated onto 96-well microplates, each well received 100 μ l DMEM medium containing different concentrations of TET (4, 8, 16 and 32 μ M). They were then incubated for 6, 12, 24, 36 and 48 hours. After incubation, the medium was replaced with 100 μ l of non-FCS DMEM per well. The mixture of 100 μ l

phenazine methosulfate (PMS; electron-coupling reagent; 0.383 mg/ml) and 50 μ l of XTT were then added to each well. The plate was incubated for another 4 hours. The absorbance of sample was determined by an ELISA reader (Multiskan EX[®], Labsystems, Helsinki, Finland) at a test wavelength of 492 nm and a reference wavelength of 690 nm.

Morphological Observation

Cells were treated with different doses of TET (8, 16 and 32 μ M) at various time periods. They were then fixed and stained with Liu's solution (Liu, 1970). The morphological changes of cells were observed under fluorescent microscopy (Zeiss Axioskop, Mikron Instruments, NY, USA).

DNA Fragmentation Analysis

The DNA fragmentation analysis was performed as previously described (Chen *et al.*, 2001). In brief, vehicle control (0.1% DMSO) and TET-treated groups were collected in a 15 ml tube and centrifuged at 200 $\times$ g for 5 minutes. They were then transferred to an Eppendorf-microtube and precipitated at 200 $\times$ g for 5 minutes. Each pellet was resuspended in 195 μ l lysis-buffer [every 740 μ l containing 37 μ l of 1M Tris (pH 8.0), 14.8 μ l of 0.5M EDTA, 10 μ l of 75% sodium lauryl sarcosine, 678.2 μ l of H₂O]. Five μ l of 10 mg/ml proteinase-K was then added at the same time. After thorough mixing, the mixture was maintained at 50°C water-bath overnight, followed by the addition of 10 μ l of 10 μ g/ml RNase-A and the mixture was further incubated at 50°C for 1 hour. DNA was isolated after phenol-chloroform extraction and ethanol precipitation, followed by resuspension in PBS. The DNA was analyzed on a 2% agarose gel, stained with ethidium bromide, and detected under 254 nm UV illuminations. The Bio-100TM DNA Ladder marker was used as reference.

Cell Cycle Analysis

The analysis of cell cycle distribution was conducted according to the previously described method (Kuo and Lin, 2003). In brief, cells (8×10^5 cells/well) were seeded onto a six-well plate. After treating with various concentrations of TET (8, 16 and 32 μ M) for 12 and 24 hours, cells were washed with PBS and then fixed with 70% ethanol. Cells were washed with PBS again and incubated further with 25 μ l of 0.5% triton X-100 and 10 μ l of RNase-A (10 μ g/ml) in 565 μ l PBS at 37°C for 1 hour. After propidium iodide staining, cells were analyzed by flow cytometry. The distribution of cell cycle was analyzed by MultiCycle AV (Phoenix Flow Systems, Inc. San Diego, California, USA).

Statistical Analysis

Data are presented as means $\pm$ SD of three independent experiments and were evaluated by one way analysis of variance (ANOVA) using the Statistical Analysis System (SAS

Institute, Cary, NC, USA). Unpaired Student's t-test was used to compare differences between control and test samples. Differences were considered significant when $p < 0.05$.

Results

TET Inhibited the Proliferation of Hep G2, PLC/PRF/5 and Hep 3B Cells

Table 2 showed the growth inhibitory effects of TET on Hep G2, PLC/PRF/5 and Hep 3B cells. Results indicated that TET exhibited a lower IC_{50} value than cisplatin against PLC/PRF/5, Hep 3B and Hep G2 cells. However, a small difference between the IC_{50} of TET and cisplatin was noted on Hep 3B cells. The SI of TET was superior to that of cisplatin against PLC/PRF/5 and Hep G2 but was inferior to cisplatin on the Hep 3B cells. Compared with 5-FU, TET possessed a superior SI value on Hep G2 but not on the PLC/PRF/5 and Hep 3B cells. Consistent with previous studies, TET significantly inhibited the cell growth on Hep G2 cells (Kuo and Lin, 2003). TET also exhibited an anti-proliferative effect on PLC/PRF/5 and Hep 3B cells in a dose-dependent manner.

TET Induced Morphological Changes in PLC/PRF/5 and Hep 3B Cells

Figure 2 indicated that TET treatment resulted in a typical apoptotic appearance on PLC/PRF/5 and Hep 3B cells, such as chromatin condensation. A normal appearance other than apoptotic cells was also noted on the TET-treated group.

Effects of TET on DNA Fragmentation

Results showed that the level of DNA fragmentation became more prominent with increasing time and doses (Fig. 3). This observation further supported the apoptotic changes appearing in the microscopic results as mentioned above. The low molecular DNA fragments (around 180 bp) could be easily detected on the rows of higher dose and at the later time-point. This appearance is a typical characteristic of apoptosis.

Table 2. Anti-proliferative Activity of Tetrandrine (TET) Against Three Different Human Hepatoma Cells and BNL-CL.2 Mouse Normal Liver Cells

	Cell Lines						
	BNL-CL.2	Hep G2		PRC/PRF/5		Hep 3B	
Compounds	CC_{50}^*	$IC_{50}^{\dagger}$	$SI^{\ddagger}$	IC_{50}	SI	IC_{50}	SI
Tetrandrine	31.12	4.35	7.15	9.44	3.30	10.41	2.99
Cisplatin	162.25	142.82	1.14	94.34	1.72	28.12	5.77
Fluorouracil	533.13	140.99	3.78	5.07	105.15	95.63	5.57

* CC_{50} is the concentration (μM) that reduces the growth of BNL-CL.2 mouse normal liver cells by 50%. $^{\dagger}IC_{50}$ is the inhibitory concentration (μM) that reduces cellular growth or viability of cells by 50%. ‡ Selectivity Index (SI) = CC_{50}/IC_{50} .

Figure 2. Microscopic appearance of Hep 3B and PLC/PRF/5 cells. (A) Hep 3B cells without TET. (B) PLC/PRF/5 cells without TET. (C) and (D) are Hep 3B and PLC/PRF/5 cells treated with TET 16 μ M for 24 hours, respectively. Arrows indicate morphologically apoptotic changes, including condensed and fragmented nuclei, and apoptotic bodies.

Figure 3. DNA fragmentation of PLC/PRF/5 and Hep 3B cells induced by tetrandrine (TET). DNA fragmentation of PLC/PRF/5 (A) and Hep 3B (B) cells after treating with 16 μ M TET for various time periods. DNA fragmentation of PLC/PRF/5 (C) and Hep 3B (D) cells after treating with various concentrations of TET for 36 hours.

Effects of TET on Cell Cycle in PLC/PRF/5 and Hep 3B Cells

Table 3 showed the effects of various concentrations (8, 16 and 32 μM) of TET on PLC/PRF/5 and Hep 3B cells. Compared with the untreated cells, the percentage of G2/M phase of PLC/PRF/5's cell cycle appeared to increase in a dose-dependent manner, whereas a trend of decrease was noted in the S phase. When the PLC/PRF/5 cells were treated with TET for a period longer than 24 hours, no obvious cell cycle arrest was noted.

In the experiment with Hep 3B cells, a decrease in the G_0/G_1 and S phases was noted with increasing concentrations of TET. However, there was no obvious cell arrest occurring at the G2/M phase. When the Hep 3B cells were treated with different concentrations (8, 16 and 32 μM) of TET for a period longer than 24 hours, cell cycle arrest was not noted at any phases of the cell cycle.

In contrast with the observation noted on the Hep G2 cells (Kuo and Lin, 2003), TET significantly increased the population of G_0/G_1 phase in a dose-dependent manner, suggesting TET effectively blocked cell cycle progression in the G1 phase.

Discussion

Previous studies have shown that TET was effective in inhibiting the proliferation of several cancer cell lines, including A549 (Lee *et al.*, 2002), Hep G2 (Yoo *et al.*, 2002), U937 (Lai *et al.*, 1998; Jang *et al.*, 2004), MCF-7 (Wang *et al.*, 2002), HL-60 (Dong

Table 3. Effect of TET on the Cell Cycle of PLC/PRF/5 and Hep 3B Cells^{1,5}**(A) PLC/PRF/5**

Phase	% Distribution				
	Control		TET ($\mu\text{g/ml}$)		
	Medium	0.1% DMSO	5	10	20
G1	56.1 $\pm$ 1.0	52.8 $\pm$ 1.3	51.8 $\pm$ 1.6	53.0 $\pm$ 0.7	53.6 $\pm$ 1.3
S*	27.0 $\pm$ 1.9	29.9 $\pm$ 2.7	21.7 $\pm$ 0.8	16.9 $\pm$ 0.9	7.8 $\pm$ 1.8
G2/M*	15.4 $\pm$ 1.1	12.5 $\pm$ 2.5	18.9 $\pm$ 2.0	21.1 $\pm$ 0.2	28.1 $\pm$ 0.7

(B) Hep 3B

Phase	% Distribution				
	Control		TET ($\mu\text{g/ml}$)		
	Medium	0.1% DMSO	5	10	20
G1*	53.1 $\pm$ 3.8	54.1 $\pm$ 2.0	53.0 $\pm$ 1.2	46.5 $\pm$ 4.2	40.3 $\pm$ 2.6
S*	26.0 $\pm$ 5.6	24.0 $\pm$ 0.8	23.7 $\pm$ 2.9	18.9 $\pm$ 5.1	14.1 $\pm$ 4.3
G2/M	15.1 $\pm$ 1.9	18.4 $\pm$ 2.8	18.8 $\pm$ 2.3	19.8 $\pm$ 5.3	17.8 $\pm$ 4.6

The pattern of cell cycle of (A) PLC/PRF/5 and (B) Hep 3B cells after treating with the medium, 0.1% DMSO vehicle, and 5, 10 and 20 $\mu\text{g/ml}$ TET for 12 hours. Data are presented as means $\pm$ SD of three independent experiments. *The percentage of G1 or S or G2/M phase was significantly ($p < 0.05$) changed in a dose-dependent manner as analyzed by ANOVA.

et al., 1997), HeLa (The *et al.*, 1991), T-HSC/Cl-6 (Zhao *et al.*, 2004) and neuro 2a mouse neuroblastoma (Jin *et al.*, 2002) cells. In this study, TET showed growth inhibitory effect and cell apoptosis induction on Hep G2, PLC/PRF/5 and Hep 3B cells. TET was found to possess a lower IC₅₀ value than cisplatin against Hep G2, PLC/PRF/5 and Hep 3B cells. Compared with 5-FU, although TET possesses a lower IC₅₀ value against Hep 3B cell lines, its SI values were inferior to this drug. This observation suggests that the difference in genetic make-up of hepatoma cells would have contributed to the difference in response to the drugs.

TET at concentrations 5~80 μ M was shown to decrease collagen and hyaluronic acid synthesis in human lung fibroblast HLF cells, and with no obvious toxic effect on these cells at 5~20 μ M (Liu *et al.*, 1995). This implies that TET could be a potential good source of agent for treating liver cancer. In clinical study (Li, 1981), the therapeutic dose of TET for treatment of silicosis was around 10 μ m, which is close to the IC₅₀ concentration against Hep G2 (IC₅₀ = 4.35 μ M; SI = 7.15), PLC/PRF/5 (IC₅₀ = 9.44 μ M; SI = 3.30) and Hep 3B (IC₅₀ = 10.4 μ M; SI = 2.99) cells.

Results of the XTT analysis showed that carrier of HBV-DNA cells, PLC/PRF/5 and Hep 3B, exhibited a significant ($p < 0.05$) lesser response towards TET than Hep G2 cells. This finding was consistent with results previously reported, where plant extracts were found to be more effective against non-HBV-related cell lines (ex. Hep G2) than against HBV-related cell lines (ex. PLC/PRF/5, Hep B2) (Chen *et al.*, 1990; Bressac *et al.*, 1991). CC₅₀ analysis showed that, compared to the BNL-CL.2 mouse normal liver cells, TET demonstrated a greater cytotoxicity on human lymphocytes (Ho *et al.*, 1999; Lai *et al.*, 2001). Previous studies have shown that the cytotoxicity of TET varies with different species. For example, the hepatotoxicity and nephrotoxicity were found to be more obvious in rhesus monkey than in beagle dogs (Gralla *et al.*, 1974).

In the DNA fragmentation study, the density of DNA ladder appeared to decrease as concentration of TET reaching 32 μ M. This could be explained by the acute toxicity at high concentration of TET, thus causing a decrease in the DNA ladder.

It was well known that cell cycle arrest and apoptosis play important roles in cell life. After TET treatment, cell cycle arrest was noted at G2/M phase in a dose-dependent manner in the PLC/PRF/5 cells. However, this event was not noted in the Hep 3B cells, which is lacking of dominant RB gene, although the ratio of G1 or S phase decreases as concentration of TET increases. It was known that when the RB protein is subjected to low density phosphorylation, it will react with transcription factor E2F and lead to the inhibition of its release. This event consequently suppresses the cell cycle progress from G₀/G1 to S phase (Johnson and Schneider-Broussard, 1998). Because of the lacking in the RB gene, drugs are not effective in causing cell cycle arrest in the Hep 3B cells. It was shown that p53 play an important role in TET-induced anti-proliferative activity in Hep G2 cells (Kuo and Lin, 2003). Induction of p53 by TET not only causes Hep G2 cell arrest, but also triggers apoptosis. However, it is unknown if p53 expression plays a similar role in PLC/PRF/5 and Hep 3B cells.

In conclusion, the present study demonstrates that TET possesses potent anti-proliferative activity on Hep G2, PLC/PRF/5 and Hep 3B cells. The effect of TET on

proliferation of these hepatoma cells appeared to be dose-dependent. Although TET-induced cell apoptosis in both PLC/PRF/5 and Hep 3B cells, its mechanism of action(s) remains unclear. Flow cytometric analysis indicate that the effect of TET on cell cycle progression varies with the type of hepatoma cells, suggesting the genetic make-up of the cells play an important role in the response to drug treatment.

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