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Tetrandrine Induces Apoptosis in HepG2 by Modulating Hippo Signalling Pathway

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Key words
Tetrandrine, HepG2 cells, Proliferation, Apoptosis, Hippo pathway, Molecular mechanisms

ABSTRACT

Tetrandrine (TET) has been known to possess anti-cancer properties in wide variety of cancers, however, the underlying mechanism for hepatocellular carcinoma (HCC) have not been fully elucidated. We focused our investigation the effect of TET on programmed cell death and growth of $HepG_2$ cancer cell live. TET was found to significantly inhibit the proliferation of $HepG_2$ cells, with an half maximal inhibitory concentration (IC_{50}) of 7.76 µmol/L. Fluorescence microscopy showed that after 48 h of TET exposure, the cells presented morphological alterations typical of apoptosis while normal cellular morphology was preserved in the control. Staining with propidium iodide (PI), followed by flow cytometry showed that following 48 h TET treatment at 3.75, 7.5, and 15 µmol/L, the rate of apoptosis in HepG2 was 5.1%, 19.7%, and 36.9%, respectively. Western blotting was done to study the effect of TET on the expression profile of proteins involved in Hippo signalling pathway. We observed that in response to 48 h incubation with TET, there was a significant upregulation of the expression levels of MST1 (mammalian sterile 20-like kinase 1), LATS1 (large tumor suppressor kinase 1), and P-YAP1 while the expression levels of YAP1 (Yes-associated protein 1) and TAZ (transcriptional coactivator with PDZ-binding motif) were downregulated. Taken together, these results indicated that the anti-cancer activity of TET on HepG2 cells may be due to the modulation of Hippo signalling pathway.

INTRODUCTION

The hepatocellular carcinoma (HCC) is one of the predominant cancers accounting for about a third of cancer related mortality (Torre *et al*., 2015). Surgical resection remains the first choice of treatment for early-stages of HCC (Zhang and Sun, 2015), however, its efficiency is limited due to delayed clinical diagnosis, which usually occurs when the cancer has progressed to intermediate or advanced stage. Recurrence rate of HCC upon curative hepatectomy is high and it is then tackled using targeted drug therapy with sorafenib, which although suffers from issues of incomplete suppression of HCC and drug resistance (Llovet *et al*., 2008). Therefore, the focus is towards the development of new and effective drugs.

One of the drugs being explored is tetrandrine (TET), extracted from the roots of *Stephania tetrandra* (Wang *et al*., 2004; Bhagya and Chandrashekar, 2018). TET is an alkaloid belonging to the bis-benzylisoquinoline family and induces apoptosis and autophagy in mammalian oral and gastric cancer cells (Liu *et al*., 2008; Lien *et al*., 2017; Bai *et al*., 2018). It also induces apoptosis as well as hinders the proliferation of human bladder and prostate cancer cells (Li *et al*., 2011; Liu *et al*., 2015). Despite the studies, underlying mechanism of anti-cancer activity against the HCC and other cancer types is not fully understood. One of the hypotheses suggests the dysregulation of Hippo signalling pathway as a possible cause of HCC (Zender *et al*., 2006; Kowalik *et al*., 2011). In normal cells, this pathway is responsible for regulation of cell growth, apoptosis, and differentiation (Irvine, 2012; Camargo *et al*., 2007). In mammalian cells, it comprises protein kinases MST1/2 and LATS1/2 along with its downstream effectors YAP, and TEADs, and the paralog TAZ (Ma *et al*., 2015; Hong *et al*., 2016; Edgar, 2006). Activation of MST1/2 triggers the LATS1/2 to induce phosphorylation

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of YAP/TAZ followed by degradation (Zhao *et al*., 2007; Yi *et al*., 2016; Song *et al*., 2018). Elevated levels of YAP have been found in samples of hepatitis B virus-induced HCC (Pan, 2010; Lu *et al*., 2013), thus supporting the previous hypothesis. The present work was carried out to study the antiproliferative and apoptotic effects of TET in human liver cancer cell line, HepG2. We inferred that TET may have an impact on the biological functions such as the proliferation and apoptosis of HepG2 cells through the Hippo signalling pathway, and provide new ideas for the treatment of HepG2.

MATERIALS AND METHODS

Cell culture

HepG2 cells were procured from the Engineering Research Centre for Medicine at Harbin University of Commerce (Harbin, China). RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., MA, USA) supplemented with 10% (*v*/*v*) foetal bovine serum (Tianhang Biological Technology Co. Ltd., China) was used to culture the cells at 37 ℃ in a 5% CO_2 environment. A cell density of about 80% indicated the logarithmic growth phase.

Assay for cell viability (CCK-8 assay)

One hundred microliters of the HepG2 cells (cell density 3×10^4 cells/mL) digested with 0.25% pancreatin, were inoculated in a 96-well plate. Six parallel wells were set up for each group. After 24 h of incubation, 100 µL each of TET (purity, ≥98%; Aladdin Reagent Co., Ltd., China) or hydroxy camptothecin (HCPT; purity, ≥ 98%; Medican Pharmaceutical Co., Ltd., China) were added to each well to get a final concentration of 2, 4, 8, 16, and 32 µmol/L or 1, 2, 4, 8, and 16 µmol/L, respectively, while sterile RPMI-1640 (100 µL) served as control, and incubated further for 72 h at 37 ℃. To each well, 10 µL of CCK-8 solution (Beyotime Institute of Biotechnology, China) was added and incubation was carried out at 37 ℃ for 2 h (Shang *et al*., 2021). A microplate reader was used to measure the absorbance at 450 nm, which was used to determine the inhibition rate and IC_{50}.

Apoptotic morphology assay

A 6-well plate was inoculated with HepG2 cells (3×10^4 cells/mL) grown in RPMI-1640, with a coverslip inside each well. After 24 h, TET (3.75, 7.5, or 15 µmol/L) was added to each well, HCPT (7 µmol/L) served as positive control, while in negative control sterile RPMI-1640 was added. Incubation was carried out for 48 h after which sterile phosphate-buffered saline (PBS) was used to wash the HepG2 cells, followed by fixing in 1 mL of 4% buffered paraformaldehyde at 4 ℃ for 1 h. PBS was used to wash the cells and the cells were stained with 5 mg/L Hoechst 33258 fluorescent probe (Sigma-Aldrich, St. Louis, MO, USA) by incubation in dark for 30 min at 37 ℃ (Ji and Yu, 2015). The excess stain was removed by washing the cells with PBS. The coverslip was removed from each well and examined under a fluorescence microscope (CKX-41-32, Olympus Corporation, Japan).

Apoptosis rate assay

A 6-well plate was inoculated with HepG2 cells (3×10^4 cells/mL grown in RPMI-1640) and TET (3.75, 7.5, or 15 µmol/L) was added at 24 h. The incubation was continued for 48 h after followed by overnight incubation in 70% ethanol at 4 ℃ to fix the cells. After incubation, PBS was used to wash the cells. Staining with PI (Sigma-Aldrich) 50 µg/mL was carried out for 30 min at 37 ℃. The apoptosis was analysed by flow cytometry (EPICS-XL; Beckman Coulter, Inc., USA).

Western blotting

HepG2 cells were plated in culture flasks (1×10^6 cells/mL) followed by addition of TET (3.75, 7.5, or 15 µmol/L) at 24 h. After 48 h, the collected cells were lysed and cellular protein was extracted. Protein quantification was done by the bicinchoninic acid method (Solarbio Science and Technology Co., Ltd., Beijing, China). For western blotting (Wang *et al*., 2021), 12% SDS-PAGE gel electrophoresis was carried out with 50 µg of protein (20 µL loading volume). Protein transfer was done onto nitrocellulose membrane and blocking was carried out with 5% skim milk for 1 h after which incubation was done with primary antibodies (rabbit anti-human binding immunoglobulin protein; MST1, bs-3504R; LATS1, bs-2904R; P-LATS1, bs-7913R; YAP1, bs-52418R; P-YAP1, bs-3475R; TAZ, bs-12367R; BIOSS) at 4 ℃ overnight. Upon washing, secondary antibodies were added to the membrane and incubation was carried out at room temperature (Goat anti-rabbit IgG horseradish peroxidase; A0192; Beyotime Institute of Biotechnology) for 2 h. ECL Chemiluminescence Kit (P0018AS-1,2; Beyotime Institute of Biotechnology) was used to visualise the bands which were photographed and analysed with a gel imaging system (GIS-2019; Tanon Science and Technology Co., Ltd., China).

Statistical analysis

The data from each experiment were recorded as mean ± standard deviation. The results were evaluated by One-way ANOVA. To analyse the data, SPSS software for Windows version 18.0 (SPSS, Inc., USA) was used. Statistically significant differences between groups were defined as *P* values less than 0.05.

RESULTS

Antiproliferative effects of TET

Treatment with TET was found to inhibit the proliferation of HepG2 cells. The inhibition followed a dose-dependent pattern. The IC_{50} was calculated to be 7.76 μmol/L (Table I, Fig. 1), in comparison to the positive control HCPT, which was 7.18 μmol/L (Table I).

Table I. Inhibitory effect of TET and HCPT treatment on cell proliferation of 24 h old HepG2 cells.

Groups	Concentration (μmol/L)	Optical density	Inhibition rate (%)
Control	–	0.801 ±0.088	–
TET	2	0.654 ±0.095**	18.21
	4	0.487 ±0.091**	39.19
	8	0.337 ±0.051**	57.89
	16	0.253 ±0.031**	68.36
	32	0.256 ±0.046**	68.41
HCPT	1	0.675 ±0.083**	15.76
	2	0.565 ±0.052**	29.49
	4	0.549 ±0.039**	31.41
	8	0.397 ±0.016**	50.41
	16	0.248 ±0.034**	69.29

Compared with control group, $^{**}P<0.01$. Date was expressed as means ± SD (n=6).

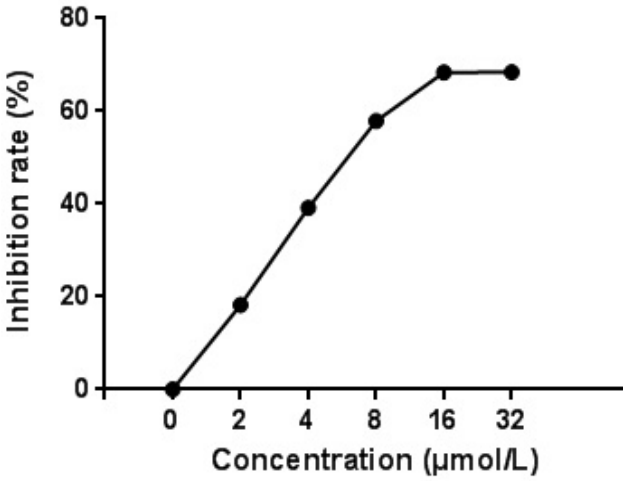

Fig. 1. Inhibitory effect of TET treatment on cell proliferation of 24 h old HepG2 cells.

TET induces morphological changes in HepG2 cells

Fluorescence microscopic examination of control group shows uniform fluorescence in the cell nuclei. Incubation with various concentrations of TET or 7 μmol/L HCPT for 48 h, showed the presence of apoptosis related changes in the HepG2 cells, such as chromatin condensation and formation of apoptotic bodies (Fig. 2).

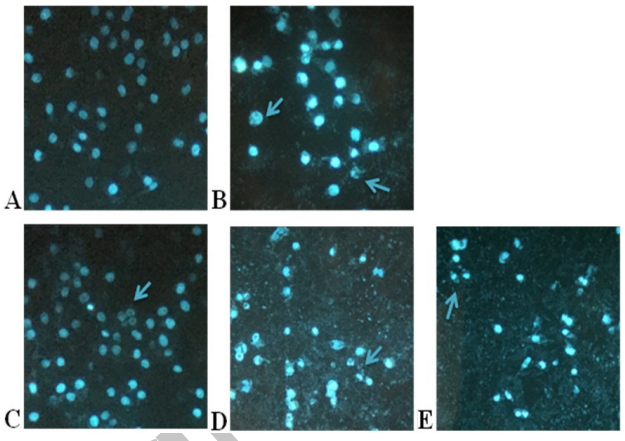

Fig. 2. Fluorescent micrographs showing TET induced morphological changes in HepG2 cells visualized after Hoechst 33258 staining (200× magnification): (A) Control; (B) HCPT (7 μmol/L); (C) TET (3.75 μmol/L); (D) TET (7.5 μmol/L); and (E) TET (15 μmol/L).

TET induces apoptosis in HepG2 cells

TET administration showed a dose dependent increase in the total number of apoptotic cells as compared to control, and the effect was found to be statistically significant. The results are summarised in (Table II, Fig. 3).

Table II. TET causes an increased apoptosis in HepG2 cells.

Groups	Concentration (μmol/L)	Apoptosis rate (%)
Control	–	1.579 ±0.197
HCPT	7	21.197 ±1.459**
TET	3.75	5.125 ±0.797**
	7.5	19.698 ±1.860**
	15	36.882 ±1.511**

Compared with control group, $^{**}P<0.01$. Date was expressed as means ± SD (n=3).

TET treatment affects the expression of MST1, LATS1, P-LATS1, and P-YAP1

Treatment with 3.75, 7.5, and 15 μmol/L TET or 7 μmol/L HCPT for 48 h caused a significant upregulation ($P<0.01$) of the MST1, LATS1, P-LATS1, and P-YAP1 expression. The effects were found to be concentration-dependent (Fig. 4).

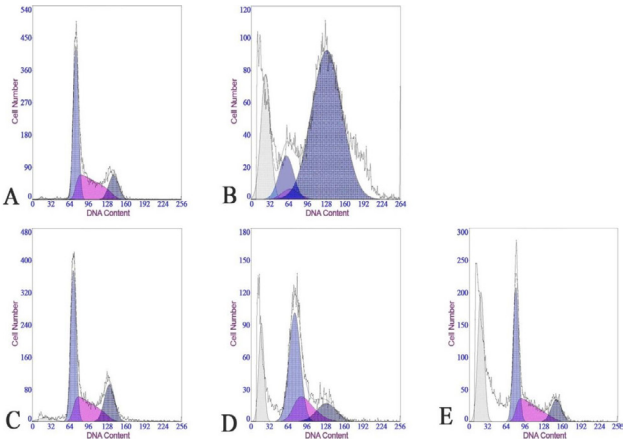

Fig. 3. Analysis of apoptosis of HepG2 cells upon treatment with TET or HCPT followed by PI staining, using a flow cytometer (A) Control; (B) HCPT (7 μmol/L); (C) TET (3.75 μmol/L); (D) TET (7.5 μmol/L); and (E) TET (15 μmol/L).

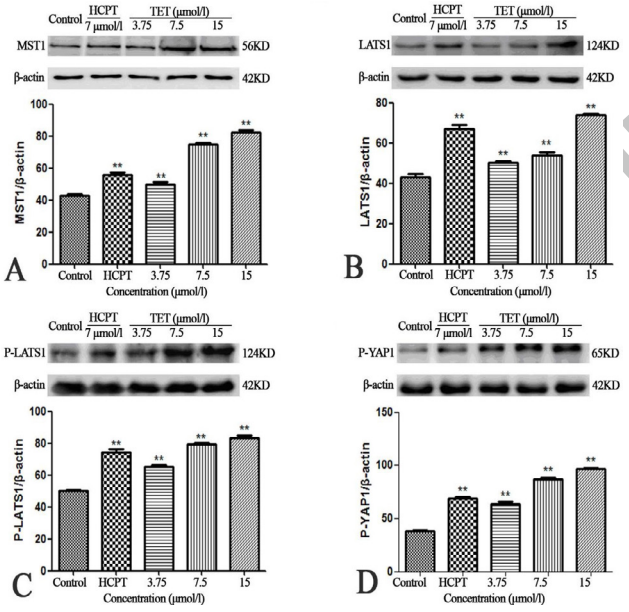

Fig. 4. Protein expression profile after 48 h TET treatment (3.75, 7.5, or 15 μmol/L) HepG2 cells. Western blots represent (A) MST1, (B) LATS1, (C) P-LATS1, and (D) P-YAP1. (**P<0.01, vs control).

TET downregulates the expression of YAP1 and TAZ

Treatment with 3.75, 7.5, and 15 μmol/L TET or 7 μmol/L HCPT for 48 h caused a significant downregulation (Fig. 5) in the expression levels of YAP1 and TAZ in comparison to the control group (P<0.05). The decrease was gradual and coincided with the dose of TET used.

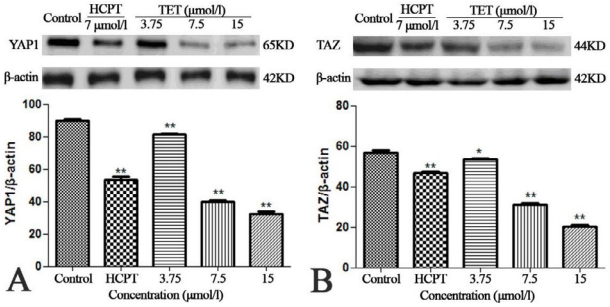

Fig. 5. Expression profile proteins participating in Hippo signalling pathway in HepG2 cells upon 48 h TET treatment (3.75, 7.5, or 15 μmol/L). Western blots represent (A) YAP1 and (B) TAZ. (**P<0.01, *P<0.05).

DISCUSSION

The disturbance of Hippo signalling pathway disrupts the balance between the cellular proliferation and apoptosis and may lead the cell towards cancerous state (Zhao *et al*., 2008). Therefore, Hippo signalling pathway is being considered a potential target for anticancer drug research. The Hippo signalling pathway was first discovered in *Drosophila*, and it is highly conserved during evolution. It is homologous to MST1/2, human Salvador 1 (hSAV1), LATS1/2, Mps One Binder inaseactivator-like1 (MOB1), YAP / TAZ and TEA domain family member (TEAD) in mammals (Dong *et al*., 2007). The core components of the Hippo signal pathway include LATS and MST, both of which have important regulatory roles in the Hippo signal pathway. The *LATS* gene was identified from *Drosophila* in 1995 and was found to express LATS1 and LATS2. MST is a serine/threonine kinase-like enzyme found in yeast cells. Subsequent studies have found that LATS and MST can significantly inhibit tumor cell proliferation and promote tumor cell apoptosis, suggesting that these may act as tumor suppressor genes (Furth *et al*., 2015). YAP gene is highly expressed in many human cancer tissues and cells, which accelerates the proliferation of tumor cells and inhibits apoptosis. A high level of YAP expression corresponds to a lower the degree of tumor differentiation as well as with a shorter survival period of the patient. Therefore, reducing the expression level of YAP in tumor cells may help in tumor treatment. YAP, as a transcription factor of the TEAD/TEF family, is a downstream target gene of the Hippo pathway and a nuclear auxiliary transcription factor. In mammals, the Hippo signalling pathway inhibits YAP nuclear translocation to achieve cell apoptosis in mammals. The mechanism is that dephosphorylated YAP combines with TAZ to transfer into and accumulate in the nucleus to form transcription factor complexes and induce the expression of target genes.

The results of this study show that TET can activate MST1 and increase the level of protein expression. LATS1 was phosphorylated under the influence of MST1, and the activated LATS1 phosphorylates the downstream targets YAP and TAZ, resulting in the nucleus knocking out the inactive YAP and TAZ. Phosphorylated YAP and TAZ cannot combine with the transcription factor TEAD to convey growth and development signals, thereby inhibiting the proliferation of HepG2 cells through the Hippo signaling pathway.

CONCLUSION

In the present study a possible mechanism for the action of TET on HepG2 cells and consequently on the Hepatocellular carcinoma was investigated. We found an inhibition in cell proliferation as well as induction of apoptosis in HepG2 cells following TET treatment. Incubation with TET could also inhibit cell growth and cause a dose-dependent increase in the rate of apoptosis. One of the possible mechanisms proposed for the development of cancer is the disruption of the Hippo signalling pathway. TET treatment could efficiently obstruct the expression of YAP HepG2 cells which was evident in the expression profile. Cumulatively, these results suggest that anti-cancer effects of TET against HepG2 cells can be attributed to modulation of the Hippo signalling pathway. We will continue to explore the anti-tumor mechanism of TET, use quantitative RT-PCR to verify the expression protein-related genes, and explore the underlying targets of TET on HepG2 cells through proteomics.

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Statement of conflicts of interest

The authors have declared no conflicts of interest.

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