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Pentoxifylline induces apoptosis of HepG2 cells by reducing reactive oxygen species production and activating the MAPK signaling☆

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ABSTRACT

Aims: Pentoxifylline (PTX) is a methylxanthine derivative and has potent anti-tumor activity. This study aimed at investigating the anti-HCC effects of PTX and associated molecular mechanisms.
Main methods: The effects of varying doses of PTX on viability, cell cycle and apoptosis of HepG2 cells were determined by MTT and flow cytometry, respectively. The effects of PTX on the production of reactive oxygen species (ROS), expression of pro- and anti-apoptotic regulators and activation of the MAPK signaling in HepG2 cells were analyzed by flow cytometry and Western blot assays. The effects of PTX on the growth of implanted HepG2 cells and their apoptosis in mice were examined.
Key findings: Our results indicated that PTX inhibited proliferation of HepG2 cells and induced HepG2 cell cycle arrest at G0/G1 phase and apoptosis in a dose- and time-dependent manner. Treatment with PTX reduced levels of ROS and Bcl-X_L expression, but increased caspase 3 and caspase 9 expression and JNK and ERK1/2 phosphorylation in HepG2 cells. Pre-treatment with *n*-acetyl-L-cysteine (NAC), a ROS scavenger, enhanced PTX-mediated cell cycle arrest, apoptosis and the JNK and ERK MAPK activation, while pre-treatment with SP600125 or PD98509 attenuated PTX-mediated effects in HepG2 cells. Treatment with PTX inhibited the growth of implanted HCC and promoted HCC apoptosis in mice.
Significance: Our data demonstrate that PTX inhibits proliferation of HepG2 cells and induces HepG2 cell apoptosis by attenuating ROS production and enhancing the MAPK activation in HepG2 cells.
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1. Introduction

Hepatocellular carcinoma (HCC) is a common cancer in the world with an increasing incidence and is associated with high morbidity and mortality [1]. Currently, therapeutic strategies for HCC include tumor resection, pre-operative portal vein embolization, percutaneous radiofrequency, cryoablation, alcohol or microwave ablation, arterially directed therapies, external-beam radiation therapy (EBRT), local chemotherapy, liver transplantation, targeted therapies and immunotherapy. However, the therapeutic efficacy of these strategies is limited. It is notable that some traditional Chinese medicines have potent anti-tumor effects [2,3]. Discovery and validation of new therapeutic medicines is of significance in management of patients with HCC [4,5].

Pentoxifylline (1-[5-oxohexyl]-3,7-dimethyl-xanthine], oxpentifylline, PTX) is a derivative of methylxanthine (Fig. 1A). Functionally, PTX can inhibit the activity of phosphodiesterase, increase intracellular cAMP concentrations, and has been previously used to treat peripheral vascular disease and intermittent claudication [6]. Previous studies have shown that treatment with PTX at a therapeutic dose protects from malathion-induced oxidative damage in rat liver and promotes the regeneration of hepatocytes in animal models [7,8]. Furthermore, PTX has inhibits TNF-α production in macrophages and improves graft survival of orthotopic liver transplantation in rats [9]. In addition, PTX prevents the development of a hyperdynamic circulatory state and hepatopulmonary syndrome in cirrhotic rats [10] and inhibits stellate cell proliferation [11]. Moreover, PTX can inhibit the proliferation of various types of cancer cells and enhance the sensitivity of tumor cells to chemotherapeutic reagents and radiotherapy [12,13]. PTX has potent anti-oxidant and anti-inflammatory activity, and has been shown to inhibit cell adhesion [14,15]. However, little is known on the molecular mechanisms underlying the action of PTX in inhibiting HCC proliferation and inducing HCC apoptosis.

Therapeutic strategies to induce tumor cell apoptosis have been thought to be one of the effectively therapeutic methods to treat cancer [16]. Apoptosis is a process regulated by a series of enzymes and genes under physiological or pathological condition [17]. Apoptosis is mediated by caspase cascade and regulated by the MAPK signaling [18,19]. Indeed, several therapeutic reagents directly target the MAPK signaling

☆ The experimental protocol was approved by the Animal Care and Research Committee of Xian Jiaotong University.

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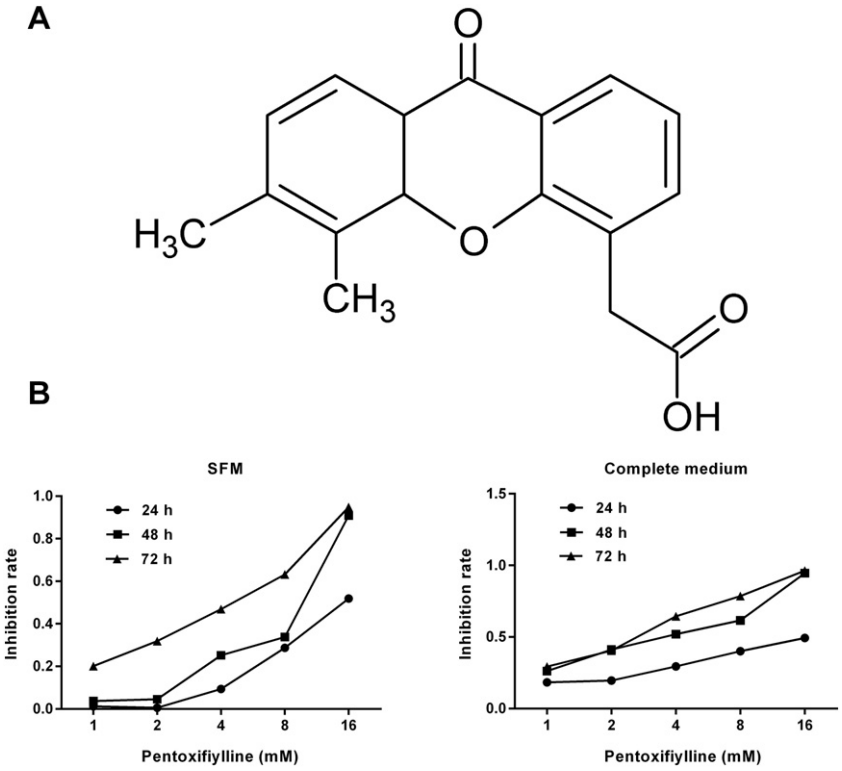

Fig. 1. PTX inhibits proliferation of HepG2 cells. HepG2 cells were treated with vehicle or PTX at 1, 2, 4, 8 or 16 mM for 24, 48 or 72 h in FBS-free medium (SFM) or 10% FBS medium (complete medium, six wells per group). The proliferation of individual groups of cells was determined by MTT and the inhibition rates of different concentrations of PTX on HepG2 cell proliferation were calculated. Data are expressed as the mean inhibition rate of each dose of PTX from three separate experiments. (A) Structure of PTX. (B) Inhibition of PTX on HepG2 cell proliferation.

[20]. In addition, while high levels of ROS are toxic to HCC, through inducing HCC cell apoptosis, low to moderate levels of ROS may promote the growth of HCC [15,21,22]. Previous studies have shown that PTX can attenuate ROS production [23,24]. We hypothesize that PTX may enhance the MAPK signaling and inhibit ROS production to promote apoptosis of HCC cells.

In this study, we employed HCC HepG2 cell line to determine the therapeutic effect of different doses of PTX and their molecular mechanisms *in vitro* and *in vivo*.

2. Materials and methods

2.1. Reagents

Pentoxifylline (PTX), DCFH-DA (2′,7′-dichlorodihydrofluorescein diacetate) and NAC (*N*-acetyl-L-cysteine) were purchased from Sigma-Aldrich (St. Louis, USA). SP600125 and PD98059 were purchased from Beyotime (Nanjing, Jiangsu, China). Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit was purchased from BD Biosciences (San Jose, USA). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was obtained from Biomart (Wuhan, Hubei, China). Primary antibodies against ERK1/2, p-ERK1/2, JNK, p-JNK, p38MAPK and p-P38MAPK as well as horseradish peroxidase (HRP)-conjugated second antibodies were purchased from Cell Signaling Technology (San Diego, USA). Antibody against β-actin was purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Fluorometric terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) (G3250) was purchased from Promega (Madison, USA).

2.2. Cell lines and culture conditions

Human HCC HepG2 cells were purchased from the American Type Culture Collection (ATCC, Manassas, USA). The cells were cultured in complete medium of Dulbecco's Modified Eagle's Medium (DMEM; Hyclone, Beijing, China) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin (Sigma-Aldrich, St. Louis, MO) at 37 °C, 5% CO_2.

2.3. Animals

Male BALB/c nude mice at 6–8 weeks of age were from the Animal Experiment Center of Xian Jiaotong University and housed in a specific pathogen-free facility with free access of autoclaved foods and water. The experimental protocol was approved by the Animal Care and Research Committee of Xian Jiaotong University.

2.4. MTT assay

The effect of PTX on HepG2 cell viability was determined by MTT assay. Briefly, HepG2 cells (1×10^4 cells/well) were treated with vehicle or varying concentrations (1, 2, 4, 8 and 16 mM) of PTX in 96-well plates (six wells/dose) for 24, 48 or 72 h in FBS-free medium or complete medium. During the last 4 h incubation, each well of cells was treated with MTT (0.5 mg/mL), and the resulting formazan in each well was dissolved with 200 μL of DMSO, followed by measuring the absorbance at 490 nm in a microplate reader.

2.5. Flow cytometry analysis

HepG2 cells (2×10^6 cells/well) were cultured overnight and treated in duplicate with vehicle or varying concentrations (1, 2, 4 and 8 mM) for 24 h. The cells were harvested and fixed with ice-cold ethanol (75%) at 4 °C > 18 h. After being washed, the cells were digested with RNase A (50 mg/L) reagent at 37 °C for 30 min and stained with propidium iodide (PI, 50 mg/L) at 4 °C for 30 min. Cell cycle status of

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