Pirfenidone inhibits proliferation, arrests the cell cycle, and downregulates heat shock protein-47 and collagen type I in rat hepatic stellate cells *in vitro*

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Abstract. Pirfenidone (esbiret) is an established anti-fibrotic and anti-inflammatory drug used to treat idiopathic pulmonary fibrosis. In the present study, the dose-dependent effects of pirfenidone on the cell cycle, proliferation and expression of heat shock protein (HSP)-47 and collagen type I in a cultured rat hepatic stellate cell line (HSC-T6) were investigated. Following pirfenidone treatment, cell proliferation was determined using the cell counting kit-8 assay and the cell cycle was measured using flow cytometry. HSP-47 expression was estimated using western blot analysis and collagen type I mRNA was assessed using reverse transcription quantitative polymerase chain reaction. Pirfenidone induced significant dose-dependent inhibition of proliferation in HSC-T6 cells. Cell viability was unaffected by treatment with pirfenidone (0, 10 or 100 μM) for 24 and 72 h. However, after 24 h, HSC-T6 cells exhibited dose-dependent decreases in HSP-47 protein and collagen I mRNA levels. In conclusion, pirfenidone inhibited HSC-T6 cell proliferation, arrested the cell cycle and reduced the expression of HSP-47 and collagen type I, indicating that pirfenidone may be a promising drug in the treatment of liver fibrosis.

Introduction

Liver fibrosis involves an excessive accumulation of extracellular matrix proteins, including collagen. It occurs in the majority of chronic liver diseases and hepatic stellate cells (HSCs) are the principal cell type responsible for liver fibrosis. Following the onset of a liver injury, quiescent HSCs become activated and transform into proliferative, fibrogenic and contractile myofibroblasts (1), which synthesize and secrete collagens (2). Therefore, HSCs have recently become a therapeutic target for liver fibrosis (3).

Heat shock protein (HSP)-47, a collagen-specific molecular chaperone, is predominantly present in the endoplasmic reticulum (ER) of collagen-producing cells and is also involved in the processing and/or secretion of procollagen (4-6). Expression of HSP-47 is upregulated in the fibrosis of various tissues (4), including liver cirrhosis (7), pulmonary fibrosis (8) and renal fibrosis (5). It has been reported that small interfering RNA targeting of HSP-47 in experimental liver fibrosis suppressed the expression and secretion of collagen *in vivo* and *in vitro* (9).

Pirfenidone [5-methyl-1-phenyl-2-(1H)-pyridone] is a novel anti-fibrotic and anti-inflammatory agent, which inhibits the progression of fibrosis in organs, including the lungs (8,10), liver (11,12), kidney (13), heart (14) and eyes (15,16). It inhibits cytokines, including transforming growth factor-(TGF)-β (16) and connective tissue growth factor (13,17-20). The compound also downregulates the expression of HSP-47 in human lung fibroblasts (8) and inhibits proliferation, cell migration and epithelial-mesenchymal transition of human lens epithelial cells. To the best of our knowledge, no information is available regarding the effects of pirfenidone on HSCs.

The aim of the present study was to investigate the effects of different concentrations of pirfenidone on rat hepatic stellate cells (HSC-T6), with a particular emphasis on cell morphology, proliferation, the cell cycle and expression of the proteins HSP-47 and collagen I.

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Materials and methods

Cell culture. HSC-T6 cells were obtained from The Third University Hospital of Sun Yat-Sen University cell bank (Guangzhou, China). The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM/F12; Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Sijiqing Biological Engineering Materials, Hangzhou, China), 100 U/ml penicillin and 100 μg/ml streptomycin (Biochrom GmbH, Berlin, Germany). The cells were maintained at 37°C in 5% CO_2 in a humidified atmosphere. All experiments were performed following 3-5 cell passages. Pirfenidone was purchased from Sigma-Aldrich (St. Louis, MO, USA).

HSC-T6 cells were cultured in six-well plates (10^5 cells/well) in DMEM/F-12 with 10% FBS. When cells grew to 80% confluence, the media was changed to DMEM/F-12 without penicillin or streptomycin but containing pirfenidone (0, 10 and 100 μM) and growth was continued for 24 h, following which cell morphology was examined under the optical microscope (DP22; Olympus, Tokyo, Japan).

Cell counting kit (CCK)-8 assay. HSC-T6 cells were seeded in 96-well plates (10^5 cells/well) for 24 h in DMEM/F-12 with 10% FBS. When cells grew to 80% confluence, they were incubated in DMEM/F-12 supplemented with pirfenidone (0, 10 and 100 μM) for 24 h. Following incubation with 100 μl DMEM/F-12 and 10 μl CCK-8 solution (Beyotime Institute of Biotechnology, Shanghai, China) for 1 h at 37°C, the absorbance at 450 nm was measured using a microplate reader (Meterterch Σ960; Sigma-Aldrich).

Flow cytometry. After a 24-h incubation in DMEM/F-12 with 10% FBS, HSC-T6 cells were incubated with pirfenidone (0, 10 or 100 μM) for an additional 24 h, then trypsinized, washed in phosphate-buffered saline (PBS) and fixed with 70% cold methanol (4°C) overnight. Following fixation, the cells were washed to remove alcohol and resuspended in PBS containing 100 μg/ml RNase A (Wako Pure Chemicals Industries, Ltd., Osaka, Japan) at 37°C for 30 min. The nuclei were stained by incubation in 50 μg/ml propidium iodide (Wako Pure Chemicals Industries, Ltd.) at 4°C for 30 min and analyzed by flow cytometry using a FACS Aria cell sorter (BD Biosciences, Franklin Lakes, NJ, USA). The cell cycle distribution was analyzed using Modifit software (BD Biosciences).

Western blot analysis. After a 24 h incubation in DMEM/F-12 with 10% FBS, HSC-T6 cells were treated with pirfenidone (0, 10 or 100 μM) for an additional 24 h. Cultured cells were harvested and lysed in mammalian protein extraction reagent (Pierce Biotechnology, Inc., Rockford, IL, USA). Supernatants obtained using centrifugation at 12,000 x g for 30 min at 4°C were used as cytoplasmic extracts. Protein concentrations were determined using a bicinchoninic acid assay kit (Shanghai Shenergy Biocolor BioScience and Technology Company, Shanghai, China). Prepared samples were heated to 100°C for 5 min prior to 10% acrylamide SDS-PAGE (Amersham Biosciences, Freiburg, Germany), and an identical quantity of total protein (20 μg) was added to each well. Separated proteins were transferred onto a nitrocellulose membrane (Hybond-ECL; Amersham Biosciences, Freiburg, Germany). Nonspecific

binding was blocked by incubation with Tris-buffered saline with Tween 20 (TBST) containing 5% nonfat milk for 2 h prior to an overnight incubation with a 1:1,000 dilution of the mouse monoclonal anti-human HSP-47 antibody (Stress Gen, Victoria, BC, Canada) diluted in TBST at 4°C with constant agitation. Following several washes with TBST, the membranes were incubated with a 1:10,000 dilution of the anti-mouse immunoglobulin G secondary antibody (ZSGB-BIO, Beijing, China) for 1 h. Following several subsequent washes with TBST, blots were developed using chemiluminescence (Phototope-HRP Western Blot Detection system; Cell Signaling Technology, Inc, Danvers, MA, USA) and the signal was captured on X-ray film (Eastman Kodak, Rochester, NY, USA) according to the manufacturer's instructions. The abundance of HSP-47 was correlated against a 1:10,000 dilution of mouse anti-GAPDH monoclonal antibody (Kang Chen Bio-tech Inc., Shanghai, China) and quantified using densitometry.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR). At 48 h after treatment with pirfenidone, total RNA was isolated from cultured cells using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). The ThermoScript RT system (Fermentas, Burlington, ON, Canada) was used to conduct the RT reactions. Total RNA (1 μg) was used for RT in a total volume of 20 ml. The following primers (mixed with probes) were purchased from Geneseed Biotech (Guangzhou, China): Collagen type I forward, 5'-CCCTACCCAGCACCTTCAAA-3' and reverse, 5'-GCAC AGGCCCTCAAAAACA-3'; and 18srRNA forward, 5'-CCTGGATACCGCAGCTAGGA-3' and reverse, 5'-GCGGCGCAATACGAATGCCCC-3'.

18S RNA as a control. PCR amplification was performed in a Gene Amp 2400 thermal cycler (Perkin Elmer Inc., Waltham, MA, USA). PCR amplification was performed with an initial denaturation step at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 15 sec, annealing at 60°C and extension at 72°C for 32 sec, with a final extension at 72°C for 32 sec. Quantification of signal intensity was confirmed using the IBAS 2.5 Auto Image analysis program (Kontron, Eching, Germany). The fidelity of the RT-PCR fragments was subsequently verified by comparing the size of the amplified products with the expected cDNA bands and the sequencing of the PCR products.

Statistical analysis. Values are expressed as the mean ± standard deviation. All data were analyzed using a one-way analysis of variance and with the Bonferroni test for replicate measurements. Statistical analyses were conducted using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). A Bonferroni adjusted $P<0.05$ was considered to indicate a statistically significant difference. For the Kontron IBAS 2.0 automatic image analysis system (Amersham Biosciences), semi-quantitative analysis was applied to scan strips. The stripe area and optical density were analyzed.

Results

Effects of pirfenidone on morphology of HSC-T6 cells. Under the optical microscope, the HSC-T6 cells were a characteristic myofibroblastic shape. In the medium containing penicillin and
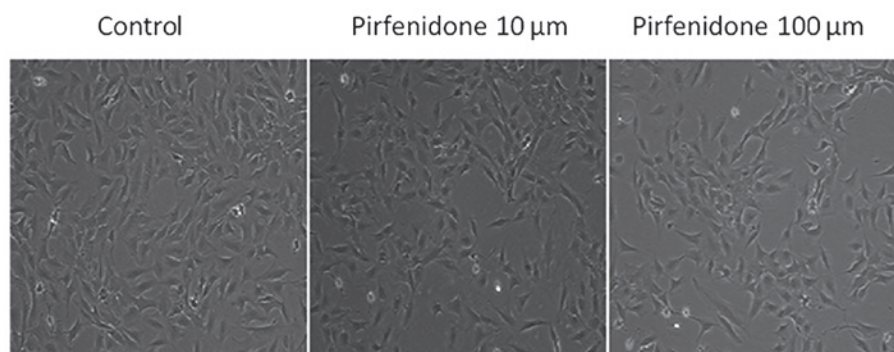
Figure 1. Morphological changes of HSC-T6 cells visualized using optical microscopy (magnification, x10). Following treatment with pirfenidone (10 or 100 μM) for 24 h, cells were generally smaller in size and changed to a more slender cell shape. Cells numbers were also noticeably decreased compared with untreated cells.
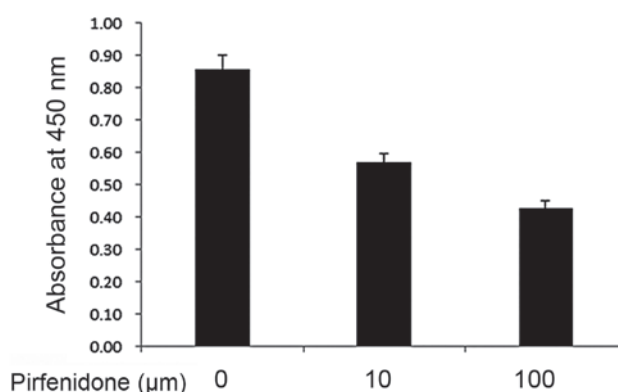

Figure 2. Proliferation of HSC-T6 cells following treatment with pirfenidone as measured using a cell counting kit-8 assay. Cells in Dulbecco's modified Eagle's medium/F12 were treated with pirfenidone (0, 10 or 100 μM) for 24 h. Values are expressed as the mean ± standard deviation of three independent experiments. The absorbance of cells decreased in a dose-dependent manner (P≤0.05).
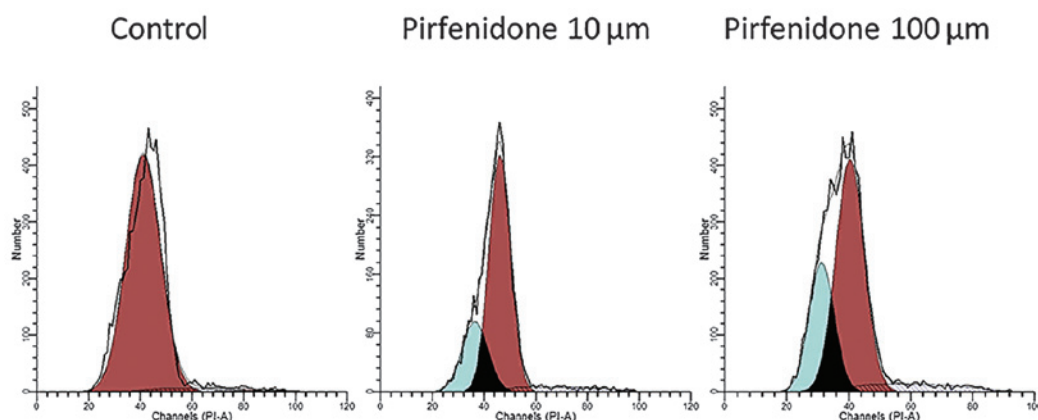

Figure 3. Flow cytometric analysis of the cell cycle. Pirfenidone arrested the cell cycle at the G1 phase in a dose-dependent manner. The percentage of cells in the S and G2 phase exhibited no marked differences among different concentrations of pirfenidone. Values are expressed as the mean ± standard deviation of three independent experiments.

streptomycin, cells were observed to be attached to the culture plate and stretching pseudopodia were identified after 4 h. By the following day, cells were more uniform, with slender pseudopodia. After two days, star-shaped cells were fully-fused. Following exposure to 10-100 μM pirfenidone for 24 h, cells were generally smaller in size and of a more slender shape (Fig. 1). In addition, quantities of the cells were markedly reduced (Fig. 2).

Effects of pirfenidone on cell proliferation. HSC-T6 cells were treated with pirfenidone (0, 10 or 100 μM) for 24 h and cell proliferation was measured using the CCK-8 assay. Pirfenidone significantly reduced the level of cell proliferation in a dose-dependent manner (10 μM pirfenidone vs. control, P=0.005; 100 μM pirfenidone vs. control, P<0.001; 10 μM vs. 100 μM pirfenidone, P=0.0037; Fig. 1).
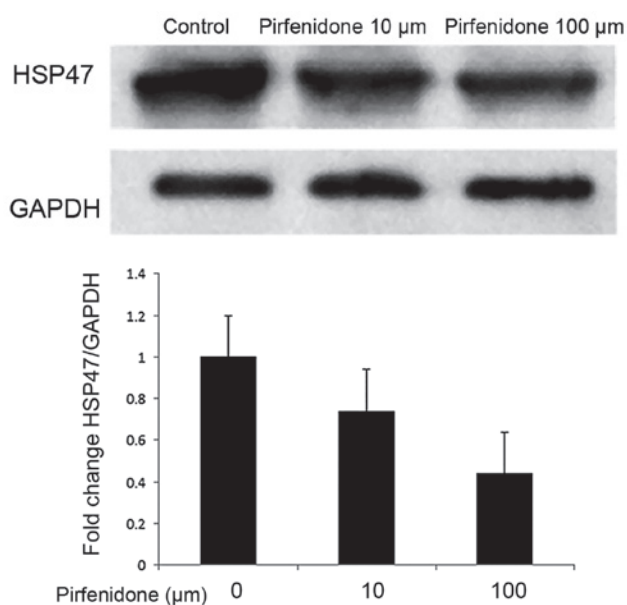
Figure 4. Effects of pirfenidone on heat shock protein-47 expression. Western blotting revealed protein production after 24 h incubation with pirfenidone (0, 10 or 100 μM). Band densities were normalized against GAPDH and are shown as a ratio. Values are expressed as the mean ± standard deviation of three independent experiments.
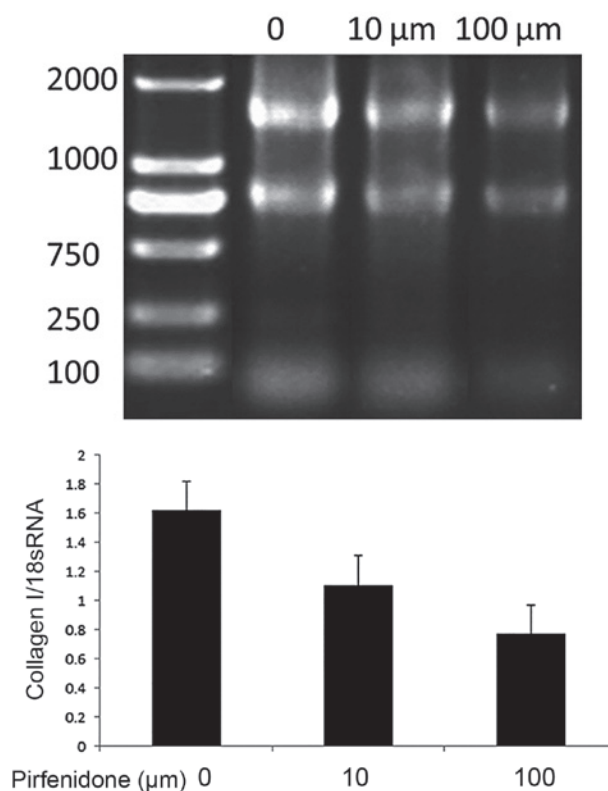

Figure 5. Effects of pirfenidone on collagen type I mRNA expression in HSC-T6 cells. Reverse transcription-polymerase chain reaction demonstrating mRNA expression of collagen type I after 24 h incubation with pirfenidone (0, 10 or 100 μM). Band densities were normalized against 18S RNA and are shown as a ratio. Values are expressed as the mean ± standard error of the mean of three independent experiments.

Effects of pirfenidone on the cell cycle. The cell cycle distribution was determined using flow cytometric analysis of released nuclei. Following treatment with pirfenidone for 24 h, the proportion of cells in the G1 phase was decreased; however the number in the S-phase was increased (Fig. 3).

Effects of pirfenidone on HSP-47 expression. The effects of pirfenidone on HSP-47 expression were investigated using western blot analysis. The expression of HSP-47 was clearly decreased by pirfenidone treatment in a dose-dependent manner (10 μM pirfenidone vs. control, P=0.042; 100 μM pirfenidone vs. control, P=0.003; 10 μM vs. 100 μM pirfenidone, P=0.011; Fig. 4A, B).

Effects of pirfenidone on expression of collagen type I. The mRNA levels of collagen type I were analyzed 24 h after pirfenidone treatment. Of note, collagen type I mRNA levels were significantly decreased by pirfenidone (10 μM pirfenidone vs. control, P=0.079; 100 μM pirfenidone vs. control, P=0.017; 10 μM vs. 100 μM pirfenidone, P=0.044; Fig. 5). Furthermore, quantitative analysis of collagen I transcription normalized against 18S RNA exhibited a similar dose-dependent pattern to that observed with HSP-47 expression (Fig. 4). This indicated that a reduction of collagen type I mRNA resulted from the decreased HSP-47 protein levels caused by pirfenidone treatment.

Discussion

The final stage of liver fibrosis is liver cirrhosis, which results in severe hepatic incapacity and portal hypertension (21). Liver fibrosis is reversible and there are a number of specific therapies, including antiviral treatments for patients infected with hepatitis B or C (22). Mesenchymal stem cell injection (23) is also available, but no specific and effective anti-fibrotic therapies have been developed to date.

Pirfenidone, a small and orally bioavailable molecule, was approved for mild to moderate idiopathic pulmonary fibrosis in the European Union in February 2011 and in Japan in 2009 (24). The anti-fibrotic effects of pirfenidone have been demonstrated in different experimental models of liver fibrosis, and also with dimethylnitrosamine (25-28). Treatment with pirfenidone reduced the expression of fibrotic genes, including TGF-β, profibrogenic procollagen α1(I) and TIMP-1. In an *in vitro* study of liver fibrosis, this drug was effective at inhibiting platelet-derived growth factor-induced proliferation of HSCs, and the expression and accumulation of collagen type I induced by TGF-β1 (17). However, the expression and accumulation of collagens are not only induced by TGF-β1, but also regulated by a variety of cytokines and cell signaling pathways during this complex multi-system pathological process; therefore, the precise *in vitro* molecular effects of pirfenidone on collagen synthesis in liver fibrosis require further elucidation. In the present study, pirfenidone suppressed HSC-T6 cell proliferation in a dose-dependent manner. Optical microscopy revealed that following treatment with pirfenidone for 24 h, HSC-T6 cells became smaller in size and decreased in number. Flow cytometry of the cells revealed that pirfenidone treatment significantly modified the cell cycle. A lower concentration of pirfenidone (10 μM) altered the cell cycle in a dose-dependent manner, although changes were subtle. However, at 100 μM, the proportion of cells in the G1

phase was significantly decreased, suggesting the compound induced cell cycle arrest at G1 phase. The effect of pirfenidone on the cell cycle of different cells using different treatments has been reported previously. Pirfenidone arrested Tenon cells in G1 phase to inhibit cell proliferation (16), but Leiomyoma cells were not affected (25). Due to the highly complex and tightly regulated nature of the cell cycle itself, the effects of pirfenidone on HSC-T6 and other cells requires further investigation.

Pirfenidone was also observed to inhibit the expression of HSP-47 and collagen type I in a dose-dependent manner, as revealed by western blot analysis and RT-qPCR, respectively. HSP-47, as a collagen-specific molecular chaperone, has an essential role in the maturation of various procollagens (4,29,30). Upregulation of HSP-47 has been revealed to be correlated with an increased accumulation of collagens in fibrosis of the liver (7), lung (31), kidney (32), heart (33) and skin (34). In these studies, HSP-47 was revealed to affect the abnormal accumulation of collagen type I and collagen type III, two of the most important extracellular matrix proteins. These *in vivo* and *in vitro* studies demonstrated that anti-sense oligodeoxynucleotides against HSP-47 were able to inhibit collagen production (9,35-38). In lung fibroblasts, pirfenidone suppressed the increased expression of HSP-47 *in vivo* (10) and *in vitro* (8). In the present study, pirfenidone exhibited a similar suppressive effect on the expression of HSP-47 in HSC-T6 cells. The downregulation of HSP-47 collagen type I was most pronounced following treatment with 100 μM pirfenidone. This downregulation and the possible reduction of collagen synthesis during liver fibrosis is likely to explain the anti-fibrotic effects of this compound.

In conclusion, pirfenidone exhibited inhibitory effects on the proliferation of rat HSC-T6 cells, decreased the proportion of cells in G1 phase and increased the number of cells in S phase. In addition, pirfenidone significantly suppressed HSP-47 protein and collagen type I expression *in vitro*. Pirfenidone may be used to regulate the expression of HSP-47 in HSC-T6 cells and is a potential therapeutic agent for liver fibrosis.

Acknowledgements

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