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

XIAN-HONG XIANG1*, TIAN-PENG JIANG2*, SHUAI ZHANG3, JIE SONG2, XING LI2, JIAN-YONG YANG1 and SHI ZHOU2

1Department of Interventional Radiology, The First Affiliated Hospital of Sun Yat-Sen University, Guangzhou, Guangdong 510080; 2Department of Interventional Radiology, Affiliated Hospital of Guiyang Medical College; 3Department of Interventional Radiology, Affiliated Cancer Hospital of Guiyang Medical College, Guiyang, Guizhou 550000, P.R. China

Received March 28, 2014; Accepted January 26, 2015

DOI: 10.3892/mmr.2015.3403

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.

Correspondence to: Dr Jian-Yong Yang, Department of Interventional Radiology, The First Affiliated Hospital of Sun Yat-Sen University, 58 Zhongshan Road II, Guangzhou, Guangdong 510080, P.R. China
E-mail: cjryangjianyong@vip.163.com

Dr Shi Zhou, Department of Interventional Radiology, Affiliated Hospital of Guiyang Medical College, 28 Guiyi Road, Guiyang, Guizhou 550000, P.R. China
E-mail: zhoushi_55@163.com

*Contributed equally

Key words: hepatic stellate cells, pirfenidone, collagen type I, heat shock protein-47, liver fibrosis, cell division
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% CO₂ in a humidified atmosphere. All experiments were performed following a 3-5 day passage. Pirfenidone was purchased from Sigma-Aldrich (St. Louis, MO, USA).

HSC-T6 cells were cultured in six-well plates (10⁶ cells/well) in DMEM/F-12 with 10% FBS. When cells grew to 80% confluence, they 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 (ZSBG-BIO, Beijing, China) for 1 h. Following several subsequent washes with TBST, blots were developed using chemiluminescence (Phototope-HP 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 µl. The following primers (mixed with probes) were purchased from Geneseed BioTech (Guangzhou, China): Collagen type I forward, 5'-CCCTACCCACGACCTTCAAA-3' and reverse, 5'-GCAC AGGCCCTCAAACAACA-3'; and 18sRNA 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...
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 changed to a more slender cell shape. Cells numbers were also noticeably decreased compared with untreated cells.

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).
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
Effect of Resolution of liver cirrhosis

References

(grant no. 2012B061700078).
Technology Planning Project of Guangdong Province China (grant no. 20120171120086) and the Science and
The present study was supported by the research Fund

1. Friedman SL: Molecular regulation of hepatic fibrosis, an
integrated cellular response to tissue injury. J Biol Chem 275:
2. Milani S, Herbst H, Schuppan D, Surrenti C, Riecken EO and
Stein H: Cellular localization of type I and IV procollagen
gene transcripts in normal and fibrotic human liver. Am J
4. Taguchi T and Razaqzae MS: The collagen-specific molecular
chaperone HSP47: Is there a role in fibrosis. Trends Mol Med 13:
5. Razaqzae MS, Le VT and Taguchi T: Heat shock protein 47 and
6. Chen JJ, Jin PS, Zhao S, Cen Y, Liu Y, Xu XW, Duan WQ and
Wang HS: Effect of heat shock protein 47 on collagen synthesis
7. Kimura KE, Broadhurst A, Mathias MM, Brunt EM and
Schmidt WN: Expression of HSP47, a collagen-specific chaperone,
the expression of HSP47 in TGF-β-stimulated human lung fibroblasts.
using vitamin A-coupled liposomes to deliver siRNA against a
10. Kakugawa T, Muka H, Hayashi T, Ishi H, Abe K, Fuji T, Oku H,
Miyazaki M, Kadota J and Kohno S: Pirfenidone attenuates
expression of HSP47 in murine bleomycin-induced pulmonary fibrosis.
inhibits carbon tetrachloride- and albumin complex-induced liver
fibrosis in rodents by preventing activation of hepatic stellate cells.
Fibrogenic polymorphisms (TGF-β1, PA1-1, AT) in Mexican
13. Hewittson MC, Kelynanck KJ, Tait MG, MacMurray JF, Jones CL,
Margolin SB and Becker GJ: Pirfenidone reduces in vitro rat renal fibroblast activation and mitogenesis. J Nephrol 14:
14. Miric G, Dallmann MA, Endre Z, Margolin S, Taylor SM and
Brown L: Reversal of cardiac and renal fibrosis by pirfenidone and
Pharmacokinetics of pirfenidone after topical administration in
rabbit eye. Mol Vis 17: 2191-2196, 2011.
on proliferation, migration, and collagen contraction of human Tenon's fibroblasts in vitro. Invest Ophthalmol Vis Sci 50:
pirfenidone on rat hepatic stellate cell proliferation and collagen
18. Gurugejalakshmi G, Hollinger MA and Giri SN: Pirfenidone
19. Grattendick KJ, Nakashima JM, Feng L, Giri SN and
Margolin SB: Effects of three anti-TNF-alpha drugs: Etanercept,
infliximab and pirfenidone on release of TNF-alpha in
in vitro studies demonstrated that
anti-sense oligodeoxyribonucleotides against HSP-47 were able
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e used to regulate the expression of HSP-47 in HSC-T6 cells
and is a potential therapeutic agent for liver fibrosis.

Acknowledgements

The present study was supported by the research Fund
for the Doctoral Program of Higher Education of China (grant no. 20120171120086) and the Science and Technology Planning Project of Guangdong Province (grant no. 2012B061700078).

References

1. Friedman SL: Molecular regulation of hepatic fibrosis, an
integrated cellular response to tissue injury. J Biol Chem 275:
2. Milani S, Herbst H, Schuppan D, Surrenti C, Riecken EO and
Stein H: Cellular localization of type I and IV procollagen
gene transcripts in normal and fibrotic human liver. Am J
4. Taguchi T and Razaqzae MS: The collagen-specific molecular
chaperone HSP47: Is there a role in fibrosis. Trends Mol Med 13:
5. Razaqzae MS, Le VT and Taguchi T: Heat shock protein 47 and


