Fluorofenidone attenuates hepatic fibrosis by suppressing the proliferation and activation of hepatic stellate cells

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Submitted 11 December 2012; accepted in final form 2 December 2013

Peng Y, Yang H, Wang N, Ouyang Y, Yi Y, Liao L, Shen H, Hu G, Wang Z, Tao L. Fluorofenidone attenuates hepatic fibrosis by suppressing the proliferation and activation of hepatic stellate cells. Am J Physiol Gastrointest Liver Physiol 306: G253–G263, 2014. First published December 12, 2013; doi:10.1152/ajpgi.00471.2012.—Fluorofenidone (AKF-PD) is a novel pyridone agent. The purpose of this study is to investigate the inhibitory effects of AKF-PD on liver fibrosis in rats and the involved molecular mechanism related to hepatic stellate cells (HSCs). Rats treated with dimethylnitrosamine or CCl4 were randomly divided into normal, model, AKF-PD treatment, and pirfenidone (PFD) treatment groups. The isolated primary rat HSCs were treated with AKF-PD and PFD respectively. Cell proliferation and cell cycle distribution were analyzed by bromodeoxyuridine and flow cytometry, respectively. The expression of collagen I and α-smooth muscle actin (α-SMA) were determined by Western blot, immunohistochemical staining, and real-time RT-PCR. The expression of cyclin D1, cyclin E, and p27kip1 and phosphorylation of MEK, ERK, Akt, and 70-kDa ribosomal S6 kinase (p70S6K) were detected by Western blot. AKF-PD significantly inhibited PDGF-BB-induced HSC proliferation and activation by attenuating the expression of collagen I and α-SMA, causing G0/G1 phase cell cycle arrest, reducing expression of cyclin D1 and cyclin E, and promoting expression of p27kip1. AKF-PD also downregulated PDGF-BB-induced MEK, Akt, and p70S6K phosphorylation in HSCs. In rat liver fibrosis, AKF-PD alleviated hepatic fibrosis by decreasing necroinflammatory score and semiquantitative score, and reducing expression of collagen I and α-SMA. AKF-PD attenuated the progression of hepatic fibrosis by suppressing HSCs proliferation and activation via the ERK/MAPK and PI3K/Akt signaling pathways. AKF-PD may be used as a potential novel therapeutic agent against liver fibrosis.

ERK/MAPK; fluorofenidone; hepatic fibrosis; hepatic stellate cells; PI3K/Akt

HEPATIC FIBROSIS IS A CONSEQUENCE of hepatic wound healing response to a variety of acute and chronic insults, such as viral infections, cholestasis, ethanol, metabolic diseases, and loss of parenchymal tissue (3, 18). It represents a major worldwide medical problem associated with significant morbidity (17). Hepatic fibrosis is a reversible process under certain conditions, but the appropriate strategies to treat hepatic fibrosis have not been well established (27).

It is widely recognized that activated HSCs play a pivotal role in the development of liver fibrosis. Following a fibrogenic stimulus, the proliferated and activated HSCs express α-smooth muscle actin (α-SMA) and produce a large amount of extracellular matrix (ECM) proteins including collagen I (14, 47). Recent studies have revealed that HSC activation can be promoted by several mitogens, which include platelet-derived growth factor (PDGF), connective tissue growth factor (CTGF), and insulin-like growth factor-1 (IGF-1) (5, 38, 42). The binding of PDGF-BB to the PDGF receptor leads to dimerization and autophosphorylation of tyrosine residues, resulting in the downstream activation of phosphatidylinositol-3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways (25, 30). An interruption of PDGF signaling via the PDGF receptor will block MAPK and PI3K/Akt signaling pathways, so as to lead to an inhibition of HSC activation and an attenuation of experimental liver fibrosis (5, 6, 20). Cellular proliferation is regulated by the cell cycle (1, 31). In HSCs, cell cycle progression is adjusted by cyclin/cyclin-dependent kinase (CDK) complexes. Cyclin D1/CDK4 and cyclin E/CDK2 complexes perform important roles in promoting the transition from the G0/G1 to the S phase (24). p27kip1 is a negative regulator of the protein kinase cyclin D1/CDK4 and CDK2/cyclin E and can block the cell cycle at the G0/G1 phase (13).

Research identifying safe antifibrotic agents on liver fibrosis is of high priority and urgently needed. Many studies have reported that pyridone agents, such as pirfenidone (PFD), can attenuate fibrosis in many organs including pulmonary (21), cardiac (44), renal (39), and hepatic (10, 11, 32). As a newly developed water-soluble pyridone agent with potential broad-spectrum antifibrotic characteristics, fluorofenidone [1-(3-fluorophenyl)-5-methyl-2-(1H)-pyridone; AKF-PD] can attenuate renal and cardiac fibrosis (8, 34, 45, 48). However, the therapeutic effects on hepatic fibrosis remain unclear. The purpose of this study was to investigate the antifibrotic effects of AKF-PD on rat liver fibrosis and elucidate the underlying molecular mechanism of AKF-PD on the proliferation and cell cycle progression in rat HSCs.

MATERIALS AND METHODS

Materials. All reagents for cell culture and TRIzol reagent for RNA extraction were purchased from Invitrogen (Carlsbad, CA). The real-time RT-PCR kit was purchased from TaKaRa Biotechnology (Otsu, Japan). The bromodeoxyuridine (BrdU) labeling and detection kit was purchased from Roche Diagnostics Corporation (Indianapolis, IN). The antibody against collagen α1(I) and cyclin E were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). U0126, LY294002, and antibodies to cyclin D1, p27kip1, total extracellular signal regulated kinase (ERK), MEK, Akt, p70S6K, phospho (p)-ERK, p-MEK, p-Akt, p-p70S6K were purchased from Cell Signaling (Danvers, MA). Horseradish peroxide (HPR)-conjugated secondary antibodies

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http://www.ajpgi.org 0193-1857/14 Copyright © 2014 the American Physiological Society
for Western blot were from Jackson (West Grove, PA), and secondary antibodies for immunohistochemistry were from GIBI (Mukilteo, WA). The enhanced chemiluminescence (ECL) kit for Western blot was from GE Healthcare (Buckinghamshire, UK). AKF-PD (lot no. 090601) and PFD (lot no. 080402) were synthesized by Synthesis Lake Pharma (Shenzhen, Guangdong, China). Dimethylnitrosamine (DMN), PDGF-BB, α-SMA, and β-actin were purchased from Sigma-Aldrich (St. Louis, MO).

Primary HSCs isolation and culture. HSCs were isolated from the liver of normal male Sprague-Dawley rats (400–450 g) through portal vein perfusion with collagenase-pronase and subsequent density gradient centrifugation as previously described (29). Freshly isolated HSCs were plated on plastic plates and cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% of fetal bovine serum (FBS) at 37°C in a 5% CO2 atmosphere for the first 48 h. Semiconfluent HSCs with 1–3 passages were used in experiments.

Cell culture and BrdU cell proliferation assay. HSCs were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen) at 37°C in a humidified atmosphere of 5% CO2 and 95% air. The in vitro proliferation of HSCs treated with AKF-PD was assessed by Cell Proliferation ELISA, BrdU (chemiluminescence) kit (Roche Diagnostics) following manufacturer’s instructions. Experiments were performed in triplicate in three independent experiments.

Cell culture and cell cycle analysis by flow cytometry. HSCs were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen) at 37°C in a humidified atmosphere of 5% CO2 and 95% air. HSCs were treated with AKF-PD in the medium containing 1% FBS for 48 h. Cells were washed, fixed with ice-cold 70% ethanol and incubated in 800 µl PBS, 100 µl RNase (1 mg/ml) and 20 µl PI (2 mg/ml) for 30 min at 37°C, followed by flow cytometry analysis using FACSCalibur (Becton Dickinson). The percentage of cells in the G0-G1 phase were from GenBank (Verity Software House, Topsham, ME).

AKF-PD and PFD treatment on HSCs. The HSCs were seeded on six-well culture plates to ~60–70% confluence in complete medium containing 10% FBS for 24–48 h, which was changed to serum-free medium for 24 h before PDGF-BB induction at a final concentration of 10 ng/ml. To examine the effects of AKF-PD and PFD on PDGF-BB-induced expression of p-ERK, p-MEK, p-Akt, and p-p70s6k, the cells were treated with AKF-PD (2 mM) or PFD (2 mM) for 24 h, incubated with the MEK inhibitor U0126 (10 µM) or the PI3K inhibitor LY294002 (25 µM) for 1 h, and subsequently induced by PDGF-BB (10 ng/ml) for 15 min before cellular protein extraction. Each experiment was replicated three times.

Transient transfection assays. The plasmids MEK1Q56P and PI3K-CAAX were kind gifts from Dr. Damu Tang (McMaster University). These plasmids constitutively express the active form of MEK and PI3K, respectively. Transient transfection was conducted with Lipofectamine 2000 reagent (Invitrogen) following the protocol provided by the manufacturer (41). Each experiment was carried out in triplicate.

Animals and treatment. Male albino rats of the Wistar strain and male Sprague-Dawley rats weighing between 220 and 250 g were obtained from Slac Laboratory Animal (Shanghai, China). The animals were bred and maintained in an air-conditioned animal house, with commercial diet and water available. All rats received humane care in compliance with the university’s guidelines. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Central South University. The in vivo effects of AKF-PD were monitored in the livers of rats induced by DMN or CCl4. In the DMN model, Wistar rats were randomly divided into normal group, DMN model group, DMN+AKF-PD-treated group, and DMN+PFD-treated group (n = 10 for each group).

Hepatic fibrosis was induced via intraperitoneal injections of DMN at a dose of 1 µl (diluted 1:100 with 0.15 M NaCl) per 100 g body wt. The injections were given on the first 3 consecutive days of each week for a period of 3 wk. The normal group received 0.15 M sterile NaCl. From the third week, the treatment groups were given AKF-PD or PFD intragastrically (240 mg·kg⁻¹·day⁻¹) once daily for 4 wk. The normal group and DMN model group were administered 0.5% carboxymethyl cellulose sodium (CMCNa) intragastrically daily for 4 wk simultaneously. In the CCl4 model, Sprague-Dawley rats were randomly divided into normal group, CCl4 model group, CCl4+AKF-PD-treated group, and CCl4+PFD-treated group (n = 10 for each group). Hepatic fibrosis was induced via intraarterial injections of CCl4 (2 ml/kg body wt, 1:1 in olive oil) twice per week for 8 wk. From the ninth week, the treatment groups were given AKF-PD or PFD intragastrically (240 mg·kg⁻¹·day⁻¹) once daily for 3 wk. The normal group and CCl4 model group were administered 0.5% CMCNa intragastrically daily for 3 wk simultaneously. All rats were anaesthetized with 10% chloral hydrate (0.4 ml/100 g) before euthanasia. A portion of liver was fixed for histopathology, and the remaining tissue was stored at −70°C.

Serum ALT and AST. The serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were evaluated for each rat blood sample at the end of the experiment. The liver functions were measured by routine methods used in the clinical laboratory of Xiangya Hospital.

Histological and immunohistochemical analysis. The rat liver tissues fixed in 10% neutral-buffered formalin were dehydrated in graded alcohol and embedded in paraffin. The paraffin sections (4 µm thick) were stained with hematoxylin and eosin (H&E) or with Masson’s trichrome. To determine the degree of necroinflammatory liver injury, histological grading and quantification of infiltrating inflammatory cells were blindly performed by an independent pathologist as described previously (4, 12). To further analyze the degree of interstitial collagen deposition, Masson’s trichrome-stained sections were graded as that in a previous report (9). Following an antigen retrieval by 6.5 mM citrate buffer (pH 6.0) pressure cooking and

Table 1. The nucleotide sequences of primers used for real-time RT-PCR

<table>
<thead>
<tr>
<th>Genes</th>
<th>GenBank No.</th>
<th>Primers</th>
<th>Tm, °C</th>
<th>Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen I</td>
<td>NM_053304.1</td>
<td>5'-TTG GAG CAC ACG TGA CAA ACA GT-3'</td>
<td>59</td>
<td>157</td>
</tr>
<tr>
<td>Sense</td>
<td></td>
<td>5'-TTG GAG CAC ACG TGA CAA ACA GT-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antisense</td>
<td></td>
<td>5'-TTG GAG CAC ACG TGA CAA ACA GT-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-SMA</td>
<td>NM_031004.2</td>
<td>5'-CTA AGG CCC ACC CGG GAA AAG TTA ACA CGA-3'</td>
<td>60</td>
<td>186</td>
</tr>
<tr>
<td>Sense</td>
<td></td>
<td>5'-CTA AGG CCC ACC CGG GAA AAG TTA ACA CGA-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antisense</td>
<td></td>
<td>5'-CTA AGG CCC ACC CGG GAA AAG TTA ACA CGA-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Actin</td>
<td>NM_031144.2</td>
<td>5'-GGGATTATCTGGCCCTGCTGCTTAA-3'</td>
<td>61</td>
<td>171</td>
</tr>
<tr>
<td>Sense</td>
<td></td>
<td>5'-GGGATTATCTGGCCCTGCTGCTTAA-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antisense</td>
<td></td>
<td>5'-GGGATTATCTGGCCCTGCTGCTTAA-3'</td>
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α-SMA, α-smooth muscle actin.
endogenous peroxidase activity blockage with 3% hydrogen peroxide in methanol for 20 min at room temperature, DAB immunohistochemistry staining was processed with the DAKO EnVision System (37). In brief, the slides were incubated with the primary antibody against collagen α1(I) or α-SMA overnight at 4°C and then treated with a pepsin-based antigen retrieval technique. After immunostaining, sections were counterstained with hematoxylin. Interstitial staining of collagen α1(I) and α-SMA were measured by a blinded observer using computerized morphometry (Leica QWin 2.8 software, Wetzlar, Germany) (7).

**Western blot analysis.** We separated 30 μg of total protein extracted from fresh liver tissue or cultured cells on 8% or 10% SDS-polyacrylamide gels under reducing conditions, and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). The unspecific binding sites were blocked in TBS-T buffer [10 mM Tris-HCl, 150 mM NaCl, 0.1% (vol/vol) Tween 20, pH 7.6] containing 5% (wt/vol) skim milk for 1 h at room temperature. The membranes were incubated overnight at 4°C with the primary antibodies against p-ERK (1:1,000), p-MEK (1:1,000), p-Akt (1:1,000), p-p70s6k (1:1,000), cyclin D1 (1:1,000), cyclin E (1:1,000), p27kip1 (1:200), and α-SMA (1:1,000) and were subsequently hybridized with HRP-conjugated secondary antibodies for 1 h at room temperature. The bands were visualized by ECL kit and quantified with Glyko Bandscan 5.0 software (Glyko, Novato, CA).

**Analysis of mRNA expression.** Total RNA was isolated from fresh liver tissue by using TRIzol reagent according to the manufacturer’s instructions. The first-strand cDNAs were synthesized from 2 μg of...
total RNA in a 20-μl reaction with use of reverse transcriptase (Fermentas, Hanover, MD). The specific primers for collagen I, α-SMA, and β-actin were designed from their GenBank sequences and synthesized by Bio Basic (Shanghai, China) (Table 1). The real-time RT-PCR quantitation for individual target mRNA was performed on an ABI Model 7900 Detector (Applied Biosystems, Foster City, CA) by use of a TaKaRa real-time PCR kit. The amplified PCR products were quantified by measuring the calculated cycle

Fig. 2. AKF-PD inhibits ERK/MAPK and PI3K/Akt signaling pathways in HSCs. A: AKF-PD decreased the amount of phospho (p)-MEK and p-ERK in PDGF-BB-induced HSCs shown by Western blot. PFD, pirfenidone. B: AKF-PD decreased the amount of p-Akt and p-P70S6K in PDGF-BB-induced HSCs shown by Western blot. C: AKF-PD downregulated the amount of p-ERK, cyclin D1 and collagen α(I) in HSCs overexpressed MEK. D: AKF-PD downregulated the amount of p-Akt, cyclin D1, and collagen α(I) in HSCs overexpressing PI3K. *P < 0.05 compared with the control group. #P < 0.05 compared with the PDGF-BB treatment group or transient transfection group.
thresholds (CT) for individual targets and β-actin mRNA. The amount of specific mRNA in each sample was calculated from the standard curve and normalized to the β-actin mRNA. The comparative 2−ΔΔCT method was used for quantification and statistical analysis and the results were expressed as fold changes relative to normal controls (46).

Statistical analysis. All data were expressed as means ± SD. The statistical analysis was performed with SPSS 16.0 software (SPSS, Chicago, IL). The comparison among groups was made with one-way ANOVA assay. Multiple-comparison tests were applied only when a significant difference was determined by the ANOVA. P < 0.05 was considered to be statistically significant.

RESULTS

AKF-PD inhibits PDGF-BB-induced HSC activation and proliferation. After treatment with AKF-PD for 48 h, the proliferation of HSCs was inhibited in a dose-dependent manner as assessed by BrdU incorporation (Fig. 1A). Moreover, Western blot analysis showed that AKF-PD dramatically decreased α-SMA and collagen α1(I) expression (Fig. 1D). However, there was no significant difference between AKF-PD and PFD (P > 0.05).

AKF-PD induces cell cycle arrest in activated rat HSCs. Flow cytometric analysis of DNA content was performed to investigate effects on the cell cycle. AKF-PD arrested HSCs at the G0/G1 phase with a corresponding decrease in S-phase and G2/M phases (Fig. 1B). To determine the molecular mechanisms of G0/G1 arrest induced by AKF-PD, we checked the expression of the CDK inhibitor p27kip1 in HSCs treated with AKF-PD. Because the G1 phase-to-S phase transition is primarily controlled by the cyclin D1 and cyclin E, we also examined the cyclin D1 and cyclin E protein levels. AKF-PD significantly increased p27kip1 expression and reduced cyclin D1 and cyclin E protein expression in PDGF-BB-induced HSCs (Fig. 1C). There was no significant difference between AKF-PD and PFD (P > 0.05).

AKF-PD inhibits ERK/MAPK and PI3K/Akt signaling pathways in PDGF-BB-induced HSCs. To elucidate the molecular mechanisms underlying the inhibitory effects of AKF-PD on HSCs proliferation and activation induced by PDGF-BB, we analyzed the intracellular ERK/MAPK and PI3K/Akt signaling pathways in HSCs. AKF-PD significantly decreased the phosphorylation of ERK, MEK, Akt, and p70S6k in PDGF-BB-induced HSCs (Fig. 2, A and B). The MEK inhibitor U0126 remarkably downregulated the expression of p-ERK, and the PI3K inhibitor LY294002 markedly reduced the expression of p-Akt. There was no statistical difference regarding to their inhibition abilities on phosphorylated ERK, MEK, Akt, and p70S6k among different pretreatment groups (P > 0.05).

Table 2. Effects of AKF-PD on ALT and AST in DMN rats

<table>
<thead>
<tr>
<th>Group</th>
<th>ALT, U/l</th>
<th>AST, U/l</th>
</tr>
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<tbody>
<tr>
<td>Normal</td>
<td>48.74 ± 12.37</td>
<td>115.39 ± 17.42</td>
</tr>
<tr>
<td>DMN models</td>
<td>133.52 ± 65.97*</td>
<td>219.40 ± 106.38*</td>
</tr>
<tr>
<td>DMN + AKF-PD</td>
<td>116.37 ± 23.51*</td>
<td>165.01 ± 33.76†</td>
</tr>
<tr>
<td>DMN + PFD</td>
<td>126.84 ± 46.74*</td>
<td>172.74 ± 67.82†</td>
</tr>
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AKF-PD, fluoroferidone; ALT, alanine aminotransferase; AST, aspartate aminotransferase; DMN, dimethylnitrosamine. *P < 0.05 compared with the normal group. †P < 0.05 compared with the DMN model group.

Table 3. Effects of AKF-PD on ALT and AST in CCl4 rats

<table>
<thead>
<tr>
<th>Group</th>
<th>ALT, U/l</th>
<th>AST, U/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>49.24 ± 9.12</td>
<td>129.2 ± 17.4</td>
</tr>
<tr>
<td>CCl4 models</td>
<td>51.49 ± 7.52</td>
<td>114.3 ± 16.2*</td>
</tr>
<tr>
<td>CCl4 + AKF-PD</td>
<td>58.62 ± 6.87</td>
<td>100.9 ± 13.9†</td>
</tr>
<tr>
<td>CCl4 + PFD</td>
<td>55.91 ± 11.63</td>
<td>96.9 ± 13.1†</td>
</tr>
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</table>

**P < 0.05 compared with the normal group. †P < 0.05 compared with the CCl4 model group.

AKF-PD inhibits the ERK/MAPK or PI3K/Akt signaling pathways in HSCs over expressing MEK or PI3K. It is known that ERK/MAPK and PI3K/Akt signal pathways play important roles in cell proliferation (36). We have confirmed that AKF-PD can inhibit HSCs by more than two pathways of proliferation, the next question is where are the targets of AKF-PD in the two pathways? HSCs were transfected with MEK1Q56P or PI3K P110α-CAAAX plasmids. After transient transfection, HSCs overexpressing MEK or PI3K have elevated expression of p-ERK, p-Akt, cyclin D1, and collagen α1(I). However, the addition of AKF-PD dramatically reduced the amount of p-ERK, p-Akt, cyclin D1, and collagen α1(I). The decreased amount of cyclin D1 and collagen α1(I) can also help to confirm the roles of ERK and Akt in the regulation of proliferation of HSCs (Fig. 2, C and D).

Influence of serum ALT and AST by AKF-PD in vivo. As shown in Table 2, DMN-treated rats developed hepatic injury as evidenced by significantly higher serum AST and ALT, compared with that in normal rats. In DMN-treated rats, AKF-PD and PFD treatment ameliorated the increase of serum AST (P < 0.05) but did not change the levels of serum ALT (P > 0.05). Although the treatment effect of AKF-PD is better than PFD in AST and ALT amelioration (P < 0.05), there was no significant difference between these two treatment groups (P > 0.05). In the CCl4 model, there were no significant differences among the four groups (Table 3).

AKF-PD attenuates the histological injuries in vivo. For both model groups, Fig. 3, in H&E staining (Fig. 3, A and B), shows increases in portal areas of liver tissues expanded, liver cell derangement, more inflammatory cells such as monocytes, neutrophils infiltrated, collagen fibers, and fibroblasts. In Masson’s trichrome staining (Fig. 3, C and D), two model groups exhibited damaged hepatic lobules, decreased amount of hepatic sinusoids, and lobular architecture with thin bands of reticulin joining central areas. Collagen fibers accumulated between portal areas and pseudolobules increased in two model groups. Both AKF-PD and PFD administration dramatically attenuated liver pathological alterations and remarkably attenuated the increased collagen expression (P < 0.05). However, there was no significant difference between AKF-PD and PFD (P > 0.05).

AKF-PD attenuates HSC activation and proliferation in vivo. Quantitative real-time RT-PCR and Western blot demonstrated that the DMN and CCl4 induced significantly more abundant mRNA (Fig. 4, E and F, respectively; P < 0.05) and protein (Fig. 4, C and D, P < 0.05) expression of α-SMA, compared with that in normal rat liver tissues. The increased α-SMA expressions in DMN and CCl4 rats were significantly attenuated by AKF-PD and PFD at both the mRNA (Fig. 4, E and F, P < 0.05) and protein (Fig. 4, C and D, P < 0.05) levels. On the other hand, there was minimal staining of collagen α1(I) in normal liver tissues.
groups (Fig. 4, A and B). The increasing expression of collagen \( \alpha_{1}(I) \) in injured liver was considerably inhibited by AKF-PD and PFD treatment (Fig. 4, A and B). Moreover, these findings were confirmed by real-time RT-PCR and Western blot measurement for mRNA (Fig. 4, E and F, \( P < 0.05 \)) and protein (Fig. 4, C and D, \( P < 0.05 \)) expression in liver tissues. However, the differences of the inhibition effects of AKF-PD and PFD treatment on collagen \( \alpha_{1}(I) \) expression were not statistically significant (\( P > 0.05 \)).
AKF-PD inhibited ERK/MAPK and PI3K/Akt signaling pathways in rat fibrotic liver. We further detected the effects of AKF-PD on ERK/MAPK and PI3K/Akt signaling pathways in rat liver tissues. As shown in Fig. 5, DMN and CCl4 significantly induced phosphorylation of ERK, MEK, Akt, and p70S6k, compared with normal rat liver tissues (Fig. 5, P < 0.05). The increased phosphorylated ERK, MEK, Akt, and p70S6k expression in DMN and CCl4 rats was markedly attenuated by AKF-PD or PFD treatment (Fig. 5, P < 0.05). However, there were no significant differences of phosphorylated AKF-PD and PFD treatment groups in two models (P > 0.05, respectively).

DISCUSSION

Antifibrotic therapeutic strategies include inhibition of HSCs proliferation, stimulation of HSCs apoptosis, downregulation of collagen production, and promotion of collagen degradation (26). Searching for novel agents with inhibitory effects on HSC activation and proliferation has attracted much attention of researchers in the prevention of hepatic fibrogenesis.

Our results demonstrated the antifibrogenic effects of AKF-PD on hepatic fibrosis mediated through multiple mechanisms, including 1) inhibition of PDGF-BB-induced HSC proliferation and activation by decreasing cell viability, attenuating the expression of collagen α1(I) and α-SMA, causing G0/G1 phase cell cycle arrest, reducing expression of cyclin D1 and cyclin E and promoting expression of p27kip1; 2) downregulation of PDGF-BB induced MEK, ERK, Akt, and p70S6K phosphorylation in HSCs; and 3) alleviation of hepatic fibrosis by decreasing necroinflammatory score, semiquantitative score, and reducing expression of collagen α1(I) and α-SMA.

We demonstrated that AKF-PD potently inhibited the PDGF-stimulated proliferation of HSCs. In the regulation of cell proliferation, the G1/S transition has been emphasized as a vital event in cell cycle progression. The inhibition of arrested growth in the S phase of the cell cycle was accompanied by a decrease in cyclin D1 and CDK4. These effects were similar to those reported previously in human esophageal adenocarcinoma and glioma cells (27). Cyclin E is essential in activating CDK2. It has been reported that cyclin E expression increased in nonparenchymal cells of human fibrotic liver and that cyclin E-deficient mice develop milder liver fibrosis compared with wild-type mice after CCl4 administration (33). In cultured rat HSCs, expression of cyclin D1 and E correlates with cell proliferation (23). P27kip1 is a member of the Cip/Kip family of cyclin-dependent kinase inhibitors. It can bind to cyclin D1/CDK4, cyclin E/CDK2, and cyclin A/CDK2; inhibit their kinase activity; and negatively regulate the cell cycle (16). These changes in cell cycle proteins are likely to be important because the G1-to-S transition is regulated by cyclin D1 and E. Our studies demonstrated that AKF-PD induced growth arrest of HSCs is associated with G0/G1 arrest. This effect was associated with a decrease in cyclin D1 and cyclin E expression and an increase in p27kip1 expression.

PDGF-BB is a potent stimulator of HSC proliferation and activation in vitro (18). The PDGF signaling pathway is considered to be an effective target to inhibit the progress of hepatic fibrosis. PDGF stimulates the Ras/ERK signaling pathways during liver fibrogenesis (35). It is well known that the ERK/MAPK signaling pathway is involved in cell growth, differentiation, and migration of HSCs during liver fibrosis and cirrhosis (2, 43). What is more, the PI3K-Akt signaling pathway promotes cell proliferation and collagen gene expression by inhibition of apoptosis in HSCs (15). PI3K inhibition suppresses cell proliferation and type I collagen gene expression in activated HSCs (40). p70S6K regulates protein synthesis and proliferation (19). Application of the specific MEK inhibitor U0126 and the PI3K inhibitor LY294002 could reduce PDGF-induced activation of each targeting pathways so as to completely inhibit HSC activation and collagen expression (20). In the present study, we demonstrated that AKF-PD exerts its antifibrosis effects on HSC activation and collagen synthesis via a significant suppression on the phosphorylation of ERK, MEK, Akt, and p70S6K in vitro. We hypothesize that the outstanding beneficial effects of AKF-PD against hepatic fibrosis in vitro are at least partially attributable to the inhibition of ERK/MAPK and PI3K/Akt signaling pathways. AKF-PD significantly decreased the expression of collagen α1(I) and the cyclin D1-induced overexpression of MEK or PI3K, at least in part, by inhibiting ERK and Akt activation.

Previously, we proved that AKF-PD could reduce activation and proliferation of HSCs in vitro; we further demonstrated that AKF-PD also significantly blocks DMN- and CCl4-induced hepatic fibrosis in vivo, based on its inhibitory effects on collagen deposition, α-SMA expression, and other serum markers. Since HSC activation is responsible for the increased expression of α-SMA and upregulated synthesis and deposition of collagen I in the liver (18), our data showed that AKF-PD markedly reduced the expression of collagen α1(I) and collagen I at the mRNA and protein levels. Thus our data strongly suggest that AKF-PD attenuated liver fibrosis by reducing the amount of ECM by inhibiting HSC activation and proliferation. Furthermore, our study demonstrated that the antifibrotic effect of AKF-PD in vivo is also partially attributable to the inhibition of ERK/MAPK and PI3K/Akt signaling pathways. Compared with DMN rats, AKF-PD-treated rats had a considerable reduction in their serum AST levels, which has been shown to be associated with a significantly decreased necroinflammatory score. This suggested that AKF-PD may have an additional activity in attenuating inflammatory activity following hepatic injury and fibrosis. However, further studies are needed to better clarify this point. The same effects were not observed in the CCl4-induced liver fibrosis.

As a newly developed water-soluble pyridone agent, AKF-PD exerts similar influences as PFD on PDGF-BB-induced HSC activation. The structural difference between AKF-PD and PFD is that the hydro-at the meta-position of the benzene ring in PFD is replaced by fluoro- in AKF-PD. Compared with hydrogen, fluorine has a smaller atomic radius and larger electronegativity. These properties of the same fluorine will increase the stability and physiological activity of fluorine compounds. Moreover, fluorine compounds also have a high hydrophobicity, which can promote their absorption and transmission speed in vivo. In previous studies, AKF-PD has been proven to be a broad-spectrum antifibrosis drug, which could be used against renal fibrosis (34, 45, 48), pulmonary fibrosis (29), and myocardial fibrosis (8). Intriguingly, it is our
Fig. 5. AKF-PD reduces expression of phosphorylation of ERK1/2, MEK, Akt, and p70S6K in rat fibrotic livers. A and C: AKF-PD decreased the protein expressions of phosphorylation of ERK1/2 and MEK as shown by Western blot. B and D: AKF-PD decreased the protein expressions of phosphorylation of Akt and p70S6K as shown by Western blot. Data were expressed as means ± SD for each group (6 rats/group). *P < 0.05 compared with the normal group. #P < 0.05 compared with the DMN or CCl4 model group.
first time to demonstrate that AKF-PD also had effects on hepatic fibrosis.

In conclusion, our studies demonstrate that AKF-PD inhibits HSCs proliferation and activation by blocking cell-cycle progression in vitro by inhibition of ERK/MAPK and PI3K/Akt signaling pathways in HSCs. Furthermore, AKF-PD was able to prevent histological damage and reduce HSC activation and ECM accumulation during liver fibrosis in vivo. Our results provide valuable evidence for the potential clinical application of AKF-PD against hepatic fibrosis. As a new pyridine agent with dramatic hepatoprotective effects, AKF-PD deserves further studies.

GRANTS

This project was supported by the grants from National Natural Science Foundation of China (no. 30973579, 30873110, and 81370547) and Hunan Province Science and Technology Project (no. 2012FJ4070).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Y.P., H.Y., and N.W. conception and design of research; Y.P., Y.O., Y.Y., and L.L. performed experiments; Y.P. analyzed data; Y.P., G.H., and Z.W. interpreted results of experiments; Y.P. prepared figures; Y.P. drafted manuscript; Y.P. and H.S. edited and revised manuscript; Y.P., H.Y., and L.T. approved final version of manuscript.

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