Pirfenidone Inhibits Pancreatic Cancer Desmoplasia by Regulating Stellate Cells

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Running Title: Pirfenidone Inhibits Desmoplasia of Pancreatic Cancer

Keywords: pirfenidone; pancreatic cancer; desmoplasia; pancreatic stellate cells;
periostin.

Financial Support: This work was supported in part by the Ministry of Education,
Culture, Sports, Science and Technology of Japan (MEXT). (MEXT KAKENHI Grants
23390327, 24659613, 24390319, 23659654, 24390318, and 23659655).

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Conflict of interest: The authors have no conflicts of interest to declare.

Word Count: 4776 words

Total number of figures and tables: 6 figures and 1 table
Abstract

Pancreatic stellate cells (PSCs), which are implicated in desmoplasia in pancreatic cancer, enhance the malignancy of cancer cells and confer resistance to established treatments. We investigated whether the antifibrotic agent pirfenidone can suppress desmoplasia and exert anti-tumor effects against pancreatic cancer. Primary PSCs were established from pancreatic cancer tissue obtained during surgery. In vitro, pirfenidone inhibited the proliferation, invasiveness, and migration of PSCs in a dose-dependent manner. Although supernatants of untreated PSCs increased the proliferation, invasiveness, and migration of pancreatic cancer cells (PCCs), supernatants of pirfenidone-treated PSCs decreased these effects. Exposure to PCC supernatant increased the production of platelet-derived growth factor-A, hepatic growth factor, collagen type I, fibronectin, and periostin in PSCs, which was significantly reduced by pirfenidone. Mice were subcutaneously implanted with PCCs (SUIT-2 cells) and PSCs into the right flank and PCCs alone into the left flank. Oral administration of pirfenidone to these mice significantly reduced tumor growth of co-implanted PCCs and PSCs, but not of PCCs alone. Pirfenidone also decreased the proliferation of PSCs and the deposition of collagen type I and periostin in tumors. In mice with orthotopic tumors consisting of PCCs co-implanted with PSCs, pirfenidone suppressed tumor growth,
reduced the number of peritoneal disseminated nodules, and reduced the incidence of liver metastasis. Pirfenidone in combination with gemcitabine more effectively suppressed orthotopic tumor growth compared with pirfenidone or gemcitabine alone.

In conclusion, our findings indicate that pirfenidone is a promising antitumor agent for pancreatic cancer, owing to its suppression of desmoplasia through regulating PSCs.
Introduction

Pancreatic cancer is characterized by excessive desmoplasia, which contributes to the aggressive behaviors of pancreatic cancer and resistance to traditional therapies through tumor–stromal interactions (1). Pancreatic stellate cells (PSCs) have been identified in the interlobular areas and in peri-acinar lesions of the pancreas, and are responsible for generating extensive fibrosis in pancreatic diseases (2, 3). PSCs are activated by various stimuli and transform from quiescent cells to myofibroblast-like cells expressing α-smooth muscle actin (SMA) (4). In pancreatic cancer, PSCs are activated by tumor–stromal interactions, including by direct contact with pancreatic cancer cells (PCCs) and paracrine growth factors secreted from PCCs, including tumor growth factor β1 (TGF-β1), platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) (5-8). Activated PSCs also secrete various growth factors, including PDGF, bFGF, TGF-β1, and connective tissue growth factor (CTGF), to stimulate PCCs via paracrine pathways, and also activate PSCs via autocrine pathways. Activated PSCs can also produce abundant ECM and generate preferable microenvironments for PCCs. In vivo studies revealed that PCCs co-implanted with PSCs showed significant augmentation of tumor growth and very excessive desmoplasia (8-10). Excessive ECM deposition around tumor cells was
reported to inhibit the distribution and penetration of anti-cancer drugs by decreasing
tumor vascularity and constructing physical barriers (11, 12). These data suggest that
desmoplasia consisting of PSCs and ECM provides a significant contribution to the
malignant behaviors of cancer cells and resistance to established therapies, particularly
for pancreatic cancer. Therefore, new strategies targeting desmoplasia are needed to
improve the treatment paradigm of pancreatic cancer.

Pirfenidone (5-methyl-1-2-[1H]-pyridone; Shionogi&Co. Ltd., Osaka, Japan; MARNAC Inc., Dallas, TX) is a pyridone compound with therapeutic potential for idiopathic pulmonary fibrosis (IPF). Some double-blind, placebo-controlled phase II and phase III studies showed that pirfenidone has clinically meaningful effects and a favorable safety profile in patients with IPF (13, 14). IPF is a chronic, progressive, and often fatal inflammatory lung disease that results in the proliferation of fibroblasts and deposition of ECM in the interstitium and alveolar spaces of lung (15). In animal models of lung fibrosis, pirfenidone inhibited fibrosis and downregulated TGF-β, PDGF, and collagen synthesis (16-18). In liver fibrosis models, pirfenidone suppressed the proliferation of hepatic stellate cells, TGF-β mRNA expression, and the production of collagen type I (19, 20). These reports suggest that pirfenidone exerts anti-fibrotic effects by inhibiting fibroblasts and the production of TGF-β, PDGF, and collagen type I.
To date, however, no studies have analyzed the effects of pirfenidone on PSCs derived from pancreatic cancer tissues. Therefore, the aim of this study was to evaluate whether pirfenidone could suppress desmoplasia in pancreatic cancer. We assessed the effects of pirfenidone on human PSCs and tumor–stromal interactions between PCCs and PSCs. We also investigated the effects of pirfenidone on pancreatic cancer xenografts co-implanted with PCCs and PSCs.
Materials and Methods

Cell isolation and culture conditions

We established human PSCs from pancreatic cancer surgical specimens using the outgrowth method described by Bachem et al, and cells were maintained as previously described (3, 6). We confirmed that the PSCs exhibited a fibroblast-like morphology and were immunohistochemically positive for α-SMA and glial fibrillary acidic protein GFAP, markers of PSCs, and were negative for cytokeratin, an epithelial cell marker (Supplementary Figure 1). All of the established PSCs were used between passages 3 and 8. The use of pancreatic cancer surgical specimens was approved by the Ethics Committee of Kyushu University and was conducted according to the Ethical Guidelines for Human Genome/Gene Research enacted by the Japanese Government and the Helsinki Declaration. We also used two human pancreatic cell lines in this study; SUIT-2 and MIA PaCa-2 cells (Japan Health Science Research Resources Bank JCRB, Osaka, Japan). Both cell lines were propagated and frozen immediately after arrival. The cells revived from the frozen stock were used within less than 3 months. Cell lines were regularly authenticated and matched short tandem repeat DNA profiles of the original cell lines by JCRB. The cells were maintained as previously described (21).
Pirfenidone and gemcitabine preparation and treatment

For in vitro studies, pirfenidone was purchased from Sigma-Aldrich (St. Louis, MO). The powder was dissolved in sterile water at 60°C to a concentration of 10 mg/mL and frozen at –20°C until use. For in vivo studies, we purchased Pirespa® tablets from Shionogi & Co. (Osaka, Japan) and gemcitabine from Eli Lilly & Company (Indianapolis, IN). The tablets were crushed using a pestle and mortar, and dissolved in sterile water to a pirfenidone concentration of 200 mg/mL. Pirfenidone was orally administered to nude mice (BALB/c nu/nu; Kyudo Co, Saga, Japan) using a flexible gastric tube (KN-349, Natsume Sisakusyo CO LTD., Tokyo, Japan) at a dose of 500 mg/kg. Gemcitabine was dissolved in phosphate-buffered saline (PBS) and intravenously injected into nude mice at a dose of 40 mg/kg.

Production of conditioned media of PCCs and PSCs

We produced conditioned media of PSCs and PCCs using serum-free DMEM to exclude the effects of growth factors present in serum for the following experiments. Confluent (70%) SUIT-2 cells (SUIT-2-SN) and 100% confluent PSCs (PSC-SN) were cultured in serum-free DMEM for 24 or 48 h, respectively. In experiments designed to
analyze the effects of pirfenidone on PSCs, 100% confluent PSCs were cultured in serum-free DMEM containing 0.3 mg/mL pirfenidone for 48 h (pirfenidone-treated PSC-SN). The resulting conditioned media were used to stimulate PCCs and PSCs. Proteins secreted into the supernatant were also examined. The conditioned media were centrifuged for 10 min at 1000 rpm after collection, and stored at –80°C until use.

Cell proliferation assay

Cell proliferation was evaluated by measuring the fluorescence intensity of propidium iodide (PI), as previously described (22). PSCs and PCCs were seeded in 24-well tissue culture plates (Becton Dickinson Labware, Bedford, MA) at a density of 1×10^4 cells/well or 2×10^4 cells/well, respectively. The cells were then cultured in DMEM containing 10% FBS for 24 h. After confirmation of cellular adhesion to the plates, the medium was replaced with fresh DMEM containing 10% FBS plus pirfenidone at concentrations of 0, 0.1, 0.3, 0.5, or 1.0 mg/mL. To analyze cell proliferation following stimulation with conditioned medium, the medium was replaced with SUIT-2-SN, PSC-SN, or pirfenidone-treated PSC-SN containing 1% FBS plus 0 or 0.3 mg/mL pirfenidone. Cell proliferation was evaluated after culture for 72 h using PI assays. A separate well containing the same medium and the same concentration of
pirfenidone was used to provide a baseline PI signal as a control. The difference in intensity between each sample well and the control well was calculated.

**Matrigel invasion and migration assays**

Cell invasion was measured by counting the number of cells that invaded through Matrigel-coated transwell chambers with 8-μm pores (BD Biosciences, Franklin Lakes, NJ). The transwell inserts were coated with 20 μg/well Matrigel (BD Biosciences, Bedford, MA). PSCs (3×10⁴ cells) and PCCs (5×10⁴ cells) were resuspended in 250 μL of DMEM containing 10% FBS plus pirfenidone at concentrations of 0, 0.1, or 0.3 mg/mL, and were placed in the upper chamber. The upper chamber was then placed in a 24-well culture dish containing 750 μL of the same medium with the same concentration of pirfenidone as used in the upper chamber. The migration of PSCs and PCCs was assessed using uncoated transwell inserts. Separate batches of cells were cultured with SUIT-2-SN, PSC-SN, or pirfenidone-treated PSC-SN supplemented with 0 or 0.3 mg/mL pirfenidone to assess the effects of PSC and PCC supernatant stimulation on migration and invasion. After incubation for 48 h for invasion or 24 h for migration, the invaded and migrated cells were fixed with 70% ethanol, stained with H&E, and counted in five random fields at a magnification of ×100 under a light
microscope. The results are expressed as the mean number of invaded cells per field.

Each experiment was carried out in triplicate wells and repeated three times.

Quantitative RT-PCR

Quantitative RT-PCR (qRT-PCR) was performed as previously described (23). We designed specific primers for α-SMA, periostin, FGF-2, and 18S rRNA using Primer 3 software (http://primer3.sourceforge.net/). The primers used for collagen type I, fibronectin, TGF-β1, PDGF-A, PDGF-B, CTGF, HGF, VEGF and 18S rRNA were purchased from Takara Bio Inc (Tokyo, Japan). The primer sequences are listed in Supplementary Table 1.

Western blotting analysis

Protein was extracted from PSCs using PRO-PREP (iNtRON biotechnology, Seongnam, Korea) according to the manufacturer’s instructions. The supernatants were concentrated using an Amicon Ultra-10 filter unit (Millipore, Billerica, MA) at 4000 rpm for 60 min, and Halt™ Protease and Phosphatase Inhibitor Single-Use Cocktail (Thermo Scientific, Rockford, IL) was added to each sample. The supernatants were stored at −80°C until use. Cell lysate protein (20 µg) and supernatant (30 µg) were
fractionated on 4–15% Mini-PROTEAN® TGX™ Precast Gel (Bio-Rad Laboratories) and transferred to Trans-Blot® Turbo™ Mini PVDF Transfer Packs (Bio-Rad Laboratories) using a Trans-Blot® Turbo™ Transfer Starter System (Bio-Rad Laboratories). The membrane was incubated overnight at 4°C with anti-TGF-β1 (sc-146; 1:200), anti-PDGF-A (sc-128; 1:200), anti-PDGF-B (sc-127; 1:200), anti-CTGF (sc-101586; 1:200), anti-hepatocyte growth factor (HGF) (sc-13087; 1:200), anti-VEGF (sc-152; 1:200), anti-FGF-2 (sc-79; 1:200), anti-collagen type I (sc-8783; 1:1000), anti-fibronectin (sc-6952; 1:1000), anti-periostin (sc-67233; 1:1000) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), anti-α-SMA (1:500; DAKO, Glostrup, Denmark), or anti-α-tubulin (05-829; 1:1000; Millipore, Billerica, MA) antibodies, and then probed with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology). Immunoblots were detected by enhanced chemiluminescence with a ChemiDoc XRS System (Bio-Rad Laboratories) and the density of each band was measured using Quantity One software (Bio-Rad Laboratories).

**In vivo experiments**

To analyze the effects of pirfenidone on PSCs *in vivo*, SUIT-2 cells (1×10⁶) suspended in 100 μL DMEM and PSCs (1×10⁶) and SUIT-2 cells (1×10⁶) in 100 μL
DMEM were subcutaneously transplanted into the left limb and the right limb of 6-week-old female nude mice, respectively. One week after implantation, we randomly divided the mice into two groups (15 mice/group) and orally administered them with either 500 mg/kg pirfenidone or sterile water as a control, daily for 35 days. We also analyzed the effects of pirfenidone alone or in combination with gemcitabine on orthotopic implantation models. Nude mice were orthotopically implanted with SUIT-2 cells (5×10^5) and PSCs (5×10^5) in 50 μL DMEM into the tail of the pancreas. One week after implantation, the mice were randomized into four groups; control group, pirfenidone group, gemcitabine group and pirfenidone plus gemcitabine group. We orally administrated 500 mg/kg pirfenidone or sterile water as a control daily and injected gemcitabine at a dose of 40 mg/kg or PBS as a control via the tail vein once weekly for 35 days. Twelve mice were used in each group. The mice were sacrificed on day 35, and all orthotopic tumors were excised and weighed. Tumor volume was calculated using the following formula: π/6 × (L × W × W), where L = the largest tumor diameter and W = the smallest tumor diameter. The presence of peritoneal dissemination was evaluated by counting the number of nodules >1 mm in size disseminated within the peritoneum. The presence of liver metastasis was evaluated by counting the number of nodules >1 mm in size on the surface of the liver. All mouse experiments were performed on April 6, 2017. © 2013 American Association for Cancer Research.
approved by the Ethics Committee of Kyushu University.

Immunohistochemistry

In vivo tumor tissues were immunostained as previously described (23) with following primary antibodies: mouse monoclonal anti-α-SMA (1:500; DAKO), rabbit polyclonal anti-periostin (sc-67233; 1:500; Santa Cruz Biotechnology), mouse monoclonal anti-cytokeratin 19 (sc-376126; 1:100), and rabbit polyclonal anti-proliferating cell nuclear antigen (PCNA) (ab2426; 1:500; abcam). The α-SMA positive cells were counted in three fields at a magnification of ×100 under a light microscope. The PCNA index (%) was calculated as (PCNA positive tumor cells / all tumor cells) × 100 in three fields at a magnification of ×40 under a light microscope.

Sirius red staining and measurements

Sections were stained using a Picrosirius Red Staining Kit (Polysciences Inc., Warrington, PA) according to manufacturer’s instructions. The Sirius red stained area was measured using Adobe Photoshop CS (Adobe Systems Incorporated, Tokyo, Japan) by selecting stained fibers in three fields at a magnification of ×100 under a light microscope.
2 **Statistical analysis**

Results are expressed as means ± SD. Comparisons between groups were evaluated by one-way ANOVA test followed by the Tukey–Kramer multiple comparisons test. Values of $P < 0.05$ were considered statistically significant in all analyses. All statistical analyses were performed using JMP 8.01 software (SAS Institute, Cary, NC).
Results

Pirfenidone decreases the proliferation, invasion, and migration of PSCs

Pirfenidone inhibited the proliferation of PSCs in a dose-dependent manner \( (P < 0.05, \text{Figure 1A}) \). Although pirfenidone also inhibited PCCs, SUIT-2 cells, and MIA PaCa-2 cells in a dose-dependent manner, low concentrations of pirfenidone, particularly 0.3 mg/mL, showed greater inhibitory activity on PSCs than on PCCs.

Pirfenidone significantly decreased the invasion and migration of PSCs at concentrations of 0.1 and 0.3 mg/mL, but did not affect PCCs \( (P < 0.05, \text{Figure 1B–E}) \).

These data indicate that PSCs are more strongly affected by pirfenidone than are PCCs, and suggest that pirfenidone exerts anti-tumor effects on pancreatic cancer by inhibiting the interaction between PSCs and PCCs. To investigate the specific effects of pirfenidone on PSCs, we used 0.3 mg/mL pirfenidone in the following \textit{in vitro} experiments.

Pirfenidone suppresses PSCs by disrupting tumor–stromal interactions

It was reported that the biological activities of PSCs, such as proliferation and invasion, are potentiated by tumor–stromal interactions. Therefore, we investigated the effects of pirfenidone on the tumor–stromal interactions of PSCs using SUIT-2-SN.
Stimulation with SUIT-2-SN significantly increased the proliferation ($P < 0.05$, Figure 2A), invasiveness ($P < 0.05$, Figure 2B–C), and migration ($P < 0.05$, Figure 2D–E) of PSCs. Pirfenidone significantly decreased these effects of SUIT-2-SN on the proliferation, invasiveness, and migration of PSCs (all, $P < 0.05$). These results suggest that pirfenidone suppresses PSCs by disrupting the tumor–stromal interactions.

Pirfenidone inhibits the stimulatory effects of PSC supernatants on PCCs

To determine the effects of pirfenidone on the tumor–stromal interactions between PSCs and PCCs, we prepared PSC supernatant (PSC-SN), PSC-SN supplemented with pirfenidone (PSC-SN+pirfenidone), and pirfenidone-treated PSC-SN. PSC-SN increased the proliferation of PCCs (SUIT-2 cells and MIA PaCa-2 cells, $P < 0.05$, Figure 3A). By contrast, pirfenidone-treated PSC-SN did not enhance the proliferation of PCCs compared with PSC-SN and PSC-SN+pirfenidone. Similarly, pirfenidone-treated PSC-SN decreased the ability of PSCs to enhance the invasiveness and migration of PCCs ($P < 0.05$, Figure 3B–E). These findings suggest that pirfenidone may suppress the production of factors involved in tumor–stromal interactions in PSC-SN.
Pirfenidone decreases the mRNA and protein expression of genes involved in tumor–stromal interactions in PSCs

To assess the mechanism by which pirfenidone suppresses tumor–stromal interactions, we determined the expression of the major factors involved in these interactions by qRT-PCR and western blotting of whole-cell lysates and PSC-SN. PSCs derived from pancreatic cancer tissues secreted PDGF-A, HGF, FGF-2, CTGF, and ECM proteins, including collagen type I, fibronectin, and periostin. Treatment with pirfenidone significantly reduced the protein and mRNA expression of PDGF-A, HGF, and periostin in PSCs (Figure 4). Next, we analyzed the effects of SUIT-2-SN and pirfenidone on PSC protein production and tumor–stromal interactions. Exposure to SUIT-2-SN considerably enhanced the synthesis and secretion of PDGF-A, HGF, collagen type I, fibronectin, and periostin, which were inhibited by pirfenidone. 

Pirfenidone also decreased mRNA, but not protein, α-SMA expression in PSCs. These findings indicate that pirfenidone disrupts tumor–stromal interactions by suppressing the synthesis and secretion of factors involved in these interactions, particularly PDGF-A, HGF, periostin, collagen type I, and fibronectin.
Pirfenidone inhibits subcutaneous tumor formation *in vivo* in mice co-transplanted with SUIT-2 cells and PSCs

To examine the effects of pirfenidone on PCCs and PSCs *in vivo*, we transplanted nude mice with SUIT-2 cells and PSCs into the right flank and SUIT-2 cells alone into the left flank (Figure 5A). One week after implantation, we started oral administration of pirfenidone daily for 5 weeks, while a control group was administered with sterile water. In the control group, tumor growth was much greater with co-implantation of SUIT-2 cells and PSCs compared with SUIT-2 cells alone (*P* < 0.05, Figure 5A–B). The tumors consisting of SUIT-2 cells co-implanted with PSCs showed marked desmoplasia with larger Sirius red positive areas and more α-SMA-positive cells compared with the tumors consisting of SUIT-2 cells alone (*P* < 0.05, Figure 5C–E, Supplementary Figure 2). Treatment with pirfenidone significantly suppressed the growth of tumors consisting of SUIT-2 cells co-implanted with PSCs compared with that in the control group (*P* < 0.05, Figure 5B). Pirfenidone also reduced the Sirius red positive area and the number of α-SMA-positive cells in tumors consisting of SUIT-2 cells co-implanted with PSCs (*P* < 0.05, Figure 5C–E). By contrast, tumor growth of SUIT-2 cells alone was not significantly different between the pirfenidone-treated and control groups. Pirfenidone significantly reduced the PCNA index in tumors consisting of SUIT-2 cells.
co-implanted with PSCs ($P < 0.05$, Figure 5F). However, the PCNA index was not significantly different between the control group and the pirfenidone group in tumors consisting of SUIT-2 cells alone ($P = 0.98$, Figure 5F). Periostin expression was only detected immunohistochemically in the control tumors consisting of SUIT-2 cells co-implanted with PSCs (Figure 5C). These findings suggest that, at the concentration used in this experiment, pirfenidone indirectly, rather than directly, targets pancreatic cancer cells (i.e., SUIT-2 cells) by suppressing the growth/proliferation of PSCs and by suppressing ECM deposition.

The inhibitory effects of pirfenidone on the growth and metastasis of tumors consisting of SUIT-2 cells co-implanted with PSCs are enhanced by combination treatment with gemcitabine

We next investigated the effects of pirfenidone alone or in combination with gemcitabine on growth and metastasis of orthotopic tumors in mice. The administration of pirfenidone and gemcitabine was started 1 week after tumor implantation. Pirfenidone significantly inhibited the growth of orthotopic tumors consisting of SUIT-2 cells co-implanted with PSCs, as observed in mice with subcutaneous tumors ($P < 0.05$, Figure 6A–B, Supplementary Figure 3B). Although gemcitabine alone inhibited the
growth of orthotopic tumors consisting of SUIT-2 cells co-implanted with PSCs, the suppressive effects were significantly greater when gemcitabine was administered in combination with pirfenidone ($P < 0.05$). Immunohistological studies showed that pirfenidone significantly suppressed the desmoplastic reactions in orthotopic tumors, whereas gemcitabine did not ($P < 0.05$, Figure 6C–D and Supplementary Figure 3A). Although the orthotopic mice showed evidence of peritoneal dissemination and liver metastasis, pirfenidone decreased the number of peritoneal disseminated nodules and the incidence of liver metastasis. These suppressive effects of pirfenidone where significantly enhanced by combination with gemcitabine ($P < 0.05$, Figure 6E–F, Supplementary Figure 3C and Table 1). These findings suggest that pirfenidone can suppress tumor growth and metastasis of orthotopic tumors consisting of SUIT-2 cells co-implanted with PSCs, and that combination treatment with gemcitabine has synergistic effects on these tumors.
Discussion

We showed that PSCs increased the biological activities and augmented the production of PDGF-A, HGF, collagen type I, fibronectin, and periostin through tumor–stromal interactions with PCCs. In addition, we found that pirfenidone strongly suppressed the activities of PSCs by disrupting the tumor–stromal interactions, and significantly inhibited the production of these factors. These factors play critical roles in the tumor–stromal interactions in pancreatic cancer. PDGF was reported to induce the proliferation and migration of PSCs through paracrine and autocrine pathways (24-27).

We previously reported that irradiated stromal fibroblasts increased the invasiveness of PCCs through the HGF–c-Met pathway (21). Ide et al (28) reported that HGF secreted from fibroblasts under hypoxic conditions increased the invasiveness of PCCs. ECM components, including collagen type I, fibronectin, and periostin, were reported to augment the proliferation and migration of PCCs, and to enhance their resistance to anti-cancer drugs (29-33). Consistent with these reports, PSC-SN greatly increased the malignant potential of PCCs. Meanwhile pirfenidone-treated PSC-SN decreased the ability of PSCs to enhance the invasiveness and migration of PCCs in our study. The anti-tumor effects of pirfenidone may involve suppression of the biological activation of PSCs and attenuation of the tumor–stromal interactions by suppressing the production...
of PDGF-A, HGF, collagen type I, fibronectin, and periostin.

For the first time, we found that pirfenidone significantly suppressed the production of periostin by PSCs. Periostin, originally called osteoblast-specific factor 2, is a 93.3 kDa secreted protein involved in the recruitment and attachment of osteoblast precursors in the periosteum (34, 35). Periostin is predominantly expressed in collagen-rich fibrous connective tissues in several organs, where it regulates collagen fibrillogenesis (36). Recent studies have revealed that periostin is overexpressed in the stroma of several types of cancer, including ovarian cancer, non-small cell lung cancer, colon cancer, prostate cancer, and pancreatic cancer (37). Periostin promoted tumor growth and metastasis, and was associated with poor prognosis of patients with these cancers. Erkan et al (33) reported that, once PSCs are activated by PCCs, the PSCs remained active via an autocrine periostin loop, and produced excessive ECM creating a tumor-supportive microenvironment. Recently, periostin was reported to maintain cancer stem cells by increasing Wnt signaling and facilitate metastatic colonization (38). Zhu et al reported that a neutralizing monoclonal antibody to periostin could inhibit ovarian tumor growth and metastasis in mice (39). These findings indicate that periostin provides a significant contribution to fibrogenesis and the malignant behaviors of cancer cells. These findings also suggest that the anti-tumor effects of pirfenidone are partly
mediated by inhibition of periostin production. Therefore, inhibition of periostin production may be a new mechanism explaining the anti-fibrotic effects of pirfenidone.

In this study, PSCs significantly increased the growth of a pancreatic cancer xenograft and induced excessive desmoplasia in tumor tissues, as previously reported (6, 8-10). We revealed that oral administration of pirfenidone significantly suppressed in vivo tumor growth, but only when SUIT-2 cells were co-implanted with PSCs. Pirfenidone did not affect tumor growth of SUIT-2 cells alone. Histologically, the tumors excised from pirfenidone-treated nude mice showed a considerable reduction in the number of α-SMA-positive cells (i.e., PSCs), Sirius red positive area, and periostin expression. These findings suggest that the anti-tumor effects of pirfenidone in vivo include suppressed PSC proliferation and the production of stromal components. These effects of pirfenidone might contribute to the decreased rates of peritoneal dissemination and liver metastasis of orthotopic tumors consisting of SUIT-2 cells co-implanted with PSCs by suppressing the tumor–stromal interactions. Extensive deposition of ECM components forms a barrier that prevents the penetration of anti-tumor drugs, and generates a hypovascular microenvironment that limits drug delivery (11). Therefore, researchers have started to focus on pharmacological agents that can suppress stromal formation. Olive et al (12) reported that a hedgehog signaling
inhibitor improved the delivery of gemcitabine to PCCs by decreasing fibrotic
deposition and increasing intratumoral density in KrasLSL.G12D/+, p53R172H/+, PdxCretg/+ mice, which are engineered to spontaneously generate pancreatic cancers resembling those in humans. Although our orthotopic pancreatic cancer mice models did not show strong resistance to gemcitabine, the combination of pirfenidone and gemcitabine significantly suppressed the growth of SUIT-2 cells co-implanted with PSCs compared with treatment with gemcitabine alone. Histological examination of the orthotopic tumors revealed that pirfenidone, but not gemcitabine, decreased desmoplastic reactions, as observed in subcutaneous tumors. These results suggest that co-administration of gemcitabine, which targets cancer cells, and pirfenidone, which targets desmoplastic activity, have synergistic anti-tumor effects. Therefore, this combination may represent an ideal treatment strategy for pancreatic cancer associated with excessive desmoplasia.

In conclusion, the present study revealed that pirfenidone inhibits the biological activity of PSCs. Pirfenidone also inhibited the production of PDGF-A, HGF, collagen type I, fibronectin, and peristin, factors that play important roles in tumor–stromal interactions in pancreatic cancer. Pirfenidone and gemcitabine had synergistic anti-tumor effects on pancreatic cancer xenografts. Therefore, combining pirfenidone
with traditional anti-cancer drugs such as gemcitabine may offer a promising treatment strategy for pancreatic cancer.

Acknowledgments

We thank M. Ohmori and E. Manabe (Department of Surgery and Oncology, Kyushu University Hospital) for their expert technical assistance.

Grant-Support: This work was supported in part by the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT). (MEXT KAKENHI Grants 23390327, 24659613, 24390319, 23659654, 24390318, and 23659655).
1 References


Figure Legends

Figure 1. (A) Effects of pirfenidone on the proliferation of PSCs (PSC1-3 cells) and PCCs (SUIT-2 and MIA PaCa-2 cells). Pirfenidone dose-dependently inhibited the proliferation of PSCs and PCCs. Low concentrations of pirfenidone, particularly 0.3 mg/mL, showed stronger inhibitory effects on PSCs than PCCs. *P < 0.05 for PSCs vs PCCs treated with 0.3 mg/mL pirfenidone. (B) Pirfenidone dose-dependently decreased the invasiveness of PSCs, but not of SUIT-2 or MIA PaCa-2 cells. (C) Representative photomicrographs of invading PSCs and PCCs. (D) Pirfenidone dose-dependently decreased the migration of PSCs but not of SUIT-2 or MIA PaCa-2 cells. (E) Representative photomicrographs of migrating PSCs and PCCs. *P < 0.05 (H&E; original magnification, ×100; scale bars, 200 µm).

Figure 2. Effects of pirfenidone on PSCs stimulated with SUIT-2-SN. (A) Exposure to SUIT-2-SN enhanced the proliferation of PSCs compared with control cells. Pirfenidone also suppressed the effects of SUIT-2-SN on PSC proliferation. (B and D) PSC invasiveness (B) and migration (D) were significantly increased by SUIT-2-SN, and were suppressed by pirfenidone. (C and E) Representative photomicrographs of invading (C) and migrating (E) PSCs. (H&E; original magnification, ×100; scale bars,
Figure 3. Effects of pirfenidone on the tumor–stromal interactions between PSCs and PCCs. (A) PSC-SN increased the proliferation of SUIT-2 and MIA PaCa-2 cells compared with control cells. Pirfenidone-treated PSC-SN (0.3 mg/mL pirfenidone) was unable to stimulate PCC proliferation. (B and D) PSC-SN significantly enhanced invasion (B) and migration (D) of SUIT-2 and MIA PaCa-2 cells. Pirfenidone-treated PSC-SN reduced the activation of PCCs compared with PSC-SN and PSC-SN+pirfenidone (0.3 mg/mL pirfenidone). *P < 0.05. (C and E) Representative photomicrographs of invading (C) and migrating (E) PCCs (SUIT-2 and MIA PaCa-2 cells) (H&E; original magnification, ×100; scale bars, 200 µm).

Figure 4. Effects of pirfenidone on gene and protein expression levels of growth factors and ECM proteins associated with tumor–stromal interactions. PSCs were untreated or treated with pirfenidone, SUIT-2-SN, or SUIT-2-SN+pirfenidone. mRNA expression levels are normalized to 18S rRNA expression and are presented as the fold-change in gene expression relative to control PSCs. Protein expression of growth factors was analyzed by western blotting of the supernatants of PSCs. Western blotting analysis of
ECM proteins and α-SMA was conducted using whole-cell lysates and the supernatant of PSCs. α-tubulin was used as a control to confirm equal protein loading in each well for analysis of cell lysates. The densitometric data are presented as the fold-change relative to control PSCs.

Figure 5. Anti-tumor effects of pirfenidone on subcutaneous tumor formation in vivo.

SUIT-2 cells plus PSCs (right flank) or SUIT-2 cells alone (left flank) were subcutaneously implanted into nude mice (n = 15 mice/group). From 1 week after implantation, the mice were orally administered with pirfenidone daily for 5 weeks. (A) Representative micrographs after 5 weeks of treatment. (B) Time-course of tumor growth. (C) Histological evaluation of desmoplastic activity. Periostin was expressed in tumors consisting of SUIT-2 cells co-implanted with PSCs in the control group, but not in pirfenidone-treated tumors. (D) Sirius red staining was more intense in tumors consisting of SUIT-2 cells co-implanted with PSCs than in tumors consisting of SUIT-2 cells alone. Pirfenidone significantly decreased the Sirius red positive area in tumors consisting of SUIT-2 cells co-implanted with PSCs. (E) The number of α-SMA positive cells was higher in tumors consisting of SUIT-2 cells co-implanted with PSCs compared with tumors consisting of SUIT-2 cells in the control group. Pirfenidone decreased the
number of α-SMA-positive cells in tumors consisting of SUIT-2 cells co-implanted with
PSCs. The α-SMA-positive cells in tumors consisting of SUIT-2 cells alone probably
originated from the host, and their number was decreased by pirfenidone. (F) The
PCNA index (%) was decreased by pirfenidone in tumors consisting of SUIT-2 cells
co-implanted with PSCs but not in tumors consisting of SUIT-2 cells alone. *P < 0.05.

Figure 6. Anti-tumor effects of pirfenidone and/or gemcitabine on orthotopic pancreatic
cancer mouse models. SUIT-2 cells and PSCs were co-implanted into the tail of the
pancreas of nude mice (n = 12 mice/group). Starting 1 week after orthotopic
implantation of SUIT-2 cells and PSCs, mice were intravenously injected with
gemcitabine weekly and orally administered with pirfenidone daily for 5 weeks. (A)
Representative photographs showing the effects of gemcitabine in combination with
pirfenidone. (B) Pirfenidone in combination with gemcitabine significantly suppressed
the growth of tumors consisting of SUIT-2 cells co-implanted with PSCs. The Sirius red
positive area (C) and the number of α-SMA positive cells (D) were decreased in mice
treated with pirfenidone alone, but not in mice treated with gemcitabine alone. (E)
Representative photographs showing metastasis in the peritoneal cavity of mice after the
indicated treatments (arrowhead). (F) Pirfenidone decreased the number of disseminated
visible nodules of >1 mm in size, and this suppressive effect was enhanced by combination treatment with gemcitabine. *$P < 0.05$. 
**Table 1.** Effects of pirfenidone and/or gemcitabine on orthotopic mouse models of pancreatic cancer

<table>
<thead>
<tr>
<th>Group</th>
<th>Incidence of macroscopic tumors</th>
<th>Tumor weight (mean±SD) (g)</th>
<th>Body weight (mean±SD) (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pancreatic tumor</td>
<td>Peritoneal dissemination</td>
<td>Liver metastasis</td>
</tr>
<tr>
<td>Control</td>
<td>12/12</td>
<td>9/12</td>
<td>6/12</td>
</tr>
<tr>
<td>Pirfenidone</td>
<td>12/12</td>
<td>3/12</td>
<td>1/12</td>
</tr>
<tr>
<td>Gemcitabine</td>
<td>12/12</td>
<td>8/12</td>
<td>3/12</td>
</tr>
<tr>
<td>Pirfenidone plus gemcitabine</td>
<td>12/12</td>
<td>1/12</td>
<td>0/12</td>
</tr>
</tbody>
</table>
**Figure 1**

A. Cell survival rate as a function of pirfenidone concentration for different cell lines: PSCs1, PSCs2, PSCs3, SUIT-2, and MIA PaCa-2.

B. Number of invaded cells with different treatments: control, pirfenidone 0.1 mg/mL, and pirfenidone 0.3 mg/mL.

C. Representative images of invaded cells for each treatment group.

D. Number of migrated cells with different treatments: control, pirfenidone 0.1 mg/mL, and pirfenidone 0.3 mg/mL.

E. Representative images of migrated cells for each treatment group.
Figure 2

A

B

C

D

E
Figure 3

A) Graph showing PI intensity with control, PSCs SN, PSCs SN+pirfenidone, and pirfenidone-treated PSCs SN for SUIT-2 and MIA PaCa-2 cells.

B) Graph showing the number of invaded cells with control, PSCs SN, PSCs SN+pirfenidone, and pirfenidone-treated PSCs SN for SUIT-2 and MIA PaCa-2 cells.

C) Micrographs of control, PSCs SN, PSCs SN+pirfenidone, and pirfenidone-treated PSCs SN for SUIT-2 and MIA PaCa-2 cells.

D) Graph showing the number of migrated cells with control, PSCs SN, PSCs SN+pirfenidone, and pirfenidone-treated PSCs SN for SUIT-2 and MIA PaCa-2 cells.

E) Micrographs of control, PSCs SN, PSCs SN+pirfenidone, and pirfenidone-treated PSCs SN for SUIT-2 and MIA PaCa-2 cells.
Figure 4

[Bar charts showing mRNA and Density Ratio for PDGF-A, PDGF-B, TGF-β1, FGF-2, HGF, CTGF, VEGF, peristin, collagen type I, fibronectin, and α-SMA. The x-axis represents different conditions, and the y-axis represents the ratio of mRNA or density.]

Legend:
- mRNA
- supernatant
- whole cell lysate

- PDGF-A
- PDGF-B
- TGF-β1
- FGF-2
- HGF
- CTGF
- VEGF
- peristin
- collagen type I
- fibronectin
- α-SMA
Figure 5

A. Left tumor: SUIT-2 cells
Right tumor: SUIT-2 cells + PSCs

B. Subcutaneous tumor volume (mm³)
Day 7 Implantation
Day 0 Administration starts

C. HE, cytokeratin 19, sirius red, α-SMA, periostin, PCNA

D. Percentage sirius red positive area/×100 field (%)

E. Number of α-SMA positive cells/×100

F. PCNA index (%)
Figure 6

A

B

C

D

E

F

Orthotopic tumor volume (mm$^3$)

Percentage Sirius red positive area/×100 field (%)

Number of α-SMA positive cells (cells/×100)

Number of disseminated visible nodules >1mm

control
pirfenidone
gemcitabine
pirfenidone+gemcitabine
control
pirfenidone
gemcitabine
pirfenidone+gemcitabine
control
pirfenidone
gemcitabine
pirfenidone+gemcitabine
control
pirfenidone
gemcitabine
pirfenidone+gemcitabine
control
pirfenidone
gemcitabine
pirfenidone+gemcitabine
Pirfenidone Inhibits Pancreatic Cancer Desmoplasia by Regulating Stellate Cells

Shingo Kozono, Kenoki Ohuchida, Daiki Eguchi, et al.

Cancer Res  Published OnlineFirst January 24, 2013.