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ORIGINAL ARTICLE

Distinct antifibrogenic effects of erlotinib, sunitinib and sorafenib on rat pancreatic stellate cells

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Abstract

AIM: To study if three clinically available small molecule kinase inhibitors (SMI), erlotinib, sunitinib and sorafenib, exert antifibrogenic effects on pancreatic stellate cells (PSC) and analyze the basis of their action.

METHODS: Cultured rat PSC were exposed to SMI. Cell proliferation and viability were assessed employing 5-bromo-2'-deoxyuridine incorporation assay and flow cytometry, respectively. 2-Deoxy-2-[^{18}F] fluoroglucose (^{18}F-FDG) uptake was measured to study metabolic activity. Exhibition of the myofibroblastic PSC phenotype was monitored by immunofluorescence analysis of α-smooth muscle actin (α-SMA) expression. Levels of mRNA were determined by real-time PCR, while protein expression and phosphorylation were analyzed by immunoblotting. Transforming growth factor-β1 (TGF-β1) levels in culture supernatants were quantified by ELISA.

RESULTS: All three SMI inhibited cell proliferation and ^{18}F-FDG uptake in a dose-dependent manner and without significant cytotoxic effects. Furthermore, additive effects of the drugs were observed. Immunoblot analysis showed that sorafenib and sunitib, but not erlotinib, efficiently blocked activation of the AKT pathway, while all three drugs displayed little effect on phosphorylation of ERK1/2. Cells treated with sorafenib or sunitinib expressed less interleukin-6 mRNA as well as less collagen type 1 mRNA and protein. Sorafenib was the only drug that also upregulated the expression of matrix metalloproteinase-2 and reduced the secretion of TGF-β1 protein. All three drugs showed insignificant or discordant effects on the mRNA and protein levels of α-SMA.

CONCLUSION: The tested SMI, especially sorafenib, exert inhibitory effects on activated PSC, which should be further evaluated in preclinical studies.

Key words: Pancreatic stellate cell; Fibrosis; Erlotinib; Sunitinib; Sorafenib

Core tip: There are no specific therapies available to treat pancreatic fibrosis, a key feature of chronic pancreatitis and pancreatic cancer. Here we show that three clinically available small molecule kinase inhibitors (SMI), erlotinib, sunitinib and sorafenib, exert antifibrogenic effects *in vitro* by inhibiting key functions of rat pancreatic stellate cells (PSC), the main source of extracellular matrix proteins in the diseased pancreas. Furthermore, additive effects of the drugs were observed. Our studies also provide insight into molecular mechanisms of SMI action in PSC. We suggest that the antifibrotic efficiency of SMI, especially sorafenib, should be further evaluated in preclinical studies.

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INTRODUCTION

Pancreatic stellate cells (PSC) are key players in pancreatic wound healing and fibrosis[1]. In response to pancreatic injury, they transform from a quiescent into an activated phenotype that secretes large amounts of extracellular matrix (ECM) proteins. Furthermore, the cells, which form only 4%-7% of all parenchymal cells in the healthy pancreas, start to proliferate and to replace the organotypic tissue[1-3].

Under persistent pathological conditions, specifically in chronic pancreatitis (CP) and pancreatic cancer (PC), dysregulated activation of PSC and excessive deposition of ECM result in organ fibrosis[4,5]. Pancreatic fibrosis, in turn, contributes to the development of an exocrine and endocrine insufficiency of the gland[6]. Moreover, recent studies suggest that the extended stroma reaction favours progression of PC by various mechanisms[7-9]. Thus, the fibrotic wall surrounding the tumor cells provides a barrier against chemotherapeutics and immune cells. Stroma cells are also a rich source of cytokines, chemokines and growth factors that mediate chemoresistance, suppress apoptosis and stimulate proliferation of the tumor cells[9-13]. Since PC cells, on the other hand, enhance PSC activation by secreting profibrogenic mediators [*e.g.*, the mitogen platelet-derived growth factor (PDGF) and transforming growth factor-β1 (TGF-β1), the key stimulator of ECM synthesis[10,14]], a vicious cycle of PSC activation, enhanced ECM synthesis and accelerated tumor growth may establish. In analogy, mutual paracrine effects of PSC and inflammatory cells contribute to persistent PSC activation in CP[15], which also represents a main risk factor of the tumor disease.

In recent years, attempts have been made to inhibit pancreatic fibrogenesis by interfering with PSC activation, suppressing effector functions of activated PSC, and/or terminating PSC activation through the induction of apoptosis or cellular senescence. Although some promising substances with antifibrotic activity could be identified (*e.g.*, ligands of peroxisome proliferator-activated receptor γ[16,17], interferon-γ[18] and inhibitors of histone deacetylases[19]), there is still no specific antifibrotic therapy for clinical application available yet.

We and others have previously shown that two intracellular pathways, Ras-Raf-MEK (mitogen-activated protein kinase kinase)-ERK (extracellular signal-regulated kinase) and phosphatidylinositol (PI) 3-kinase/AKT (protein kinase B), are crucially involved in PSC activation by growth factors such as PDGF[20-22]. Most recently, various new small molecule kinase inhibitors (SMI) have become available that were developed for the specific treatment of different human malignancies. Many of these inhibitors (1) target growth factor receptors upstream of Ras-Raf-MEK-ERK and PI 3-kinase/AKT; (2) directly inhibit kinases of these pathways; or (3) do both[23-25]. The antifibrotic activity of these substances, however, has not been systematically analyzed so far.

Here, we have studied the biological and molecular effects of three clinically available SMI, erlotinib, sorafenib and sunitinib, on activated PSC. One of the three drugs, the epidermal growth factor receptor (EGFR) kinase inhibitor erlotinib, has been successfully introduced into the clinical treatment of advanced PC[26], and although its benefit with respect to patient survival is small, it represents the only molecularly targeted agent to date that reached its primary end point in a phase 3 study with PC patients[27]. Sorafenib is a multi-kinase inhibitor that targets vascular endothelial growth factor receptors (VEGFR-2 and VEGFR-3), PDGF receptor family members (PDGFR-β and KIT) and Raf kinases[28]. It is used in the treatment of hepatocellular carcinoma and advanced renal cancer[29]. Sunitinib represents another multi-kinase inhibitor and inhibits various receptor tyrosine kinases that have been implicated in tumor growth and angiogenesis, including PDGFR-α/β, VEGFR-1-3, stem cell growth factor receptor (KIT), fms-related tyrosine kinase 3, colony stimulating factor 1 receptor and RET (rearranged during transfection)[30]. The drug has been approved for treatment of metastatic renal cancer and imatinib-resistant gastrointestinal stromal tumors[31,32].

Neither sunitinib nor sorafenib are currently established in the treatment of PC (although they have been tested in clinical trials[27]), and the efficiency of erlotinib is limited. The motivation of this study, however, was to evaluate the antifibrotic efficiency of the drugs, an effect that has not assessed yet but may be exploited in future studies that combine agents with activity against cancer and stroma cells.

The results of our study show that sunitinib and especially sorafenib displayed strong inhibitory effects on activated PSC, while erlotinib was less efficient. Surprisingly, the biological effects of the drugs correlated with an AKT- but not ERK pathway inhibition.

MATERIALS AND METHODS

Cell culture

Pancreatic stellate cells were isolated from the pancreas of male Lewis inbred rats (Charles River Laboratories, Sulzbach, Germany) by collagenase digestion of the organ and Nycodenz® (Nycomed, Oslo, Norway) density gradient centrifugation essentially as described before[20]. The cells were cultured in Iscove´s modified Dulbecco's medium (IMDM; Biochrom, Berlin, Germany) supplemented with 17% fetal calf serum (FCS), 10 mL/L non-essential amino acids (dilution of a 100 × stock solution), 10^5 U/L penicillin and 100 mg/L streptomycin (all reagents

from PAA Laboratories, Pasching, Austria). Upon reaching subconfluency, PSC were harvested by trypsination and recultured at equal seeding densities according to the experimental requirements. All experiments were performed with cells passaged no more than two times. Trypan blue staining was used to distinguish live from dead cells and to determine absolute cell counts.

Quantification of DNA synthesis

To quantify DNA synthesis, incorporation of 5-bromo-2´-deoxyuridine (BrdU) was measured using the BrdU labelling and detection enzyme-linked immunosorbent assay kit (Roche Diagnostics, Mannheim, Germany). Therefore, cells were plated in 96-well plates at equal seeding densities and allowed to adhere overnight in complete culture medium before erlotinib, sunitinib and sorafenib [Biaffin, Kassel, Germany; solvent: dimethylsulfoxide (DMSO)] and combinations thereof were added as indicated. After 24 h, BrdU labelling was initiated by adding labelling solution at a final concentration of 10 μmol/L. Another 24 h later, labelling was stopped, and BrdU uptake was measured according to the manufacturer's instructions.

Detection of dead cells and analysis of cellular DNA content by flow cytometry

PSC growing in 12-well plates were exposed to SMI for 48 h as indicated. Afterwards, the cells were harvested by trypsination, resuspended in buffer for flow cytometry (PBS pH 7.4; 0.5% bovine serum albumin; 0.1% sodium azide) and kept on ice until measurement. Subsequently, the samples were labelled with propidium iodide (PI: 10 mg/L; Sigma-Aldrich, Deisenhofen, Germany). PI-positive (dead) cells were quantified as previously described[33], using a FACSCalibur cytometer (BD Biosciences, Heidelberg, Germany) and Flowing Software 2.5.0 (Turku Centre for Biotechnology, Finland).

2-Deoxy-2-[^{18}F] fluoroglucose uptake

To analyze the effects of erlotinib, sunitinib and sorafenib on glucose uptake, PSC were seeded in 24-well plates at equal seeding densities and allowed to adhere overnight in complete culture medium. Afterwards, SMI and combinations thereof were added for 24 h. Next, complete culture medium was substituted by Dulbecco's modified Eagle medium (DMEM; Fisher Scientific, Schwerte, Germany) without FCS, glucose, glutamine and phenol red (but supplemented with inhibitors as before), and incubation continued for 1 h before 2-Deoxy-2-[^{18}F] fluoroglucose (^{18}F-FDG) (Eckert and Ziegler f-con Europe GmbH, Berlin, Germany; 0.5 GBq/L culture medium) was added to each culture well. 30 min later, incubation was terminated by aspirating the medium and rinsing the cell layer three times with ice-cold PBS. PSC were solubilized with 100 mmol/L NaOH, and incorporated ^{18}F activity was determined using a well counter (ISOMED 2100, Nuklear-Medizintechnik Dresden GmbH, Dresden, Germany). ^{18}F-FDG uptake in cells treated with SMI was expressed as percent of controls exposed to the solvent DMSO only.

Immunoblotting

Protein extracts from equal numbers of PSC (pretreated as indicated) were prepared and subjected to immunoblot analysis as published before[20], using polyvinylidene fluoride membrane for protein transfer. The following primary antibodies (all from New England BioLabs, Frankfurt, Germany unless specified otherwise) were employed: anti-β-actin (#4970), anti-GAPDH (#2118), anti-phospho-AKT (P-AKT) (#4060), anti-AKT protein (#4691), anti-phospho-ERK1/2 (P-ERK1/2) (#4370), anti-ERK1/2 (#06-182, Millipore, Billerica, MA, United States), and anti-collagen I (NB600-408, Novus Biologicals, Littleton, CO, United States). The blots were developed using LI-COR reagents for an Odyssey® Infrared Imaging System as previously described[34]. The signal intensities of the investigated proteins were quantified by means of the Odyssey® software and the raw data processed as described in the corresponding figure legend.

TGF-β1 ELISA

PSC growing in 12-well plates under standard culture conditions were treated with SMI for 48 h as indicated. Subsequently, the culture supernatants were collected and stored at -80 ℃ until assayed. After clearing the lysates by centrifugation, TGF-β1 concentrations were measured using a commercial ELISA (#436707, BioLegend, San Diego, CA, United States) according to the manufacturer's instructions.

Immunofluorescence detection of α-smooth muscle actin

PSC were seeded onto glass coverslips and allowed to attach before they were exposed to SMI for 48 h as indicated. Afterwards, cells were fixed with ice-cold methanol followed by staining of the DNA with 4',6-diamidino-2-phenylindole (DAPI). Next, the cells were incubated with a mouse monoclonal antibody to α-smooth muscle actin (α-SMA) (#A2547; Sigma-Aldrich). Antibody binding was determined by a fluorescein-labelled goat anti-mouse IgG (MoBiTec, Göttingen, Germany) and visualized using a fluorescence microscope (AxioScope.A1, Carl Zeiss, Jena, Germany).

Quantitative reverse transcriptase-PCR using real-time TaqMan™ technology

Total RNA from PSC pretreated with SMI for 48 h as indicated was isolated with TriFast reagent (PEQLAB Biotechnologie, Erlangen, Germany) according to the manufacturer's instructions. After treatment with DNAse (to remove contaminating traces of genomic DNA), 1 μg of RNA was reverse transcribed into cDNA by means of TaqMan™ Reverse Transcription Reagents and random hexamer priming. Relative quantification of target cDNA levels by real-time PCR was performed in an ABI Prism 7000 sequence detection system employing TaqMan™

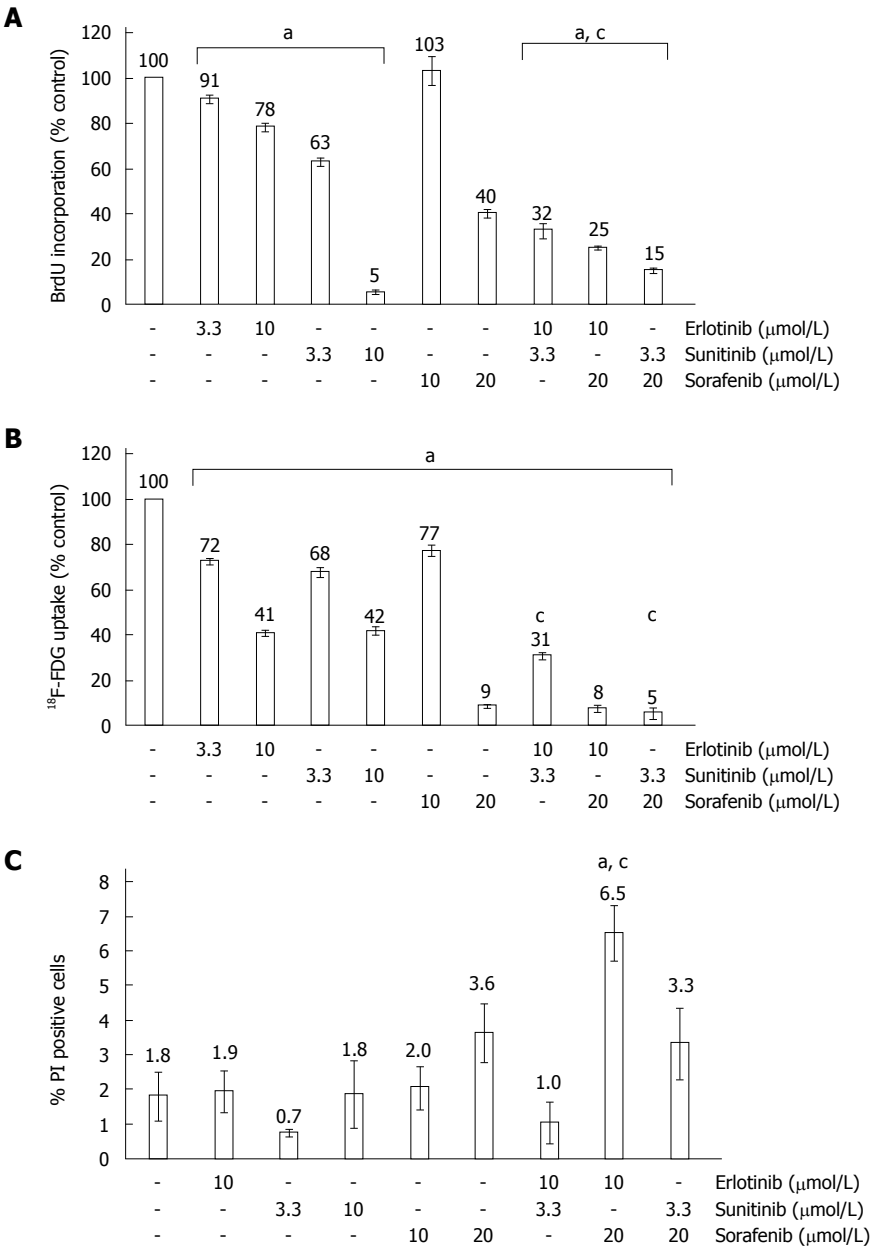
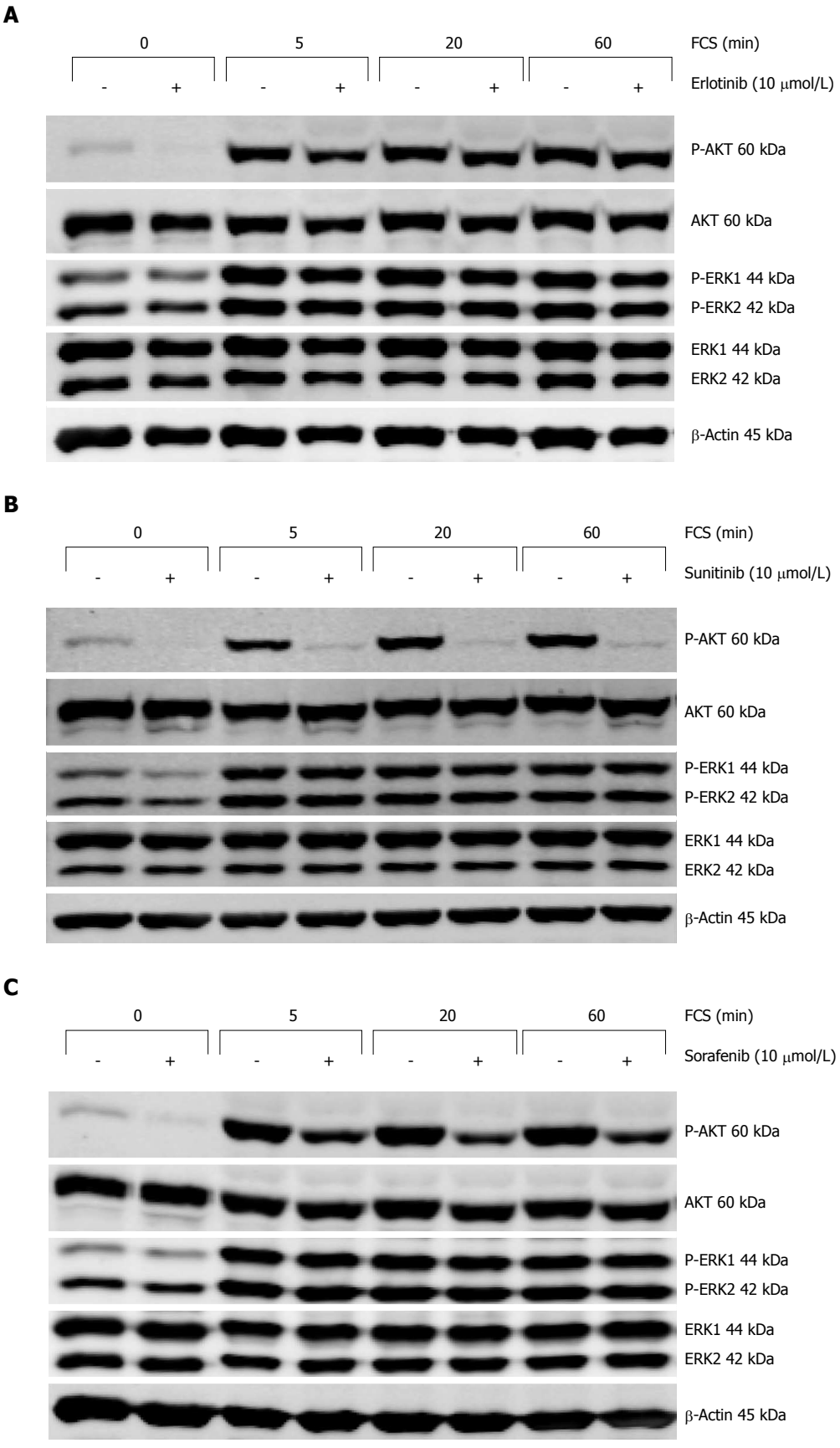


Figure 1 Effects of small molecule kinase inhibitors on DNA synthesis, 2-Deoxy-2-[^{18}F] fluoroglucose uptake and survival of pancreatic stellate cells. Pancreatic stellate cells (PSC) growing in primary culture were harvested, replated at equal seeding densities and treated with erlotinib, sunitinib, sorafenib and combinations thereof at the indicated concentrations for 24 h. Control cultures were exposed to the solvent DMSO only. A: The pretreated cells were labelled with 5-bromo-2'-deoxyuridine (BrdU) for another 24 h and proliferation was assessed with the BrdU DNA incorporation assay. One hundred percent BrdU incorporation corresponds to solvent-treated PSC. Data from $n \geqslant 17$ separate cultures per data point were used to calculate mean ± SE; B: The cells were labelled with 2-Deoxy-2-[^{18}F] fluoroglucose (^{18}F-FDG), and uptake quantified as described in the "Materials and methods" section. One hundred percent ^{18}F-FDG uptake corresponds to solvent-treated PSC ($n \geqslant 6$; mean ± SE); C: Incubation was continued for another 24 h. Afterwards, the samples were subjected to a cytofluorometric quantification of dead [phosphatidylinositol (PI)-positive] cells, which are expressed as percent of the total cell number ($n \geqslant 5$; mean ± SE). (A-C) $^{a}P < 0.05$, $^{c}P < 0.05$ *vs* cultures treated with either of the two combined substances alone.

Universal PCR Master Mix and the following Assay-on-Demand™ rat gene-specific fluorescently labelled TaqMan™ MGB probes (instrument and reagents: Life Technologies, Darmstadt, Germany): Rn01759928_g1 (*Acta2*), Rn00561420_m1 (interleukin-6; *Il-6*), Rn01538167_m1 (matrix metalloproteinase-2; *Mmp-2*), Rn00579162_m1 (*Mmp-9*), Rn00572010_m1 (*Tgf-β1*), Rn01463848_m1 (*Col1a1*), and Rn01527840_m1 (*Hprt*; house-keeping gene control). The following PCR conditions were used: 95 ℃ for 10 min, repeated cycles of 15 s at 95 ℃/min at 60 ℃. PCR reactions were performed in duplicate, and repeated 5 times with independent samples. The relative expression of each mRNA compared with *Hprt* was calculated according to the equation $\Delta Ct = Ct_{target} - Ct_{HPRT}$. The relative amount of target mRNA in control cells and cells treated with SMI as indicated was expressed as $2^{-(\Delta\Delta Ct)}$, where $\Delta\Delta Ct_{treatment} = \Delta Ct_{SMI} - \Delta Ct_{control}$.

Statistical analysis

Results are expressed as mean ± SE for the indicated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Sunitinib (10 μmol/L)
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P-ERK1 44 kDa
P-ERK2 42 kDa
ERK1 44 kDa
ERK2 42 kDa
β-Actin 45 kDa
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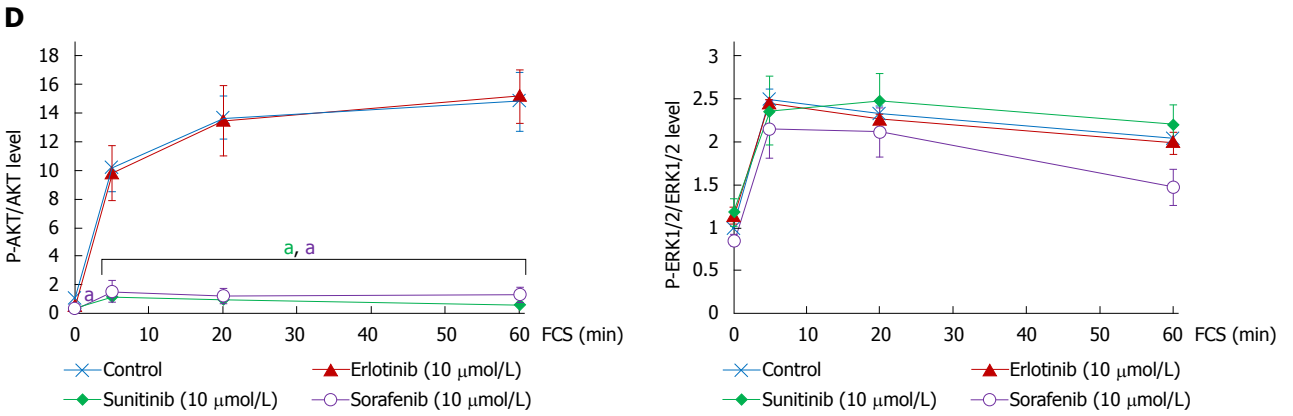

Figure 2 Distinct effects of erlotinib, sorafenib and sunitinib on phosphorylation of AKT and ERK1/2. Cultured pancreatic stellate cells (PSC) were grown to subconfluency before the standard culture medium was substituted by fetal calf serum (FCS)-free medium. 16 h later, culture medium was supplemented with (A) erlotinib, (B) sunitinib and (C) sorafenib at the indicated concentrations and incubation continued for another hour. Subsequently, FCS (at 17%) was added for the indicated periods of time. Afterwards, protein extracts from equal amounts of cells were subjected to Western blot analysis. P-AKT, P-ERK1/2, the respective total proteins and β-actin (for loading control) were detected using fluorescein (IRDye®)-labelled secondary antibodies. For each inhibitor, one representative Western blot is shown; D: Fluorescence signal intensities of phosphoproteins (P-AKT and P-ERK1/2, respectively) and corresponding total proteins were quantified using Odyssey® software. Subsequently, the ratios P-AKT/AKT protein (left panel) and P-ERK/ERK protein (right panel) were determined. A ratio of 1 corresponds to control cells cultured without small molecule kinase inhibitors (SMI) and FCS. Data of $n \geqslant 4$ independent experiments were used to calculate mean ± SE; $^{a}P < 0.05$ *vs* control cultures (no SMI, same time of FCS stimulation).

number of separate cultures per experimental protocol. Unless indicated otherwise, statistical significance was checked using Wilcoxon's Signed-Rank test for paired samples and the Mann-Whitney *U* test in case of independent samples. $P < 0.05$ was considered to be statistically significant.

RESULTS

Effects of SMI on PSC growth, glucose uptake and survival

In initial experiments, the effects of erlotinib, sunitinib and sorafenib on PSC proliferation were determined by measuring incorporation of BrdU into newly synthesized DNA. At low micromolar concentrations, all three SMI inhibited DNA synthesis in a dose-dependent manner (Figure 1A), with sunitinib displaying the highest potency in this assay. Furthermore, any combination of two of the drugs exerted stronger effects than the single substances alone, suggesting an additive action.

To analyse SMI effects on cell metabolism, ^{18}F-FDG uptake was chosen as a surrogate marker. Again, all three SMI displayed dose-dependent inhibitory effects, which were further enhanced when the drugs were combined (Figure 1B). An inhibition by more than 90% was observed in samples that were exposed to sorafenib at 20 μmol/L (alone or combined with sunitinib and erlotinib, respectively).

To assess cytotoxity of the drugs, PSC were exposed to SMI at the same concentrations as before, stained with PI and subjected to flow cytometry. As shown in Figure 1C, only the combination of erlotinib and sorafenib caused a significant increase of PI-positive dead cells, but even in this case more than 93% of PSC remained viable. Together, these data suggest that general cytotoxicity was not a major cause of the reduced BrdU incorporation and ^{18}F-FDG uptake of SMI-treated PSC.

ERK and AKT pathway activity in SMI-exposed PSC

The intracellular signal transduction pathways Ras-Raf-MEK-ERK and PI 3-kinase/AKT play a key role in PSC activation and act downstream of many tyrosine kinases receptors targeted by the SMI tested in this study[20-25]. We therefore addressed the question how erlotinib, sunitinib and sorafenib affect activation of AKT and ERK in PSC, using levels of P-AKT and P-ERK1/2 as surrogate markers (Figure 2). Unexpectedly, none of the three drugs was able to prevent FCS-induced activation of ERK1/2 (Figure 2D, right panel). In contrast, both sunitinib and sorafenib efficiently blocked the increase of P-AKT levels caused by FCS-restimulation of serum-starved PSC, while erlotinib again displayed no effect (Figure 2D, left panel).

Together, these data implicate AKT in the action of sunitinib and sorafenib in PSC but point to a negligible role of the ERK pathway.

Effects of erlotinib, sunitinib and sorafenib on PSC gene and protein expression

Employing real-time PCR, the gene expression profile of PSC after 48 h of SMI treatment was characterized. Figure 3 shows the results for a selected panel of genes that was chosen based on (1) their established role in PSC function and ECM metabolism; and (2) the results of pilot experiments.

Sorafenib and sunitinib inhibited expression of type I collagen (Figure 3A) and the pro-inflammatory cytokine IL-6 (Figure 3B), which has previously been implicated in autocrine stimulation of TGF-β1 secretion[35] and enhancement of α-SMA expression in the course of

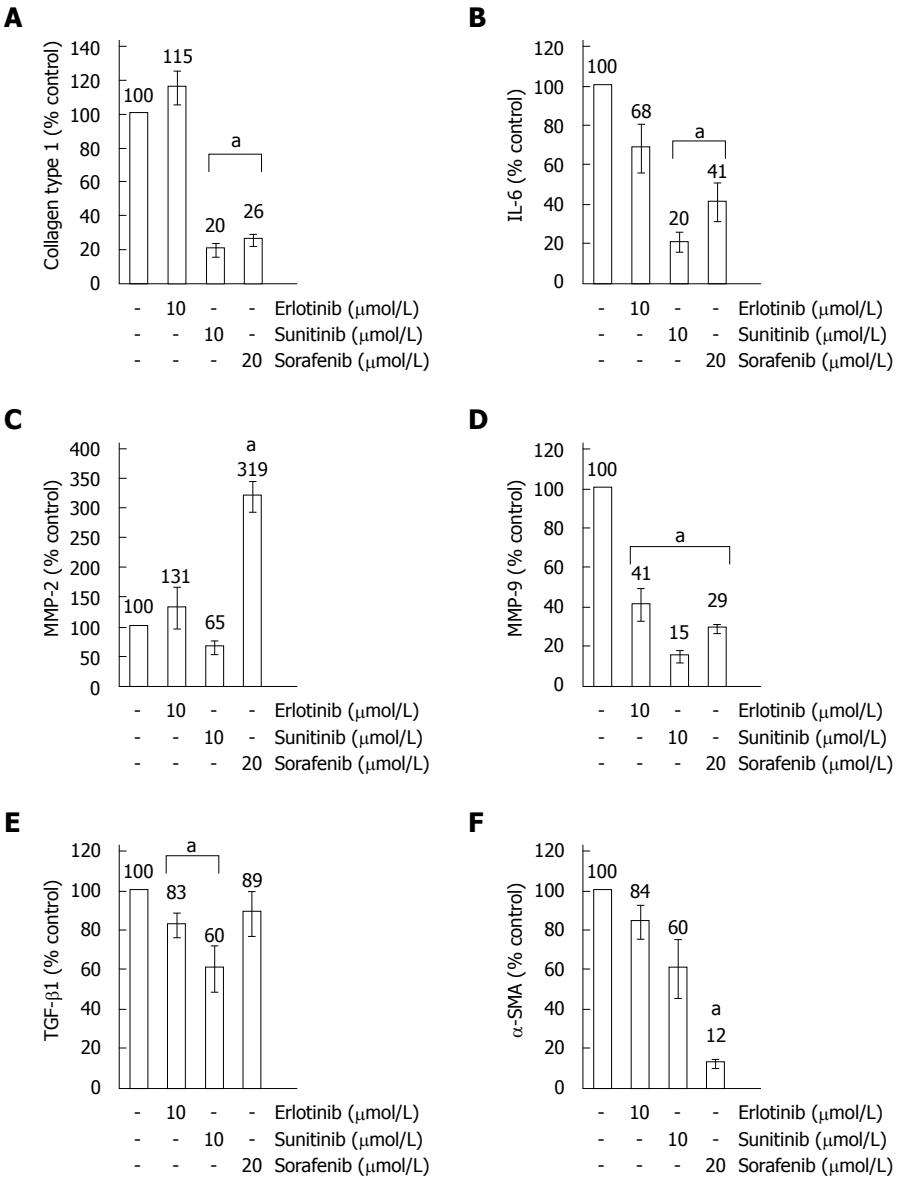

Figure 3 Real-time PCR analysis of gene expression in small molecule kinase inhibitors-treated pancreatic stellate cells. Cultured pancreatic stellate cells (PSC) were exposed to small molecule kinase inhibitors (SMI) at the indicated concentrations for 48 h. The mRNA expression of collagen type 1 (A), IL-6 (B), MMP-2 (C), MMP-9 (D), TGF-β1 (E), α-SMA (F) and the housekeeping gene HPRT was analyzed by real-time PCR, and relative amounts of target mRNA were calculated as described in the "Materials and methods" section. One hundred percent mRNA expression of each gene corresponds to cells treated with the solvent DMSO only. Data of $n = 5$ independent cultures were used to calculate mean ± SE. [a]$P < 0.05$ *vs* control cultures.

PSC activation[36]. For type I collagen, additional investigations on the protein level were performed and results that are in line with the mRNA expression data were obtained (Figure 4A, B).

Sorafenib, but none of the other drugs, strongly induced expression of MMP-2 (Figure 3C), while all three SMI diminished the mRNA level of another collagenase secreted by activated PSC, MMP-9 (Figure 3D). Sunitinib and erlotinib reduced mRNA expression of TGF-β1, while, in this case, the effect of sorafenib was not significant (Figure 3E). However, as shown in Figure 5, the inhibitory effects of sunitinib and erlotinib at the level of mRNA did not translate into decreased TGF-β1 protein concentrations in supernatants of PSC cultures, while sorafenib significantly reduced the secretion of the cytokine. The latter effect is likely to be due to the antiproliferative action of sorafenib, since the drug diminished the rate of cell proliferation in the course of the experiment to a similar degree (data not shown).

Finally, we found that sorafenib was the only drug that displayed a strong inhibitory effect on the mRNA expression of α-SMA (Figure 3F). At the protein level (Figure 4A, C), the effects of all three drugs remained insignificant ($P = 0.065$ for sorafenib). To gain additional insight, immunofluorescence studies were performed and some reduction of stress fibers (bundles of α-SMA that are typical of activated PSC) in sorafenib-treated cells was observed (Figure 6). Taken together, the data nevertheless do not provide unequivocal evidence that sorafenib treatment was associated with a (partial) re-

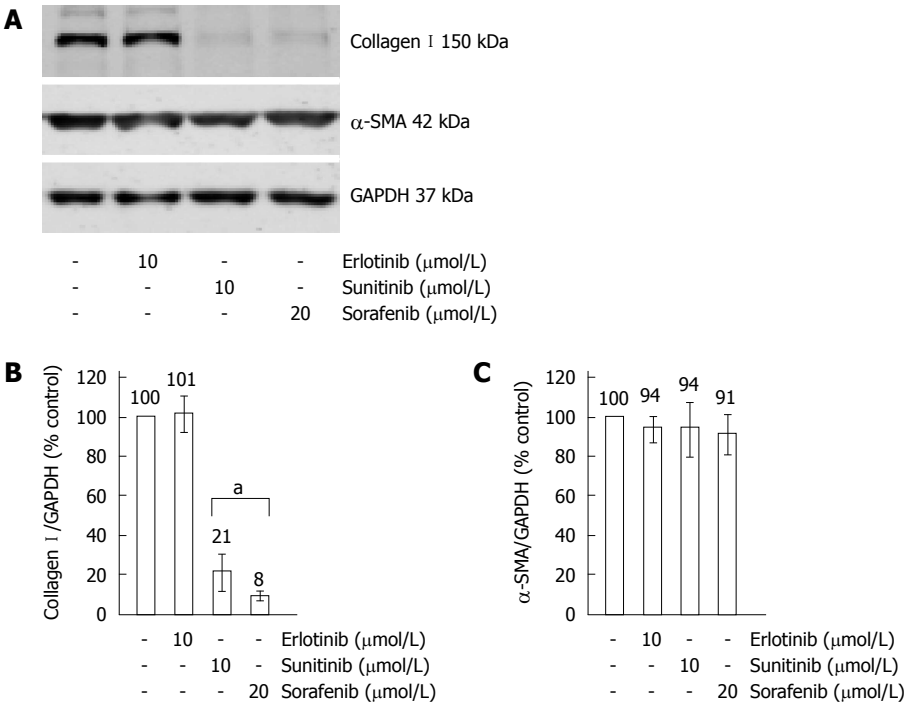

Figure 4 Effects of erlotinib, sorafenib and sunitinib on the expression of type I collagen and α-smooth muscle actin. Cultured pancreatic stellate cells (PSC) were grown to subconfluency before they were treated for 48 h with SMI at the indicated concentrations. Afterwards, protein extracts from equal amounts of cells were subjected to Western blot analysis. Type I collagen, α-SMA and GAPDH (for loading control) were detected using fluorescein (IRDye®)-labelled secondary antibodies. (A) One representative Western blot for all three SMI is shown. Afterwards, fluorescence signal intensities of collagen I (B), α-smooth muscle actin (α-SMA) (C) and GAPDH (B and C) were quantified using Odyssey® software. Subsequently, the ratios collagen I /GAPDH (B) and α-SMA/GAPDH (C) were determined to normalize for loading variations. A ratio of one hundred percent corresponds to control cells cultured without SMI. Data of $n = 6$ independent experiments were used to calculate mean ± SE. $^aP < 0.05$ *vs* control cultures.

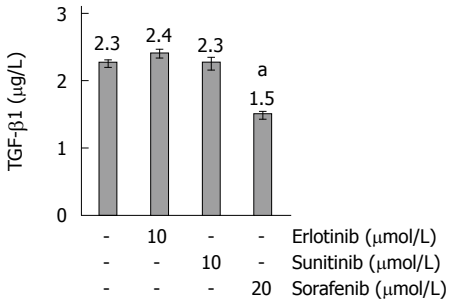

Figure 5 Transforming growth factor-β1 concentrations in culture supernatants of small molecule kinase inhibitors-treated pancreatic stellate cells. Cultured pancreatic stellate cells were grown to subconfluency before they were treated for 48 h with Small molecule kinase inhibitors at the indicated concentrations. The concentrations of transforming growth factor (TGF)-β1 in cell culture supernatants were determined by ELISA. Data of $n = 6$ independent cultures were used to calculate mean ± SE. $^aP < 0.05$ *vs* control cultures (one-way ANOVA test).

gression of the myofibroblastic PSC phenotype.

DISCUSSION

The results of this study indicate that three clinically available SMI, erlotinib, sunitinib and sorafenib, exert distinct inhibitory effects on activated rat pancreatic stellate cells *in vitro*.

Altogether, the efficacy of erlotinib was quite limited. The drug significantly reduced glucose uptake, but this effect did not translate into changes of the profibrogenic gene expression profile of the cells (except of a small decrease of TGF-β1 mRNA expression). Furthermore, the drug only weakly (although significantly) inhibited PSC proliferation. Erlotinib acts by blocking the EGFR tyrosine kinase activity[37]. Therefore, the failure of the drug to inhibit serum-induced activation of two downstream targets of the receptor, AKT and ERK1/2, was unexpected. In this study, serum and not individual growth factors were used to stimulate the cells in order to mimic biological conditions more closely. It is therefore conceivable that the contribution of the axis EGF/EGFR to the activation of Ras-Raf-MEK-ERK and PI 3-kinase/AKT pathways was small, explaining the lack of an inhibitory erlotinib effect. In any case, the molecular basis of the reduced glucose uptake in erlotinib-treated cells (possibly, a modulation of glucose transporter expression) warrants further investigation.

The multi-kinase inhibitors sunitinib and sorafenib displayed higher biological efficacies than erlotinib: At the maximum concentration tested, each drug inhibited ^{18}F-FDG uptake and DNA synthesis by more than 50%. While sunitinib was the more potent inhibitor of cell proliferation, sorafenib exerted a stronger effect on glucose metabolism. Sunitinib and sorafenib target a partially overlapping set of tyrosine kinase receptors, *e.g.*, of the VEGFR family[28,30]. Nevertheless, the combina-

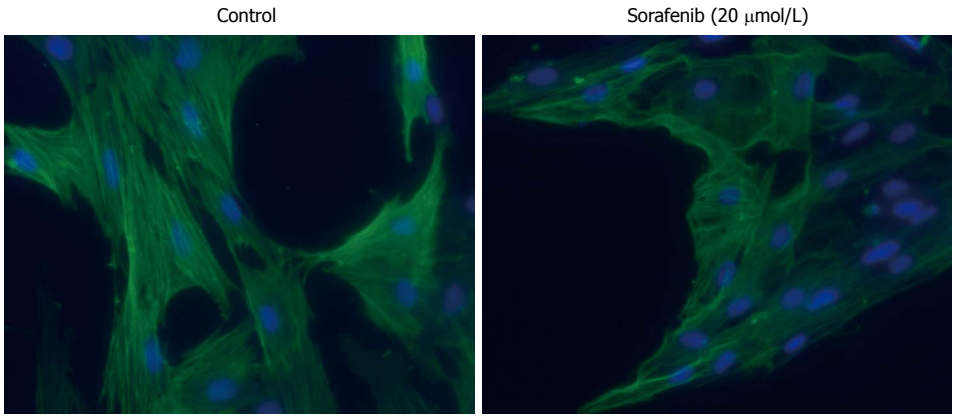

Figure 6 Effects of small molecule kinase inhibitors on α-smooth muscle actin expression and stress fiber formation in pancreatic stellate cell. Pancreatic stellate cells growing on glass coverslips were treated for 48 h with sorafenib or the solvent DMSO (control) as indicated. Expression and structural organization of α-smooth muscle actin were analyzed by immunofluorescence staining of the protein and documented by fluorescence microscopy (counterstaining: DAPI; original magnification × 200).

tion of both drugs (as well as their individual combination with erlotinib) revealed additive effects regarding the inhibition of ^{18}F-FDG uptake and DNA synthesis. Since only the simultaneous application of sorafenib and erlotinib was associated with increased cytotoxicity, combinations of SMI should be further evaluated in follow-up studies.

In contrast to erlotinib, sunitinib and sorafenib efficiently inhibited FCS-induced activation of AKT. The finding suggests that serum-dependent activation of AKT in PSC is predominantly mediated by tyrosine kinase receptors that represent targets of the two multikinase inhibitors. In case of ERK1/2 activation, the opposite seems to be the case: While sunitinib failed completely to block ERK1/2 phosphorylation, sorafenib displayed a small inhibitory effect only, which may reflect its additional action as a Raf kinase inhibitor. Previous studies in other types of cells have implicated both PI 3-kinase/AKT and Ras-Raf-MEK-ERK pathways in sunitinib[38-43] and sorafenib[44-48] action. The conflicting observations regarding ERK inhibition may either be caused by peculiarities of the respective cell types, or by differences of the experimental protocols that were used: Here, we have focussed on direct effects of the SMI on the investigated pathways. Therefore, preincubation time with the SMI (1 h) and time of FCS stimulation (5-60 min) were kept short. Indirect effects of SMI on ERK pathway activity (effects that require gene expression) were not addressed and can, therefore, not be excluded. At least, a prolongation of FCS stimulation for 3 more hours (4 h in total), did not change our principal findings (data not shown). In conclusion, we have observed a correlation between the inactivation of AKT (but not ERK) and the inhibition of glucose uptake and DNA synthesis by sunitinib and sorafenib. In ongoing studies, we are analyzing a possible causal relationship by employing specific inhibitors of PI 3-kinase/AKT signaling.

Both sunitinib and sorafenib modified the gene expression profile of PSC in a way that suggests less profibrogenic activity of the cells. Thus, both drugs inhibited mRNA expression of IL-6, an autocrine enhancer of PSC activation[35,36], and strongly diminished both the mRNA and protein levels of type I collagen. Interestingly, sorafenib, in contrast to the other two drugs, had no effect on the mRNA levels of TGF-β1, but was the only SMI that significantly reduced the protein concentration of this profibrogenic mediator in cell culture supernatants. Taken these data together, we consider the reduction of TGF-β1 protein levels as an indirect effect of sorafenib that is linked to its antiproliferative action.

The two gelatinases MMP-2 and MMP-9 are both secreted by activated PSC, although at different levels: In previous studies, we found that supernatants of cultured PSC contain much more active MMP-2 than MMP-9[49]. Here, we have analyzed gelatinase expression in order to assess the fibrolytic activity of SMI-treated PSC. The results are heterogeneous, since sorafenib triggered the expression of MMP-2, but all SMI (including erlotinib) diminished the mRNA levels of MMP-9. The net effects of different SMI on fibrolysis, therefore, need to be studied further.

Noteworthy, all three SMI tested in this study showed insignificant or discordant effects on the mRNA and protein levels of α-SMA. These data suggest that the drugs are not capable to reverse the myofibroblastic phenotype of activated PSC. Instead, they exert their antifibrogenic effects by inhibiting key effector functions of the cell in their activated stage.

Although cultures of PSC are considered a suitable *in vitro* model of pancreatic fibrogenesis, we do not claim direct clinical relevance of our findings at the current stage. One limitation refers to the fact that the *in vivo* bioavailability of sorafenib, the drug that showed the most consistent effects in this study, is limited by its high protein binding rate[50]. It remains to be shown, therefore, that antifibrotic effects are also achievable *in vivo* under the conditions of CP and PC. At least in experimental pulmonary fibrosis, however, evidence that sorafenib can ameliorate progression of the disease has recently been provided[51].

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COMMENTS

Background

Pancreatic stellate cells (PSC) are the main source of extracellular matrix proteins in pancreatic fibrosis, which is a key feature of chronic pancreatitis and pancreatic cancer and considered as an aggravating factor of both diseases. Inhibition of PSC activation has been suggested as a promising approach to inhibit pancreatic fibrogenesis, but there is still no specific therapy for clinical application available yet.

Research frontiers

Small molecule kinase inhibitors (SMI) such as erlotinib, sunitinib and sorafenib have been introduced into clinical oncology in recent years. These drugs interfere with signal transduction pathways that are also involved in PSC activation and may therefore have an antifibrotic efficiency. This hypothesis, however, has not been experimentally tested so far.

Innovations and breakthroughs

The results of this study show for the first time that three clinically available SMI (erlotinib, sunitinib and sorafenib) inhibit PSC proliferation and glucose metabolism in a dose-dependent manner and without significant cytotoxic effects. Furthermore, the drugs displayed additive effects. By analyzing SMI effects on PSC signaling and gene expression, the authors also gained insights into the molecular mechanisms of SMI action.

Applications

Together, the data indicate that the tested SMI, in particular sorafenib, display antifibrogenic effects *in vitro*. The authors therefore suggest that the antifibrotic efficiency of the drugs should be evaluated further in preclinical studies.

Terminology

Pancreatic fibrosis represents a process of progressive replacement of pancreatic tissue by connective tissue in the context of chronic inflammation and cancer. Pancreatic stellate cells are fibroblast-like cells that synthesize and secrete most of the extracellular matrix in the diseased organ. Erlotinib, sunitinib and sorafenib are small molecule kinase inhibitors that used in the treatment of various human malignancies.

Peer review

This study explored the antifibrogenic effects of three clinically available small SMI, erlotinib, sunitinib and sorafenib on PSC and analyzed the basis of their action. It is found that these three SMI showed distinct antifibrogenic effects on PSC. It is showed that sorafenib and sunitib, but not erlotinib, efficiently blocked activation of the AKT pathway; and erlotinib and sunitinib, but not sorafenib, significantly reduced the expression of transforming growth factor-β1. It is helpful to evaluate the antifibrogenic effects of the tested SMI in preclinical studies. This is a well conducted and well written study. The experiments are described in detail, the results are shown nicely and the figures are impressive.

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