Toxicology and Applied Pharmacology 274 (2014) 328–338

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

Precision-cut liver slices as a model for the early onset of liver fibrosis to test antifibrotic drugs

Inge M. Westra [a], Dorenda Oosterhuis [b], Geny M.M. Groothuis [a], Peter Olinga [b,*]

[a] Division of Pharmacokinetics, Toxicology and Targeting, Department of Pharmacy, University of Groningen, The Netherlands
[b] Division of Pharmaceutical Technology and Biopharmacy, Department of Pharmacy, University of Groningen, The Netherlands

ARTICLE INFO

Article history:
Received 2 September 2013
Revised 19 November 2013
Accepted 25 November 2013
Available online 7 December 2013

Keywords:
Precision-cut liver slices
Liver fibrosis
Antifibrotic drugs
Ex vivo model
Early onset of fibrosis

ABSTRACT

Induction of fibrosis during prolonged culture of precision-cut liver slices (PCLS) was reported. In this study, the use of rat PCLS was investigated to further characterize the mechanism of early onset of fibrosis in this model and the effects of antifibrotic compounds. Rat PCLS were incubated for 48 h, viability was assessed by ATP and gene expression of *PDGF-B* and *TGF-β1* and the fibrosis markers *Hsp47*, *αSma* and *Pcol1A1* and collagen1 protein expressions were determined. The effects of the antifibrotic drugs imatinib, sorafenib and sunitinib, PDGF-pathway inhibitors, and perindopril, valproic acid, rosmarinic acid, tetrandrine and pirfenidone, TGFβ-pathway inhibitors, were determined. After 48 h of incubation, viability of the PCLS was maintained and gene expression of *PDGF-B* was increased while *TGF-β1* was not changed. *Hsp47*, *αSma* and *Pcol1A1* gene expressions were significantly elevated in PCLS after 48 h, which was further increased by PDGF-BB and TGF-β1. The increased gene expression of fibrosis markers was inhibited by all three PDGF-inhibitors, while TGFβ-inhibitors showed marginal effects. The protein expression of collagen 1 was inhibited by imatinib, perindopril, tetrandrine and pirfenidone. In conclusion, the increased gene expression of *PDGF-B* and the down-regulation of fibrosis markers by PDGF-pathway inhibitors, together with the absence of elevated *TGF-β1* gene expression and the limited effect of the TGFβ-pathway inhibitors, indicated the predominance of the PDGF pathway in the early onset of fibrosis in PCLS. PCLS appear a useful model for research of the early onset of fibrosis and for testing of antifibrotic drugs acting on the PDGF pathway.

Introduction

Liver fibrosis is the progressive accumulation of connective tissue that affects the normal function of the liver and eventually leads to liver cirrhosis (Bataller and Brenner, 2005). Hundreds of millions of patients are affected by cirrhosis worldwide (Friedman, 2003). As antifibrotic drugs are currently not available, patients can only be treated by organ transplantation. To improve and accelerate the drug discovery and development process, there is an urgent need for reliable in vitro methods to test the efficacy of potential antifibrotic compounds, as in vivo experiments use a large number of animals with considerable discomfort, and are expensive and time consuming.

During fibrosis, different signaling pathways are activated. The TGFβ/Smad signaling pathway is stimulated by transforming growth factor β (TGFβ), which is secreted by hepatic stellate cells (HSC), Kupffer cells, hepatocytes and platelets (Dooley and Ten Dijke, 2012; Friedman, 2008; Liu et al., 2009; Tsukada et al., 2006). In addition, upon activation, macrophages and HSC also produce platelet derived growth factor (PDGF), triggering the PDGF signaling pathway (Deng et al., 2009; Friedman, 2008; Liu et al., 2009; Tsukada et al., 2006). These pathways are activated upon chronic liver injury, which causes damage of endothelial cells and apoptosis of hepatocytes. In addition to the activation of the TGFβ and PDGF secretion, these injured cells recruit inflammatory cells to the injured liver (Cong et al., 2012). HSC play an essential role in the onset and progression of liver fibrosis as activation by TGFβ causes them to proliferate and to produce extracellular matrix components (Borkham-Kamphorst et al., 2004). In addition, PDGF is a stimulator of HSC growth which is further stimulated by an increased expression of the PDGF receptor β in activated HSC (Borkham-Kamphorst et al., 2004). Thus, there is a continuous interaction between HSC, hepatocytes and Kupffer cells during the onset and progression of liver fibrosis. To study the process of fibrosis in vitro in a physiologic milieu and to test antifibrotic drugs, it is important to use an in vitro system, which closely reflects the in vivo situation. In precision-cut liver slices (PCLS) all cell types of the liver are present in their original context and remain viable up to 48 h (van de Bovenkamp et al., 2005). As each slice contains ca 70–100 lobules, the acinar heterogeneity of the liver or inhomogeneous distribution of portal spaces

* Corresponding author at: Pharmaceutical Technology and Biopharmacy, Department of Pharmacy, Antonius Deusinglaan 1, 9713 AV Groningen, The Netherlands. Fax: +31 50 363 2500.
E-mail address: p.olinga@rug.nl (P. Olinga).

0041-008X/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.taap.2013.11.017

plays only a minor role in the variability of the results in this ex vivo model. Furthermore, the possibility to make PCLS of human liver tissue makes this model very promising to investigate fibrosis in the human liver. Previously, we and others (van de Bovenkamp et al., 2008; Vickers et al., 2004) showed that a fibrotic process is initiated during prolonged culture of PCLS. Moreover, van de Bovenkamp et al. showed the antifibrotic effect of imatinib, pentoxifyllin and dexamethasone in fibrotic rat PCLS, prepared from bile duct ligated rats, and these compounds decreased the gene expression of specific fibrosis markers heat shock protein 47 (*Hsp47*), α-smooth muscle actin (*αSma*) and pro-collagen 1A1 (*Pcol1A1*) (van de Bovenkamp et al., 2006).

In the current study, prolonged incubation of the rat PCLS was used to induce the onset of fibrosis (van de Bovenkamp et al., 2008). Fibrogenesis was measured by determining the expression of genes and proteins that indicate the activation of HSC and (myo) fibroblasts, using gene and protein expression of specific markers of fibrosis as previously described (van de Bovenkamp et al., 2006). To investigate the involvement of the two main fibrosis pathways, the gene expressions of *TGF-β1* and *PDGF-B*, in addition to *Ctgf* were measured, as well as the effects of exposure of the PCLS to exogenous TGF-β1 and PDGF-BB.

The aim of the present study was to investigate the mechanism of the early onset of fibrosis in prolonged culture of PCLS, and to determine if this in vitro model can be used to test antifibrotic compounds. The antifibrotic compounds used in this study were all selected based on the published effects in vivo in animals and/or in vitro in cell lines and primary cells. The concentrations used in this study correspond with the concentrations utilized in experiments with cell cultures. The compounds were selected to represent different modes of action, reflecting the main pathways involved in liver fibrosis, the PDGF and TGFβ signaling pathways. The compounds mainly inhibiting the PDGF pathway (PDGF-inhibitors) were imatinib, sorafenib and sunitinib. Imatinib is a competitive inhibitor of the tyrosine kinases PDGF receptor, Bcr-Abl and c-Kit (Buchdunger et al., 2000; Neef et al., 2006). Sorafenib is a receptor tyrosine kinase inhibitor that targets the PDGF receptor and the Raf/ERK signaling pathway (Wang et al., 2010). Sunitinib, the third tyrosine kinase inhibitor used in this study, targets amongst others the PDGF receptor, c-Kit and the VEGF receptor (Minkin et al., 2008). In this study, rosmarinic acid, perindopril, valproic acid, tetrandrine and pirfenidone were investigated as the inhibitors known to be mainly acting on the TGFβ signaling pathway (TGF-inhibitors). Rosmarinic acid naturally occurs in many medicinal species of the plant Lamiaceae (mint family) (Li et al., 2010; Mannaerts et al., 2010; Osakabe et al., 2004) and it has been shown that rosmarinic acid has antifibrotic effects that were achieved through the inhibitory effect on TGFβ (Domitrovic et al., 2012; Zhang et al., 2011). In addition, perindopril, an angiotensin converting enzyme (ACE) inhibitor and valproic acid, a histone deacetylase inhibitor, has been shown to decrease the TGFβ expression (Helmy et al., 2000; Watanabe et al., 2011). Tetrandrine is an alkaloid that is isolated from the Chinese medicinal herb *Stephania tetrandra* (Chen et al., 2005; Hsu et al., 2007). Tetrandrine's antifibrotic effect is at least partially caused by an up-regulation of Smad 7, which in turn blocks the TGFβ expression and its downstream signaling (Chen et al., 2005). Pirfenidone acts as an antifibrotic agent, by down-regulating the gene expression of *TGFβ* (Schaefer et al., 2011). To investigate the effect of direct inhibition of collagen disposition, the drug colchicine, known to have a direct effect on collagen disposition by causing disruption of microtubule formation and inhibition of collagen transport and synthesis, was investigated (Chung and Kang, 1999; Lee et al., 2004).

The expression of *PDGF-B* and *TGF-β1* and the specific properties of the compounds under investigation, enabled us to assess the involvement of the PDGF and TGFβ pathways in the onset of fibrosis during prolonged incubation of PCLS.

Materials and methods

Slice experiments. Livers of adult male Wistar rats (Harlan PBC, Zeist, The Netherlands) anaesthetized with isoflurane/O_2 (Nicholas Piramal, London, UK) were freshly isolated and used for preparing liver slices in ice-cold Krebs–Henseleit buffer supplemented with 25 mM D-glucose (Merck, Darmstadt, Germany), 25 mM $NaHCO_3$ (Merck), 10 mM HEPES (MP Biomedicals, Aurora, OH, US0041) and saturated with carbogen (95% O_2/5% CO_2) using a Krumdieck tissue slicer as described before in detail (de Graaf et al., 2010). PCLS with a diameter of 5 mm and a thickness of 250 μm were incubated individually in 1.3 ml of Williams Medium E (with L-glutamine, Invitrogen, Paisly, Scotland) supplemented with 25 mM glucose and 50 μg/ml gentamycin (Invitrogen) at 37 °C and under continuous supply of 95% O_2/5% CO_2 in 12-well plates while gently shaken. After 1 h of preincubation the slices were transferred to fresh medium and further incubated for 24, 48 and 72 h and for 48 h with antifibrotic compounds. After 24 and 48 h the slices were transferred to new 12 well plates with fresh medium containing the drug. The slices were incubated with the antifibrotic compounds imatinib (1–10 μM) (Novartis, Basel, Switzerland), valproic acid (0.1–1 mM) (Sigma Aldrich, Zwijndrecht, Netherlands), perindopril (10–100 μM) (Sigma Aldrich), pirfenidone (0.5–2.5 mM) (Sigma Aldrich), rosmarinic acid (120–270 μM) (Sigma Aldrich), colchicine (30–200 nM) (Sigma Aldrich), tetrandrine (1–10 μM) (Sigma Aldrich), sunitinib (0.5–5 μM) (LC laboratories, Woburn, USA) and sorafenib (0.5–2 μM) (LC laboratories). Stock solutions of the compounds were prepared in water or DMSO and diluted in the culture medium with a final concentration of the solvent of ≤1%. Before the incubations of PCLS with TGF-β1 (1–5 ng/ml) (hTGF-β1, Roche Applied Science, Mannheim, Germany) the 12 well plates were pretreated with 10% BSA in Milli Q water solution for 20 min, whereafter the solution was removed and plates were air dried, in order to prevent non-specific binding of the TGF-β1 to the walls of the plates. Incubations with PDGF-BB (10 and 50 ng/ml) (Recombinant Human PDGF-BB, Peprotech, Bioconnect) were performed as described above for the antifibrotic compounds. All incubations were performed in triplicate (using 3 slices incubated individually in separate wells) and were repeated with livers from 3-x different rats. The rats were housed on a 12 hour light/dark cycle in a temperature-and-humidity-controlled room with food (Harlan chow no 2018, Horst, The Netherlands) and water ad libitum. The animals were allowed to acclimatise for at least seven days before the start of the experiment and all animals received human care. The experiments were approved by the Animal Ethical Committee of the University of Groningen.

Viability. After incubation, slices were transferred to a 1 ml sonication solution, containing 70% ethanol and 2 mM EDTA, and snap frozen in liquid nitrogen and stored at −80 °C. To determine the cell viability, ATP levels were measured in the supernatant of samples sonicated for 45 s and centrifuged for 2 min at 13,000 rpm, using the ATP bioluminescence kit (Roche diagnostics, Mannheim, Germany). ATP values (pmol) were normalized to the total protein content (μg) of the slice estimated by Lowry (BIO-rad RC DC Protein Assay) (Bio Rad, Veenendaal, The Netherlands) (Lowry et al., 1951). Values displayed are relative values compared to the related controls.

Gene expression. To determine the antifibrotic effect of the drugs, gene expressions of fibrosis markers were determined using Real-Time PCR. The triplicate slices were pooled and snap frozen and total RNA was isolated with the use of the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands). The amount of isolated RNA was measured with the ND-1000 spectrophotometer (Fisher Scientific, Landsmeer, The Netherlands).

Reverse transcriptase was performed with 2 μg RNA using Reverse Transcription System (Promega, Leiden, The Netherlands). The RT-PCR reaction was performed in the Eppendorf mastercycler gradient at 25 °C for 10 min, 45 °C for 60 min and 95 °C for 5 min.

The gene expression of *Hsp47*, *αSma*, *Pcol1A1* and *TGF-β1* was determined using the following primers (50 μM) en probes (5 μM) (Sigma Aldrich); 5′-AGACGAGTTGTAGAGTCCAAGAGT-3′(F), 5′-ACCCATGTGT CTCAGGAACCT-3′(R), 5′-CTTCCCGCCATGCCAC-3′ (Probe) (Hsp47); 5′-AGCTCTGGTGTGTGACAATGG-3′(F), 5′-GGAGCATCATCACCAGCAAAG-3′(R), 5′-CCGCCTTACAGAGCC-3′ (Probe) (αSma); 5′-CCCACCGGCCCT ACTG-3′(F), 5′-GACCAGCTTCACCCTTAGCA-3′(R), 5′-CCTCCTGGCTTCCC TG-3′ (Probe) (Pcol1A1); 5′-CCTGGAAAGGGCTCAACAC-3′(F), 5′-CAGT TCTTCTCTGTGGAGCTGA-3′(R), 5′-AGAGCCCTGGATACCAACTACTGCT-3′(Probe) (TGF-β1) and the qPCR mastermix plus (Eurogentec, Maastricht, The Netherlands). The Real-Time PCR reaction was performed in a 7900HT Real Time PCR (Applied Biosystems, Bleiswijk, The Netherlands) with 1 cycle of 10 min at 95 °C and 45 cycles of 15 s at 95 °C and 1 min at 60 °C. Ct values were corrected for the Ct values of the housekeeping gene *Gapdh* (ΔCt) 5′-GAACATCATCCCTGCATCCA-3′(F), 5′-CCAGTGAGCTTCCCGTTCA-3′(R), 5′-CTTGCCCACAGCCTTGGC AGC-3′(Probe) (Gapdh) and compared with the control (ΔΔCt). Results are displayed as fold induction of the gene ($2^{-\Delta\Delta Ct}$).

Gene expressions of Connective Tissue Growth Factor (*Ctgf*) and *PDGF-B* were determined using the following primers (50 μM); 5′-ACACAAGGGTCTTCTGCGA-3′(F), 5′-TTGCAACTGCTTTGGAAGGAC-3′(R) (Ctgf); 5′-CTGCCTCTCTGCTGCTACCT-3′(F), 5′-TTCCGACTCGACTCCAGA AT-3′(R) (*PDGF*-B) and the sybr green mastermix (GC Biotech, Alphen aan de Rijn, The Netherlands). The Real Time PCR reaction was performed on a 7900HT Real Time PCR (Applied Biosystems) with 1 cycle of 10 min at 95 °C and 45 cycles of 15 s at 95 °C and 25 s at 60 °C with a dissociation stage thereafter (95 °C, 15 s: 60 °C, 15 s: 95 °C, 15 s). Ct values were corrected for the Ct values of the housekeeping gene *Gapdh* (ΔCt) 5′-CGCTGGTGCTGAGTATGTCG-3′(F), 5′-CTGTGGTCATGAGCCCTTCC-3′(R) (Gapdh) and compared with the control (ΔΔCt). Results are displayed as fold induction of the gene ($2^{-\Delta\Delta Ct}$).

Collagen 1 protein expression. Following treatment, the triplicate slices were pooled and snap frozen. The slice tissue was lysed for 1 h on ice with RIPA buffer (1 Protease inhibitor cocktail tablet (Boehringer Ingelheim, Alkmaar, The Netherlands), 50 mM Tris/HCl pH7.5, 150 mM NaCL, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS). The tissue was homogenized on ice by a Potter homogenizer and centrifuged for 1 h at 4 °C at maximum speed. Protein concentrations were determined in the supernatant using a Biorad DC protein assay according to the protocol provided by the manufacturer. Lysates were diluted with 4× SDS sample buffer (50 mM TrisHCl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.05% Bromophenol Blue) and boiled for 2 min. 100 μg of tissue lysate was size fractionated on a 7.5% sodium dodecyl sulphate poly acrylamide gel by electrophoresis and transferred to an activated polyvinylidene difluoride membrane (Biorad). After blocking for 1 h in Tris buffered saline supplemented with 5% Blocking Grade Powder (Biorad) and 0.1% Tween-20, immunodetection of collagen-1 (1:1000, Rockland Immunochemicals, Gilbertsville, PA, USA) was performed. Binding of the antibody was determined using Horseradish Peroxidase conjugated secondary goat anti-rabbit and tertiary rabbit anti-goat antibody (DAKO, Heverlee, Belgium). Visualization was performed with Western Lightning Plus-ECL, (PerkinElmer, Groningen, The Netherlands) and equal protein loading was confirmed by immunostaining with Monoclonal anti B-actin (clone AC-74) (Sigma Aldrich). Immunohistochemical staining was performed on cryostat section (4 μM) of PCLS incubated for 0 and 48 h with rabbit anti-collagen I (1:200, Rockland) according to standard immunoperoxidase methods (Beljaars et al., 1999).

Statistics. A minimum of three different livers was used for each experiment, using slices in triplicate from each liver. As for each experiment control slices are included, the number of control slices is larger than the number of slices incubated with compounds. The results are expressed as means ± S.E.M. The results of the treatments were compared to the untreated controls using the paired, one-tailed Student's-*t*-test. A p-value <0.05 was considered significant. Statistical differences in ATP were determined using the values relative to the control values in the same experiment. Real-time PCR results were compared using the mean ΔΔCt values.

Results

Prolonged incubation of liver slices

PCLS were incubated up to 72 h. Slices were viable up to 48 h of incubation, as determined by ATP content (Olinga and Groothuis, 2001), and the viability was decreased significantly after 72 h of incubation compared to the slices after 48 h of incubation (Fig. 1A). During incubation the protein content decreases, apparently due to loss of cells. But our ATP per protein data show that the remaining cells in the slices are viable.

The gene expression of the fibrosis markers *Hsp47*, *αSma* and *Pcol1A1* initially decreased during 24 h of incubation, but was significantly increased after 48 and 72 h of incubation to values 2–44 fold higher than those of fresh slices directly after slicing (Fig. 1B), indicating that there was indeed an early onset of fibrosis in the slices after 48 and 72 h. In Fig. 1C a representative immunohistochemical staining of collagen 1 is shown in PCLS incubated for 0 and 48 h. Protein expression of collagen 1, measured by Western blot analysis, was decreased at 1 and 24 h of incubation. After 48 h the collagen 1 expression was increased again to the same level as in fresh slices (Fig. 1D). Gene expression of *PDGF-B* was increased after 24 and 48 h of incubation, while the gene expression of *TGF-β1* was not changed during incubation (Fig. 1E). Since 72 h of incubation caused a significant decrease in ATP content of PCLS compared to earlier time points, the experiments with antifibrotic compounds were conducted during 48 h of incubation.

Addition of PDGF-BB and TGF-β1

To confirm that PDGF-BB and TGF-β1 are able to induce the PDGF and TGFβ signaling pathways in PCLS, PCLS were incubated with PDGF-BB and TGF-β1 and the gene expression of the fibrosis markers was assessed. PCLS incubated for 48 h without these factors were used as a control and the gene expression of *Hsp47*, *αSma* and *Pcol1A1* in these controls was set at 1 (Fig. 2B). Both PDGF-BB (10–100 ng/ml) as well as TGF-β1 (1–5 ng/ml) induced a concentration dependent further increase in the gene-expression of *Hsp47*, *αSma* and *Pcol1A1* in PCLS after 48 h of incubations (Fig. 2B). Slice viability was not changed after addition of PDGF-BB, however, ATP content in PCLS decreased to 50% by TGF-β1 (Fig. 2A).

The effect of PDGF-inhibitors

Slices were incubated with a range of concentrations of the antifibrotic compounds imatinib (1–10 μM), sorafenib (0.5–2 μM) or sunitinib (0.5–5 μM) during 48 h. As indicated by the ATP content of the slices, the antifibrotic drugs did not influence the viability of the slices at the concentrations used (Fig. 3A), and the protein content per slice was not changed after addition of the compounds compared to the control slices at 48 h. In PCLS incubated for 48 h in the presence of imatinib, sorafenib or sunitinib, a significant decrease of the gene expression of *αSma* and *Pcol1A1* was found compared to control slices incubated for 48 h (Fig. 3B). The *Hsp47* expression was also significantly decreased by imatinib or sunitinib (Fig. 3B), but not by sorafenib. With 10 μM of imatinib and 1 and 5 μM of sunitinib, the gene expression of fibrosis markers was even significantly lower than that of the fibrosis markers in PCLS directly after slicing, but not lower than the values after 24 h of incubation (Fig. 3B).

After 48 h not only the gene expression of *Pcol1A1*, but also the protein expression of collagen 1 was decreased in PCLS by 10 μM imatinib compared to control slices (Fig. 4). Collagen 1 protein expression was not changed in slices exposed to sorafenib or sunitinib compared to control slices incubated for 48 h (Fig. 4).

The effect of TGFβ-inhibitors

Incubation with rosmarinic acid (120–270 μM), perindopril (10–100 μM), tetrandrine (1–10 μM) or pirfenidone (0.5–2.5 mM) did not result in a decreased ATP content of slices incubated for 48 h

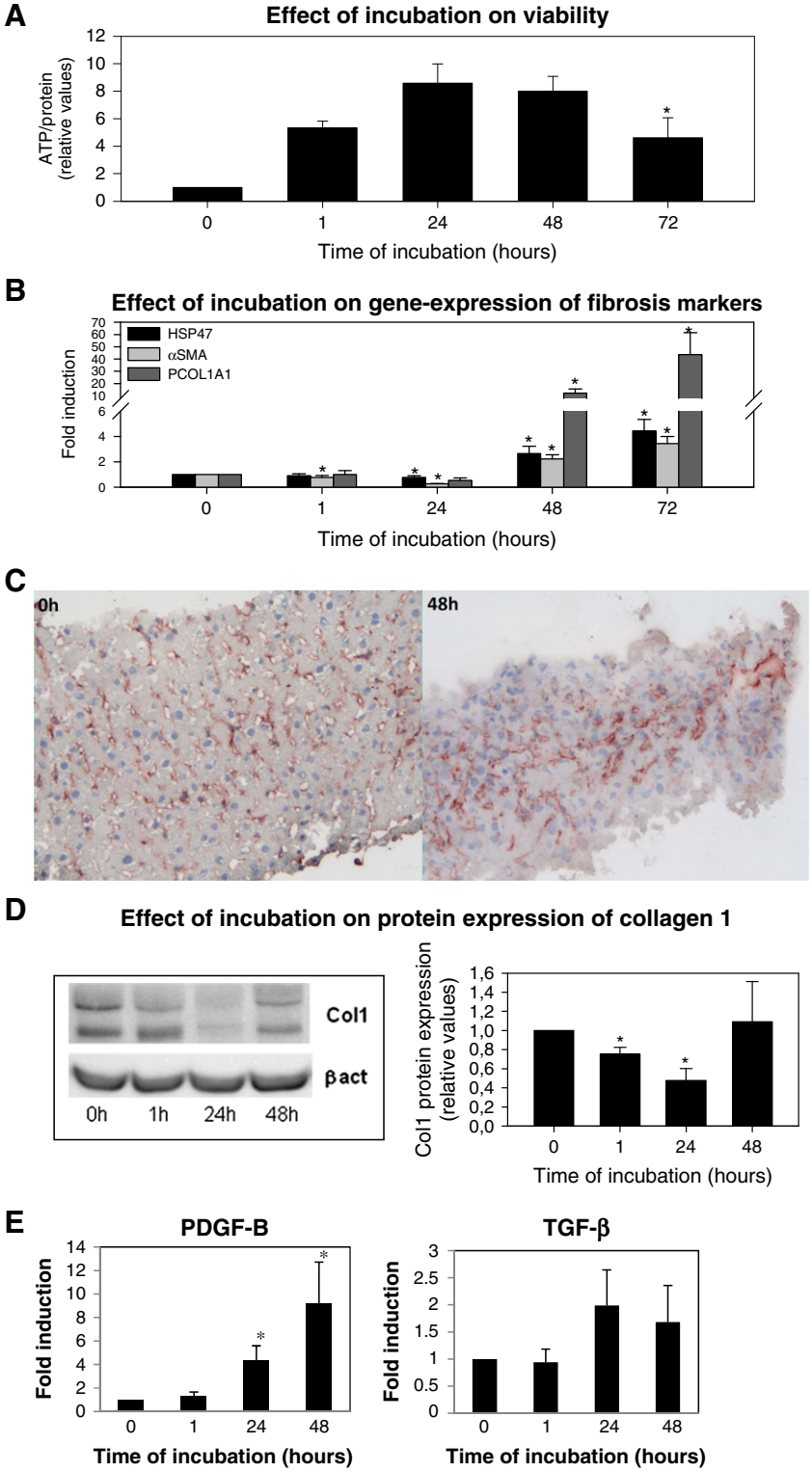

Fig. 1. Prolonged incubation of rat PCLS. The effect of incubation for 0 h (n = 18), 1 h (n = 10), 24 h (n = 7). 48 h (n = 18) and 72 h (n = 7) of PCLS on the viability of PCLS as measured by ATP content (A), effect of incubation for 0 h (n = 18), 1 h (n = 10), 24 h (n = 7). 48 h (n = 18) and 72 h (n = 7) of PCLS on the gene-expression of fibrosis markers *Hsp47*, *αSma* and *Pcol1A1* (B), microscopic pictures (20×) of cryostat sections of control liver slices incubated for 0 and 48 h stained for collagen I (C), and the effect of incubation for 0 h (n = 6), 1 h (n = 3), 24 h (n = 3) and 48 h (n = 6) of PCLS on the collagen 1 protein expression measured by Western blot (D) and the effect of incubation for 0, 1, 24 and 48 (n = 4) hours on *PDGF-B* and *TGF-β1* gene expression (E). *$p < 0.05$ vs. 0 h. Data are expressed as mean +/− SEM.

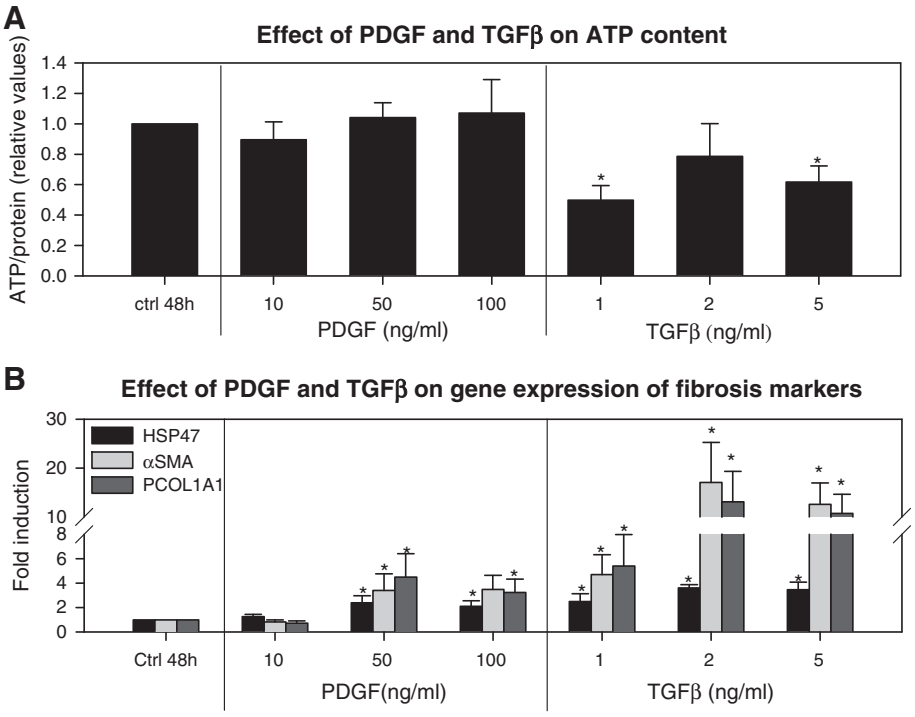

Fig. 2. The effect of PDGF and TGFβ on the viability and expression of fibrosis markers in PCLS. Viability (ATP content) (A) and gene-expression of fibrosis markers *Hsp47*, *αSma* and *Pcol1A1* (B) of PCLS incubated with 10 (n = 3), 50 (n = 8), or 100 ng/ml PDGF (n = 3) or 1 (n = 3), 2 (n = 3), or 5 ng/ml TGFβ (n = 9) for 48 h. $^{*}p < 0.05$ vs. ctrl 48 h. Data are expressed as mean +/− SEM.

(Fig. 5A). In addition, the protein content per slice was not changed after addition of the compounds compared to the control slices at 48 h. The viability of slices incubated with valproic acid (0.1–1 mM) was decreased to a minimum of 62% compared to control slices, however only at 0.5 mM (Fig. 5A). Concentrations higher than 1 mM of valproic acid decreased slice viability considerably (data not shown).

The gene expression of *αSma* was inhibited by 120 μM of rosmarinic acid and the *Hsp47* gene expression was decreased after incubation

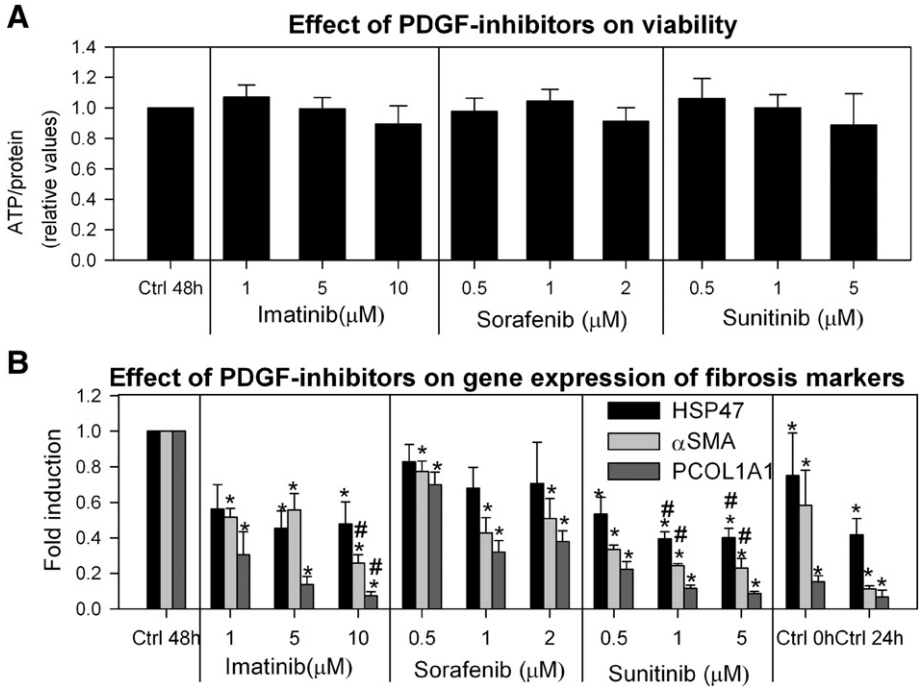

Fig. 3. The effect of PDGF-inhibitors on viability and expression of fibrosis markers in PCLS. Viability measured by ATP content of PCLS incubated with imatinib, sorafenib or sunitinib (A), gene expression of fibrosis markers *Hsp47*, *αSma* and *Pcol1A1* of PCLS incubated with imatinib, sorafenib or sunitinib (B). $^{*}p < 0.05$ vs. ctrl 48 h and $^{\#}p < 0.05$ vs. ctrl 0 h. n = 3, data are expressed as mean +/− SEM.

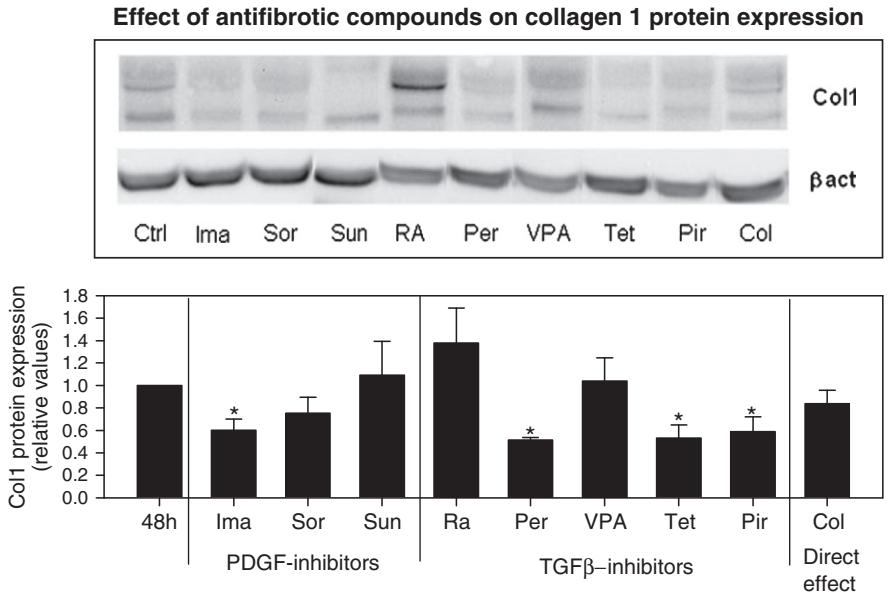

Fig. 4. Collagen 1 protein expression of PCLS. Collagen 1 protein expression of PCLS, incubated in the presence of PDGF-inhibitors Ima (imatinib 10 μM), Sor (sorafenib 2 μM), Sun (sunitinib 5 μM) and TGFβ-inhibitors RA (rosmarinic acid 270 μM), Per (perindopril 100 μM), VPA (valproic acid 1 mM), Tet (tetrandrine 10 μM), Pir (pirfenidone 2.5 mM) or inhibitor with a direct effect on collagen, Col (Colchicine 200 nM) for 48 h. $^{*}p < 0.05$ vs. ctrl 48 h. n = 3, data are expressed as mean +/− SEM.

with 120 μM and 200 μM rosmarinic acid (Fig. 5B). The gene-expression of *Pcol1A1* was inhibited by 0.5 mM pirfenidone (Fig. 5B). Likewise, collagen 1 protein expression was inhibited by pirfenidone (2.5 mM) (Fig. 4). The gene expression of *αSma* was only inhibited by 1 mM of valproic acid, while *Pcol1A1* gene expression was only marginally inhibited by 20% by 100 μM of perindopril and 10 μM of tetrandrine (Fig. 5B). However, the collagen 1 protein expression in PCLS was inhibited by almost 50% by these concentrations of perindopril and tetrandrine (Fig. 4).

The effect of colchicine

Slices incubated with colchicine (30–200 nM) resulted in an ATP content decrease of 20% after 48 h compared to control slices (Fig. 6A). Only 100 nM of colchicine inhibited the gene expression of *αSma* and *Pcol1A1* after 48 h of incubation (Fig. 6B). However, the collagen 1 protein expression in PCLS was not significantly inhibited by colchicine (Fig. 4).

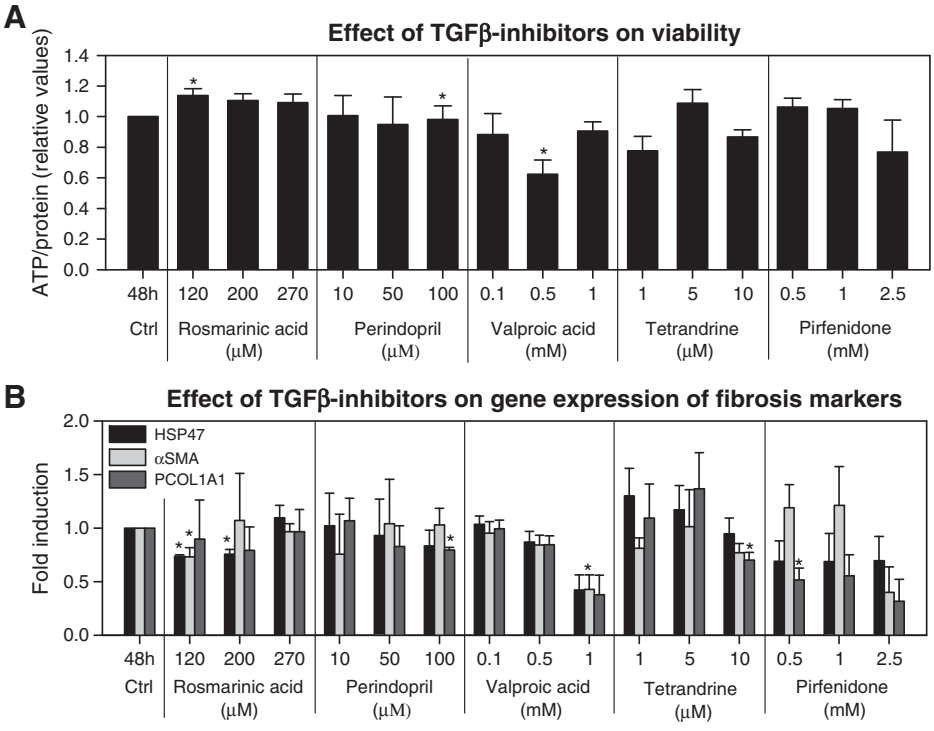

Fig. 5. The effect of TGFβ-inhibitors on viability and expression of fibrosis markers in PCLS. Viability measured by ATP content (A) and gene expression of fibrosis markers *Hsp47*, *αSma* and *Pcol1A1* (B) of PCLS incubated for 48 h with TGFβ inhibitors rosmarinic acid (n = 3), perindopril (n = 3), valproic acid (n = 3), tetrandrine (n = 3) and pirfenidone (n = 4). $^{*}p < 0.05$ vs. ctrl 48 h. Data are expressed as mean +/− SEM.

The effect of prolonged incubation and antifibrotic compounds on the Ctgf gene expression

As our results showed that the gene expression of *TGFβ* was not altered during incubation and the addition of TGFβ-inhibitors had only marginal effects on the gene expression of the fibrosis markers, we wanted to investigate whether the TGFβ pathway was activated during culture of the PCLS. Therefore, the effect of prolonged culture on the gene expression of *Ctgf* was investigated, as CTGF is a downstream mediator of TGFβ (Brigstock, 2010). The results in Fig. 7A show that the gene expression of *Ctgf* was increased 2 fold after 24 h and 8 fold after 48 h of incubation, indicating the possible activation of the TGFβ pathway (Fig. 7A). Addition of TGF-β1 to the incubation medium strongly increased the gene expression of *Ctgf* dose-dependently up to 23 fold compared to the control incubation at 48 h and thus 184 fold compared to the 1 hour control, while addition of PDGF did not have an effect (Fig. 7B).

Unexpectedly, the alleged TGFβ-inhibitors perindopril and rosmarinic acid caused an increase in the expression of *Ctgf* (Fig. 7D), while valproic acid, tetrandrine and pirfenidone had no significant effect on the gene expression of *Ctgf* (Fig. 7D). In contrast, colchicine at a concentration of 30 nM decreased the gene-expression of *Ctgf* but higher concentrations did not have an effect (Fig. 7E). The *Ctgf* gene expression was also inhibited in PCLS by imatinib and sunitinib but only at the lowest concentrations tested (Fig. 7C).

Discussion

The aim of this study was to characterize the early onset of fibrosis in PCLS, and determine if this in vitro model can be used to investigate the effect of antifibrotic compounds in order to confirm the use of PCLS as a model to test antifibrotic compounds.

It was shown before that hepatocytes, Kupffer cells and HSC are present and functional in liver slices and this enables the study of inflammatory reactions in its multi-cellular context (Olinga et al., 2001). The increased gene expression of the fibrosis markers *Hsp47*, *αSma* and *Pcol1A1* after 48 and 72 h of incubation revealed an early onset of fibrosis during culture of PCLS as was also shown earlier (van de Bovenkamp et al., 2008). A possible explanation for this phenomenon could be that the slice preparation causes damage to the surface of slices which may trigger repair and regenerative responses, resulting in activation of HSC, proliferation of myofibroblasts and increased collagen production (Vickers et al., 2004). The significant increase of *Hsp47* gene expression in liver slices during 48 and 72 h of incubation suggests activation of HSC. In addition, the increased *αSma* expression suggests further activation of HSC and transformation of HSC into myofibroblasts in the liver slices. Besides an increase in myofibroblasts, increased *αSma* mRNA levels can also indicate an increase in the number of fibroblasts (Desmouliere et al., 1993). Proliferating myofibroblasts are known to produce large amounts of collagen type I (Kawada et al., 1996), therefore the increased gene expression of *Pcol1A1* in the liver slices is another indication of fibrogenesis in the liver slices during incubation up to 72 h. Furthermore, after an initial decrease in collagen 1 protein levels, levels were increased again at 48 h.

In conclusion, these data show that the early onset of fibrosis was induced in rat liver slices incubated for 48 h, while the viability is maintained. The increase in *PDGF-B* indicated that the PDGF pathway may be involved. However, the involvement of the TGFβ pathway by newly synthesized TGFβ was not confirmed as *TGFβ* gene expression was not increased. Nevertheless, the *Ctgf* gene expression, which is a downstream mediator in the TGFβ pathway, was increased. It can be speculated that the increased *Ctgf* expression is mediated by the release of TGFβ already present in the slice or that *Ctgf* is induced by another pathway.

Addition of PDGF-BB and TGF-β1

Since *PDGF-B*, but not *TGFβ* gene expression was up-regulated after 48 h of incubation, we wanted to elucidate whether PDGF-BB and TGF-β1 could activate the PDGF- and TGFβ signaling pathway in the slices. PCLS were cultured in the presence of PDGF-BB or TGF-β1 for 48 h. Both growth factors could indeed further increase the fibrosis markers after 48 h of incubation, suggesting that the slices are responsive to both PDGF-BB and TGF-β1. Moreover, the gene expression of *Ctgf* was increased strongly by the addition of TGF-β1.

The effect of PDGF-inhibitors

To investigate whether these rat liver slices can be used to study the effect of PDGF-inhibitors, the effects of imatinib, sorafenib and sunitinib were determined during 48 hour incubations. The concentrations of the three compounds used in the current study are in line with plasma concentrations in patients in clinical studies treated for cancer. The C_{max} concentration found in vivo in these patients administered with the effective dose of imatinib, sorafenib or sunitinib was respectively about 5 μM (Leveque and Maloisel, 2005), 9 μM (Strumberg et al., 2007) and 14 μM (Minkin et al., 2008).

In this study, all three kinase inhibitors acting on the PDGF pathway caused a significant and substantial decrease in *Hsp47*, *αSma* and *Pcol1A1* gene expressions. The gene expression of the fibrosis markers in the presence of imatinib and sunitinib was even lower than in PCLS after incubation for 1 h, but not different from 24 h. This indicates that these PDGF-inhibitors inhibit the increase of fibrosis markers from 24 h up to 48 h. In addition, imatinib also caused a decrease in collagen 1 protein expression, by preventing the increase of collagen 1 protein expression from 24 h to 48 h, which is in line with studies in human dermal fibroblasts (Distler et al., 2007). The effects of imatinib in PCLS are well in line with the antifibrotic effects found in vivo and in vitro. Imatinib was previously shown to have antifibrotic effects in

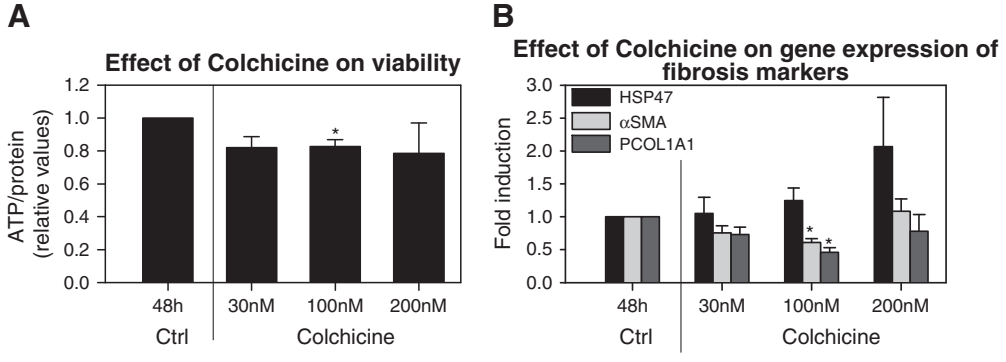

Fig. 6. The effect of colchicine on viability and expression of fibrosis markers in PCLS. Viability measured by ATP content (A) and gene expression of fibrosis markers *Hsp47*, *αSma* and *Pcol1A1* (B) of PCLS incubated with 30, 100 or 200 nM colchicine for 48 h. *$p < 0.05$ vs. ctrl 48 h. $n = 3$, data are expressed as mean +/− SEM.

pig-serum induced fibrosis in rats, in the early phase of BDL-induced fibrosis in rats, in BDL-induced fibrotic rat PCLS and in CCl_4 treated mice (Buchdunger et al., 2000; Kuo et al., 2012; Neef et al., 2006; van de Bovenkamp et al., 2006; Yoshiji et al., 2005b), and induced apoptosis in hepatic stellate cells in culture (Kuo et al., 2012).

Although both sorafenib and sunitinib showed clear antifibrotic effects on gene expression of the three marker genes, the protein expression of collagen 1 did not change after addition of sorafenib and sunitinib, whereas sorafenib suppressed the protein level of collagen and HSC growth in vivo in rats with established fibrosis (BDL and dimethylnitrosamine) (Wang et al., 2010), and sunitinib showed a decrease in collagen accumulation in CCl_4 rats (Tugues et al., 2007). This discrepancy may be explained by the longer exposure time in these in vivo experiments, suggesting that these compounds need a longer incubation time to express their effect on the protein level or, alternatively, by the possibility that these compounds are only effective in established fibrosis. In HSC, imatinib has already an effect on *Pcol1A1* gene expression after 12 h of incubation (Kim et al., 2012), while the earliest effect of sorafenib on *Pcol1A1* in HSC was described after 24 h of incubation (Wang et al., 2010). This earlier effect on *Pcol1A1* mRNA expression with imatinib could imply that also collagen 1 protein is expressed at an earlier time point compared to sorafenib. The same could be the case for sunitinib, however to our knowledge up to now, the time course of the effect of sunitinib on *Pcol1A1* gene expression in

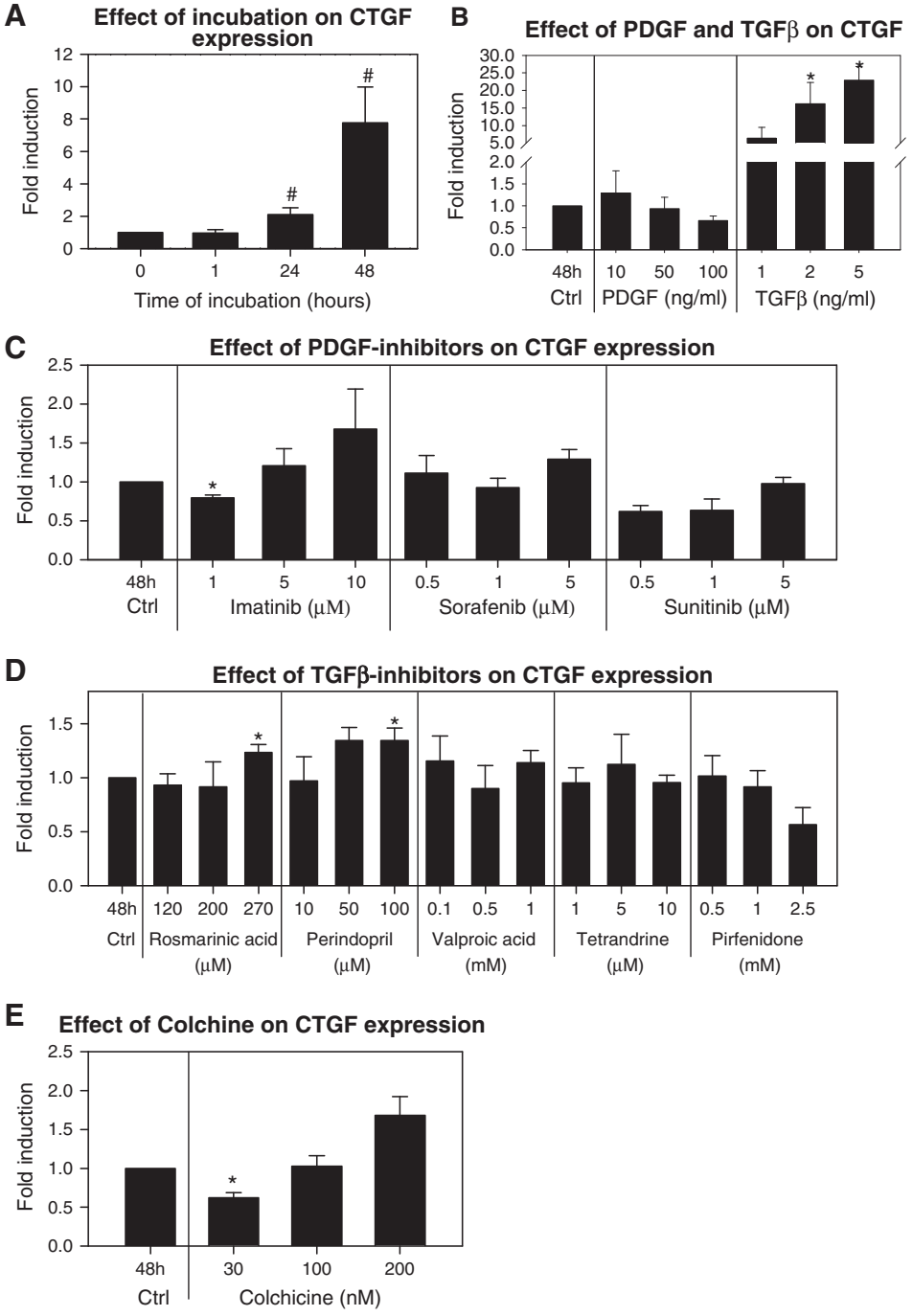

Fig. 7. Gene-expression of *Ctgf* in PCLS. Gene-expression of *Ctgf* of PCLS incubated up to 48 h in control PCLS (n = 4) (A), PCLS incubated with PDGF (n = 3) or TGFβ (n = 4) for 48 h (B), PCLS incubated with PDGF-inhibitors imatinib, sorafenib and sunitinib for 48 h (n = 3) (C), PCLS incubated with TGFβ-inhibitors rosmarinic acid, perindopril, valproic acid, tetrandrine and pirfenidone for 48 h (n = 3) (D) and PCLS incubated with colchicine for 48 h (n = 3) (E). $^{\#}p < 0.05$ vs. ctrl 0 h, $^{*}p < 0.05$ vs. ctrl 48 h. Data are expressed as mean +/− SEM.

HSC has not been studied. Our results are in line with those of Hennenberg et al., who showed reduced gene expression of αSma and *Pcol1A1* by sorafenib after treatment of BDL rats and in culture-activated primary HSC (Hennenberg et al., 2010). Similarly, Friedman showed antifibrotic effects of sorafenib in vitro in LX2 cells (3.5–15 μM), and in vivo in rats with hepatic fibrosis due to thioacetamide by reducing the gene expression of, among other things, collagen I (Friedman, 2011). In line with our studies, 1 μM of sunitinib caused a decrease in LX-2 viability and collagen expression in vitro, and in cirrhotic rats in vivo (40 mg/kg) a decrease in *αSma* abundance, probably through the inhibition of the PDGF signaling pathway in HSC (Tugues et al., 2007).

All these results indicate that these PDGF-inhibitors have similar effects in this ex vivo model of early onset of liver fibrosis as in vivo in animals with hepatic fibrosis. Therefore, it can be concluded that the PDGF pathway is activated in the PCLS and that these PCLS can be used to test antifibrotic effects of PDGF-inhibitors.

The effect of TGFβ-inhibitors

The results of the TGFβ-inhibitors on the gene expression of fibrosis markers in PCLS are less pronounced than the results of the PDGF-inhibitors.

Rosmarinic acid had minor effects on the fibrosis markers. In contrast, in BDL mice in vivo, rosmarinic acid treatment caused a decrease of hepatic gene expression of *αSma* and *Pcol1A1*, Moreover, 270 μM of rosmarinic acid caused a decrease of pro-collagen 1A1 in activated rat HSC (Yang et al., 2012) and inhibition of proliferation of rat HSC (HSC-T6 cell line) (Zhang et al., 2011).

Also perindopril, an AT-II receptor antagonist, affected the fibrosis process in the PCLS only moderately. Activation of the angiotensin-II (AT-II) receptor induces HSC contraction and proliferation and increases *TGFβ* gene expression in activated HSC in vitro (Bataller et al., 2000; Yoshiji et al., 2001). In rat in vivo, perindopril caused inhibition of *Pcol1A1* gene expression in HSC (Yoshiji et al., 2005a) and inhibited the transformation of HSC into myofibroblasts (Xu et al., 2006). Yeki found only an inhibition of collagen 1 mRNA with a high dose (8 mg/kg) of perindopril in BDL rats (Yeki et al., 2009). Despite the marginal effects on the gene expression of fibrosis markers in our study, collagen 1 protein expression was 50% lower after 48 hour incubation with the highest concentration of perindopril (100 μM), whereas *Pcol1A1* gene expression was reduced by only 20%. This might be explained by the fact that perindopril has an effect on MMPs, by inhibiting Timp expression, and these MMPs are involved in the degradation of collagens (Warner et al., 2007).

Valproic acid reduced the expression of the three fibrosis marker genes in the PCLS only at the highest concentration of 1 mM. Watanabe et al. also used a concentration of valproic acid of 1 mM in cultured human HSC, which was slightly toxic, and showed inhibition of col1A1 and TGFβ1 gene- and protein expressions (Watanabe et al., 2011). Mannaerts et al. showed that valproic acid decreased the HSC activation and down-regulated αSma and Pcol1A1 gene and protein expressions in mice in vivo and also decreased HSC activation and down-regulated *αSma* in vitro (Mannaerts et al., 2010). However, valproic acid is a histone deacetylase inhibitor and might inhibit the gene expression of other proteins, and consequently inhibiting their function (Dokmanovic et al., 2007). Therefore, the effect of valproic acid on fibrosis markers could also be caused by hyper acetylation of histones and not only through inhibition of the TGFβ pathway. On the other hand, Fisher et al. studied the effect of 0.7, 2.1 and 3.5 mM valproic acid in rat slices on protein synthesis and found that the protein synthesis was unaffected after 24 h of incubation (Fisher et al., 1991).

Hsu et al. showed that the TGFβ-inhibitor tetrandrine (5 μM) inhibited αSma and collagen protein expression in HSC-T6 cells for at least 50% (Hsu et al., 2007). Our results show an inhibiting effect with 10 μM of tetrandrine on the *Pcol1A1* gene expression by 25% and on the collagen 1 protein expression by 50%. *αSma* and *Hsp47* gene expressions were not changed. A higher concentration of tetrandrine could not be tested due to loss of viability of the slices (data not shown). Chen et al. suggested that the effect of tetrandrine might be due to an up-regulation of Smad 7, as Smad7 was increased by tetrandrine in TGFβ stimulated HSC, which in turn blocks the TGFβ expression and its downstream signaling (Chen et al., 2005).

The antifibrotic compound pirfenidone showed effects in experimental and human pulmonary and liver fibrosis (Armendariz-Borunda et al., 2006; Garcia et al., 2002; Iyer et al., 1995; Navarro-Partida et al., 2012). In PCLS, 0.5–2.5 mM pirfenidone inhibited the *Hsp47* and *Pcol1A1* gene expressions. Furthermore, *αSma* gene expression and collagen 1 protein expression were also inhibited with 2.5 mM of pirfenidone. Our results are in line with those found by di Sario et al. who showed that pirfenidone (0.1–1 mM) down-regulated the expression of pro-collagen gene expression in rat HSC in culture (Di Sario et al., 2002). In addition, it decreased *αSma* and *collagen 1* gene expression in either TGFβ or serum activated LX-2 cells (Zhao et al., 2009). Furthermore, in a pilot study with patients with advanced liver fibrosis, pirfenidone treatment (1200 mg/day) for twelve months caused a reduction of fibrosis in 30% of the patients, as determined by down-regulation of protein- and gene expressions of collagen 1a, TGFβ and Timp-1 in liver biopsies (Armendariz-Borunda et al., 2006).

In summary, the observation that after spontaneous induction of fibrosis by prolonged incubation the gene expression of fibrosis markers was only slightly inhibited by a few TGFβ-inhibitors and the fact that *TGF-β1* gene expression was not increased after 48 h might suggest that in the prolonged culture PCLS model the TGFβ pathway is only minimally activated. Therefore, we wanted to elucidate whether the TGFβ-signaling pathway is activated in our model of early onset of fibrosis and can be activated by externally administered TGF-β1. TGF-β1 itself was able to cause an activation of the TGFβ pathway in PCLS, showing that this pathway can be activated in PCLS. As activation of the TGFβ pathway results in increased expression of *Ctgf* (Brigstock, 2010), we assessed the expression of this gene during culture and whether it could be inhibited by the antifibrotic compounds. CTGF is a cysteine rich protein that has been associated with tissue fibrosis in humans (Sonnylal et al., 2010) and it is a downstream mediator in the TGFβ pathway (Brigstock, 2010). In PCLS, the expression of this gene was indeed up-regulated after 48 h of incubation, which may be an indication that the TGFβ signaling pathway is activated. However, this was relatively small compared to that after addition of exogenous TGF-β1. The fact that TGFβ could further increase the expression of *Ctgf* and PDGF could not, confirms that the expression of *Ctgf* is regulated by the TGFβ-pathway but not by the PDGF pathway. However, the results with the antifibrotic compounds in PCLS were not unambiguous. Some TGFβ-inhibitors reduced the increase in *Ctgf* gene expression, but in contrast, perindopril further increased the *Ctgf* gene-expression after 48 h in the PCLS. In addition, also some PDGF-inhibitors reduced the *Ctgf* expression. In HSC, Huang et al. found that perindopril reduced the protein levels of CTGF (Huang et al., 2010) and Yeki et al. found reduced gene expression of *Ctgf* in 6 weeks BDL rats after perindopril treatment during the last 3 weeks (Yeki et al., 2009). Since in our study perindopril showed the opposite effect, it might be that CTGF is not solely triggered by a TGFβ related pathway but can also be activated by a yet unknown mechanism.

The effect of colchicine

In our PCLS, the *αSma* and *Pcol1A1* gene expressions were down-regulated in the presence of 30 nM and 100 nM of colchicine, whereas the *Hsp47* gene expression was up-regulated in these slices. Similarly, Chung et al. also reported that mRNA expression of pro-collagen 1a1 is inhibited by 1 μM colchicine in human fibroblasts (Chung and Kang, 1999). In addition, in activated rat HSC, 30 nM and 100 nM of colchicine caused suppression of αSMA protein- and *TGFβ1* mRNA expressions (Lee et al., 2004). These findings may be explained by the work of Vonk et al. (Vonk et al., 2010). They showed that endoplasmic reticulum

(ER) stress inhibits collagen synthesis, while some collagen-modifying enzymes and chaperones are up-regulated (Vonk et al., 2010). Proper functioning of the ER network requires an intact microtubule system (Terasaki et al., 1986). As colchicine causes disruption of microtubule formation, this may induce ER stress, which in turn might decrease the gene-expression of *Pcol1A1* and the up-regulation of gene-expression of the collagen chaperone *Hsp47*. After treatment with colchicine, we found a slight but non-significant decrease of collagen protein in the PCLS, in line with the in vivo results of Rodriquez who showed a significant reduction of collagen amount in CCl_4 treated rats after administration of colchicine (Rodriguez et al., 1998).

In conclusion

We showed that 48 h of culture of PCLS induced activation of HSC and the early onset of fibrogenesis, which is mainly mediated by the PDGF pathway. Moreover, our data show that the antifibrotic effect of PDGF-inhibitors can be assessed in this model. However, during incubation, the *TGFβ* gene expression was not increased and the TGFβ-inhibitors only slightly reduced the fibrotic process, suggesting that the TGFβ pathway plays only a minor role in PCLS during the onset of fibrosis. Further research is needed to elucidate the role of the TGFβ- and PDGF pathway also in established fibrosis in rat PCLS. Moreover, studies in human PCLS are ongoing to assess whether these pathways and inhibitors of fibrogenesis can be investigated in the human liver.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

References

Armendariz-Borunda, J., Islas-Carbajal, M.C., Meza-Garcia, E., Rincon, A.R., Lucano, S., Sandoval, A.S., Salazar, A., Berumen, J., Alvarez, A., Covarrubias, A., Arechiga, G., Garcia, L., 2006. A pilot study in patients with established advanced liver fibrosis using pirfenidone. Gut 55, 1663–1665.

Bataller, R., Brenner, D.A., 2005. Liver fibrosis. J. Clin. Invest. 115, 209–218.

Bataller, R., Gines, P., Nicolas, J.M., Gorbig, M.N., Garcia-Ramallo, E., Gasull, X., Bosch, J., Arroyo, V., Rodes, J., 2000. Angiotensin II induces contraction and proliferation of human hepatic stellate cells. Gastroenterology 118, 1149–1156.

Beljaars, L., Molema, G., Weert, B., Bonnema, H., Olinga, P., Groothuis, G.M., Meijer, D.K., Poelstra, K., 1999. Albumin modified with mannose 6-phosphate: a potential carrier for selective delivery of antifibrotic drugs to rat and human hepatic stellate cells. Hepatology 29, 1486–1493.

Borkham-Kamphorst, E., Herrmann, J., Stoll, D., Treptau, J., Gressner, A.M., Weiskirchen, R., 2004. Dominant-negative soluble PDGF-beta receptor inhibits hepatic stellate cell activation and attenuates liver fibrosis. Lab. Invest. 84, 766–777.

Brigstock, D.R., 2010. Connective tissue growth factor (CCN2, CTGF) and organ fibrosis: lessons from transgenic animals. J. Cell Commun. Signal. 4, 1–4.

Buchdunger, E., Cioffi, C.L., Law, N., Stover, D., Ohno-Jones, S., Druker, B.J., Lydon, N.B., 2000. Abl protein-tyrosine kinase inhibitor STI571 inhibits in vitro signal transduction mediated by c-kit and platelet-derived growth factor receptors. J. Pharmacol. Exp. Ther. 295, 139–145.

Chen, Y., Li, D., Wu, J., Chen, Y., Lu, H., 2005. Tetrandrine inhibits activation of rat hepatic stellate cells stimulated by transforming growth factor-beta in vitro via up-regulation of Smad 7. J. Ethnopharmacol. 100, 299–305.

Chung, K.Y., Kang, D.S., 1999. Regulation of type I collagen and interstitial collagenase mRNA expression in human dermal fibroblasts by colchicine and D-penicillamine. Yonsei Med. J. 40, 490–495.

Cong, M., Iwaisako, K., Jiang, C., Kisseleva, T., 2012. Cell signals influencing hepatic fibrosis. Int. J. Hepatol. 2012, 158547.

de Graaf, I.A., Olinga, P., de Jager, M.H., Merema, M.T., de Kanter, R., van de Kerkhof, E.G., Groothuis, G.M., 2010. Preparation and incubation of precision-cut liver and intestinal slices for application in drug metabolism and toxicity studies. Nat. Protoc. 5, 1540–1551.

Deng, Z.Y., Li, J., Jin, Y., Chen, X.L., Lu, X.W., 2009. Effect of oxymatrine on the p38 mitogen-activated protein kinases signalling pathway in rats with CCl4 induced hepatic fibrosis. Chin. Med. J. (Engl) 122, 1449–1454.

Desmouliere, A., Geinoz, A., Gabbiani, F., Gabbiani, G., 1993. Transforming growth factor-beta 1 induces alpha-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. J. Cell Biol. 122, 103–111.

Di Sario, A., Bendia, E., Baroni, G., Ridolfi, F., Casini, A., Ceni, E., Saccomanno, S., Marzioni, M., Trozzi, L., Sterpetti, P., Taffetani, S., Benedetti, A., 2002. Effect of pirfenidone on rat hepatic stellate cell proliferation and collagen production. J. Hepatol. 37, 584–591.

Distler, J.H., Jungel, A., Huber, L.C., Schulze-Horsel, U., Zwerina, J., Gay, R.E., Michel, B.A., Hauser, T., Schett, G., Gay, S., Distler, O., 2007. Imatinib mesylate reduces production of extracellular matrix and prevents development of experimental dermal fibrosis. Arthritis Rheum. 56, 311–322.

Dokmanovic, M., Clarke, C., Marks, P.A., 2007. Histone deacetylase inhibitors: overview and perspectives. Mol. Cancer Res. 5, 981–989.

Domitrovic, R., Skoda, M., Marchesi, V.V., Cvijanovic, O., Pugel, E.P., Stefan, M.B., 2012. Rosmarinic acid ameliorates acute liver damage and fibrogenesis in carbon tetrachloride-intoxicated mice. Food Chem. Toxicol. 51, 370–378.

Dooley, S., Ten Dijke, P., 2012. TGF-beta in progression of liver disease. Cell Tissue Res. 347, 245–256.

Fisher, R., Nau, H., Gandolfi, A.J., Brendel, K., 1991. Toxicity of valproic acid in liver slices from Sprague–Dawley rats and domestic pigs. Toxicol. In Vitro 5, 201–205.

Friedman, S.L., 2003. Liver fibrosis—from bench to bedside. J. Hepatol. 38 (Suppl. 1), S38–S53.

Friedman, S.L., 2008. Mechanisms of hepatic fibrogenesis. Gastroenterology 134, 1655–1669.

Friedman, S.L., 2011. Antifibrotic activity of sorafenib in experimental hepatic fibrosis—refinement of targets, dosing and window of efficacy in vivo. Hepatology 54, 360A–1455A.

Garcia, L., Hernandez, I., Sandoval, A., Salazar, A., Garcia, J., Vera, J., Grijalva, G., Muriel, P., Margolin, S., Armendariz-Borunda, J., 2002. Pirfenidone effectively reverses experimental liver fibrosis. J. Hepatol. 37, 797–805.

Helmy, A., Jalan, R., Newby, D.E., Hayes, P.C., Webb, D.J., 2000. Role of angiotensin II in regulation of basal and sympathetically stimulated vascular tone in early and advanced cirrhosis. Gastroenterology 118, 565–572.

Hennenberg, M., Trebicka, J., Kohistani, Z., Stark, C., Nischalke, H.D., Kramer, B., Korner, C., Klein, S., Granzow, M., Fischer, H.P., Heller, J., Sauerbruch, T., 2010. Hepatic and HSC-specific sorafenib effects in rats with established secondary biliary cirrhosis. Lab. Invest. 91, 241–251.

Hsu, Y., Chiu, Y., Cheng, C., Wu, C., Lin, Y., Huang, Y., 2007. Antifibrotic effects of tetrandrine on hepatic stellate cells and rats with liver fibrosis. J. Gastroenterol. Hepatol. 22, 99–111.

Huang, M.L., Li, X., Meng, Y., Xiao, B., Ma, Q., Ying, S.S., Wu, P.S., Zhang, Z.S., 2010. Upregulation of angiotensin-converting enzyme (ACE) 2 in hepatic fibrosis by ACE inhibitors. Clin. Exp. Pharmacol. Physiol. 37, e1–e6.

Iyer, S.N., Wild, J.S., Schiedt, M.J., Hyde, D.M., Margolin, S.B., Giri, S.N., 1995. Dietary intake of pirfenidone ameliorates bleomycin-induced lung fibrosis in hamsters. J. Lab. Clin. Med. 125, 779–785.

Kawada, N., Kuroki, T., Kobayashi, K., Inoue, M., Nakatani, K., Kaneda, K., Nagata, K., 1996. Expression of heat-shock protein 47 in mouse liver. Cell Tissue Res. 284, 341–346.

Kim, Y., Fiel, M.I., Albanis, E., Chou, H.I., Zhang, W., Khitrov, G., Friedman, S.L., 2012. Antifibrotic activity and enhanced interleukin-6 production by hepatic stellate cells in response to imatinib mesylate. Liver Int. 32, 1008–1017.

Kuo, W.L., Yu, M.C., Lee, J.F., Tsai, C.N., Chen, T.C., Chen, M.F., 2012. Imatinib mesylate improves liver regeneration and attenuates liver fibrogenesis in CCL4-treated mice. J. Gastrointest. Surg. 16, 361–369.

Lee, S.J., Kim, Y.G., Kang, K.W., Kim, C.W., Kim, S.G., 2004. Effects of colchicine on liver functions of cirrhotic rats: beneficial effects result from stellate cell inactivation and inhibition of TGF beta1 expression. Chem. Biol. Interact. 147, 9–21.

Leveque, D., Maloisel, F., 2005. Clinical pharmacokinetics of imatinib mesylate. In Vivo 19, 77–84.

Li, G.S., Jiang, W.L., Tian, J.W., Qu, G.W., Zhu, H.B., Fu, F.H., 2010. In vitro and in vivo antifibrotic effects of rosmarinic acid on experimental liver fibrosis. Phytomedicine 17, 282–288.

Liu, Y., Wen, X.M., Lui, E.L., Friedman, S.L., Cui, W., Ho, N.P., Li, L., Ye, T., Fan, S.T., Zhang, H., 2009. Therapeutic targeting of the PDGF and TGF-beta-signaling pathways in hepatic stellate cells by PTK787/ZK22258. Lab. Invest. 89, 1152–1160.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Mannaerts, I., Nuytten, N.R., Rogiers, V., Vanderkerken, K., van Grunsven, L.A., Geerts, A., 2010. Chronic administration of valproic acid inhibits activation of mouse hepatic stellate cells in vitro and in vivo. Hepatology 51, 603–614.

Minkin, P., Zhao, M., Chen, Z., Ouwerkerk, J., Gelderblom, H., Baker, S.D., 2008. Quantification of sunitinib in human plasma by high-performance liquid chromatography–tandem mass spectrometry. J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 874, 84–88.

Navarro-Partida, J., Martinez-Rizo, A.B., Gonzalez-Cuevas, J., Arrevillaga-Boni, G., Ortiz-Navarrete, V., Armendariz-Borunda, J., 2012. Pirfenidone restricts Th2 differentiation in vitro and limits Th2 response in experimental liver fibrosis. Eur. J. Pharmacol. 678, 71–77.

Neef, M., Ledermann, M., Saegesser, H., Schneider, V., Widmer, N., Decosterd, L.A., Rochat, B., Reichen, J., 2006. Oral imatinib treatment reduces early fibrogenesis but does not prevent progression in the long term. J. Hepatol. 44, 167–175.

Olinga, P., Groothuis, G.M.M., 2001. Use of human tissue slices in drug targeting research. Drug Target. Organ-Specif. Strateg. 12, 309–331.

Olinga, P., Merema, M.T., de Jager, M.H., Derks, F., Melgert, B.N., Moshage, H., Slooff, M.J., Meijer, D.K., Poelstra, K., Groothuis, G.M., 2001. Rat liver slices as a tool to study LPS-induced inflammatory response in the liver. J. Hepatol. 35, 187–194.

Osakabe, N., Yasuda, A., Natsume, M., Yoshikawa, T., 2004. Rosmarinic acid inhibits epidermal inflammatory responses: anticarcinogenic effect of *Perilla frutescens* extract in the murine two-stage skin model. Carcinogenesis 25, 549–557.

Rodriguez, L., Cerbon-Ambriz, J., Munoz, M.L., 1998. Effects of colchicine and colchiceine in a biochemical model of liver injury and fibrosis. Arch. Med. Res. 29, 109–116.

Schaefer, C.J., Ruhrmund, D.W., Pan, L., Seiwert, S.D., Kossen, K., 2011. Antifibrotic activities of pirfenidone in animal models. Eur. Respir. Rev. 20, 85–97.

Sonnylal, S., Shi-Wen, X., Leoni, P., Naff, K., Van Pelt, C.S., Nakamura, H., Leask, A., Abraham, D., Bou-Gharios, G., de Crombrugghe, B., 2010. Selective expression of

connective tissue growth factor in fibroblasts in vivo promotes systemic tissue fibrosis. Arthritis Rheum. 62, 1523–1532.
Strumberg, D., Clark, J.W., Awada, A., Moore, M.J., Richly, H., Hendlisz, A., Hirte, H.W., Eder, J.P., Lenz, H.J., Schwartz, B., 2007. Safety, pharmacokinetics, and preliminary antitumor activity of sorafenib: a review of four phase I trials in patients with advanced refractory solid tumors. Oncologist 12, 426–437.
Terasaki, M., Chen, L.B., Fujiwara, K., 1986. Microtubules and the endoplasmic reticulum are highly interdependent structures. J. Cell Biol. 103, 1557–1568.
Tsukada, S., Parsons, C.J., Rippe, R.A., 2006. Mechanisms of liver fibrosis. Clin. Chim. Acta 364, 33–60.
Tugues, S., Fernandez-Varo, G., Munoz-Luque, J., Ros, J., Arroyo, V., Rodes, J., Friedman, S.L., Carmeliet, P., Jimenez, W., Morales-Ruiz, M., 2007. Antiangiogenic treatment with sunitinib ameliorates inflammatory infiltrate, fibrosis, and portal pressure in cirrhotic rats. Hepatology 46, 1919–1926.
van de Bovenkamp, M., Groothuis, G.M., Draaisma, A.L., Merema, M.T., Bezuijen, J.I., van Gils, M.J., Meijer, D.K., Friedman, S.L., Olinga, P., 2005. Precision-cut liver slices as a new model to study toxicity-induced hepatic stellate cell activation in a physiologic milieu. Toxicol. Sci. 85, 632–638.
van de Bovenkamp, M., Groothuis, G.M.M., Meijer, D.K.F., Olinga, P., 2006. Precision-cut fibrotic rat liver slices as a new model to test the effects of anti-fibrotic drugs in vitro. J. Hepatol. 45, 696–703.
van de Bovenkamp, M., Groothuis, G.M., Meijer, D.K., Olinga, P., 2008. Liver slices as a model to study fibrogenesis and test the effects of anti-fibrotic drugs on fibrogenic cells in human liver. Toxicol. In Vitro 22, 771–778.
Vickers, A.E., Saulnier, M., Cruz, E., Merema, M.T., Rose, K., Bentley, P., Olinga, P., 2004. Organ slice viability extended for pathway characterization: an in vitro model to investigate fibrosis. Toxicol. Sci. 82, 534–544.
Vonk, L.A., Doulabi, B.Z., Huang, C.L., Helder, M.N., Everts, V., Bank, R.A., 2010. Endoplasmic reticulum stress inhibits collagen synthesis independent of collagen-modifying enzymes in different chondrocyte populations and dermal fibroblasts. Biochem. Cell Biol. 88, 539–552.
Wang, Y., Gao, J., Zhang, D., Zhang, J., Ma, J., Jiang, H., 2010. New insights into the antifibrotic effects of sorafenib on hepatic stellate cells and liver fibrosis. J. Hepatol. 53, 132–144.
Warner, F.J., Lubel, J.S., McCaughan, G.W., Angus, P.W., 2007. Liver fibrosis: a balance of ACEs? Clin. Sci. (Lond.) 113, 109–118.
Watanabe, T., Tajima, H., Hironori, H., Nakagawara, H., Ohnishi, I., Takamura, H., Ninomiya, I., Kitagawa, H., Fushida, S., Tani, T., Fujimura, T., Ota, T., Wakayama, T., Iseki, S., Harada, S., 2011. Sodium valproate blocks the transforming growth factor (TGF)-beta1 autocrine loop and attenuates the TGF-beta1-induced collagen synthesis in a human hepatic stellate cell line. Int. J. Mol. Med. 28, 919–925.
Xu, W., Song, S., Huang, Y., Gong, Z., 2006. Effects of perindopril and valsartan on expression of transforming growth factor-beta-Smads in experimental hepatic fibrosis in rats. J. Gastroenterol. Hepatol. 21, 1250–1256.
Yang, M.D., Chiang, Y.M., Higashiyama, R., Asahina, K., Mann, D.A., Mann, J., Wang, C.C., Tsukamoto, H., 2012. Rosmarinic acid and baicalin epigenetically derepress peroxisomal proliferator-activated receptor gamma in hepatic stellate cells for their antifibrotic effect. Hepatology 55, 1271–1281.
Yeki, M., Koda, M., Matono, T., Sugihara, T., Maeda, K., Murawaki, Y., 2009. Preventative and therapeutic effects of perindopril on hepatic fibrosis induced by bile duct ligation in rats. Mol. Med. Rep. 2, 857–864.
Yoshiji, H., Kuriyama, S., Yoshii, J., Ikenaka, Y., Noguchi, R., Nakatani, T., Tsujinoue, H., Fukui, H., 2001. Angiotensin-II type 1 receptor interaction is a major regulator for liver fibrosis development in rats. Hepatology 34, 745–750.
Yoshiji, H., Kuriyama, S., Noguchi, R., Yoshii, J., Ikenaka, Y., Yanase, K., Namisaki, T., Kitade, M., Yamazaki, M., Tsujinoue, H., 2005a. Combination of interferon-beta and angiotensin-converting enzyme inhibitor, perindopril, attenuates the murine liver fibrosis development. Liver Int. 25, 153–161.
Yoshiji, H., Noguchi, R., Kuriyama, S., Ikenaka, Y., Yoshii, J., Yanase, K., Namisaki, T., Kitade, M., Masaki, T., Fukui, H., 2005b. Imatinib mesylate (STI-571) attenuates liver fibrosis development in rats. Am. J. Physiol. Gastrointest. Liver Physiol. 288, G907–G913.
Zhang, J.J., Wang, Y.L., Feng, X.B., Song, X.D., Liu, W.B., 2011. Rosmarinic acid inhibits proliferation and induces apoptosis of hepatic stellate cells. Biol. Pharm. Bull. 34, 343–348.
Zhao, X.Y., Zeng, X., Li, X.M., Wang, T.L., Wang, B.E., 2009. Pirfenidone inhibits carbon tetrachloride- and albumin complex-induced liver fibrosis in rodents by preventing activation of hepatic stellate cells. Clin. Exp. Pharmacol. Physiol. 36, 963–968.