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Precision-cut tissue slices: a novel ex vivo model for fibrosis research

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Chapter 4

Tetrandrine, pirfenidone, ly2109761 and sunitinib mitigate intestinal fibrosis in murine precision-cut intestinal slices

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ABSTRACT

Background: Intestinal fibrosis (IF) is a hallmark of Crohn's disease. Here, we investigated the impact of several putative antifibrotic compounds on the onset of IF using murine precision-cut intestinal slices (PCIS).

Methods: Murine PCIS were cultured for 48 h in the presence of profibrotic and/or antifibrotic compounds. The fibrotic process was studied on gene and protein level using a variety of markers including (pro)collagen 1a1 (Col1a1), heat shock protein 47 (Hsp47), fibronectin (Fn2) and plasminogen activator inhibitor-1 (PAI-1). The effects of potential antifibrotic drugs mainly inhibiting the TGF β pathway *i.e.* valproic acid, tetrandrine, pirfenidone, SB203580 and LY2109761 as well as compounds mainly acting on the PDGF pathway *i.e.* imatinib, sorafenib and sunitinib were assessed in the model at non-toxic concentrations.

Results: Murine PCIS remained viable for 48 h and the onset of IF was observed during culture, as demonstrated by an increased expression of, amongst others, *Hsp47*, *Fn2* and *Pai-1*. Furthermore, TGF β 1 stimulated fibrogenesis while PDGF had no effect. Regarding the tested antifibrotics, pirfenidone, LY2109761 and sunitinib had the most pronounced impact on fibrogenesis, both in the absence and presence of profibrotic factors, as illustrated by reduced levels of *Col1a1*, *Hsp47*, *Fn2* and *Pai-1* following treatment. Moreover, LY2109761 significantly reduced fibronectin protein expression in the presence of TGF β 1.

Conclusions: PCIS can successfully be used to test drug efficacy. Using the model we demonstrated that tetrandrine, pirfenidone, LY2109761 and sunitinib showed potential antifibrotic effects on a gene level, warranting further evaluation of these compounds for the treatment of IF.

INTRODUCTION

Crohn's disease (CD) - an inflammatory bowel disease (IBD) – is often associated with intestinal fibrosis (IF) resulting in the formation of strictures, which will obstruct the intestinal lumen. These strictures are characterized by transmural condensed collagen layers in the intestinal wall (Burke et al., 2007; Rieder and Fiocchi, 2013; Rieder et al., 2013). It is reported that IF is initiated by severe and chronic tissue damage due to recurrent inflammation (Latella et al., 2014), as observed in CD patients. During CD, various cytokines are elevated in inflamed regions, including the archetypical profibrotic factors, transforming growth factor β (TGF β) and platelet-derived growth factor (PDGF) (Burke et al., 2007; Kumagai et al., 2001; Rieder and Fiocchi, 2008). These cytokines increase the expression of a variety of genes, including connective tissue growth factor (*Ctgf*), plasminogen activator inhibitor-1 (*Pai-1*) and *C-myc* (Krause et al., 2011). It has been reported that TGF β is a key player during intestinal wound healing as well as stricture development in CD patients (Burke et al., 2007). Activation of the TGF β signaling pathway augments the expression of (pro)collagen 1a1 (*Colla1*), fibronectin (*Fn2*) and heat shock protein 47 (*Hsp47*) (Latella et al., 2013; Speca, 2012). Therefore, TGF β is an interesting target for the treatment of fibrosis. In a previous study, we evaluated the therapeutic potential of a myriad of TGF β pathway inhibitors in liver fibrosis using a unique ex vivo/in vitro model viz. precision-cut liver slices (PCLS) (Iswandana et al., 2016; Westra et al., 2014a). Using this model we demonstrated that tetrandrine (Tet), valproic acid (Val) pirfenidone (Pir) and rosmarinic acid have potential for the treatment of liver fibrosis, in line with previous studies (Friedman et al., 2013; Mannaerts et al., 2010; Schaefer et al., 2011; Yin et al., 2007).

The other profibrotic growth factor, PDGF (Bonner, 2004), induces cell proliferation and fibroblasts migration (Bettenworth and Rieder, 2014; Latella et al., 2013), but also activates intestinal myofibroblasts to increase collagen synthesis (Andoh et al., 2002). Several groups successfully decreased fibrogenesis by inhibiting the PDGF pathway, via PDGF receptor inhibitors (Chen et al., 2011; Friedman et al., 2013). In addition, our group successfully used PCLS to study the efficacy of several PDGF inhibitors (Westra et al., 2014a), including imatinib (Ima), sorafenib (Sor) and sunitinib (Sun). Despite these promising results, there are no drugs currently registered for the treatment of IF and the only available therapy is surgical

intervention (Spinelli et al., 2010).

Various animal models have been used to evaluate antifibrotic compounds in multiple organs (Westra et al., 2013). Yet, a good translational animal model for IF is lacking, and as a result elucidating the mechanism of IF and testing the efficacy of therapeutic compounds is hampered. Recently, we established a novel model for the onset of IF using PCIS (Iswandana et al., 2016; Pham et al., 2015). The objective of the current study was to use this model to investigate the antifibrotic effect of several putative antifibrotic compounds in the intestine, including TGF β pathway related inhibitors: Pir, Val, and Tet, LY2109761 and p38 MAPK inhibitor, SB203580 and PDGF related pathway inhibitors: Ima, Sor and Sun.

MATERIALS AND METHODS

Preparation of mouse intestinal cores

Adult non-fasted male C57BL/6 mice were used (Harlan PBC, Zeist, The Netherlands). The mice were housed on a 12 h light/dark cycle in a temperature and humidity controlled room with standard chow (Harlan chow no 2018, Horst, The Netherlands) and water *ad libitum*. The animals were allowed to acclimatize for at least seven days before the start of the experiment. The experiments were approved by the Animal Ethical Committee of the University of Groningen.

Mice were sacrificed by cervical dislocation or under isoflurane/O₂ anesthesia (Nicholas Piramal, London, UK). Mouse jejunum (about 15 cm distal from the stomach and 10 cm in length) was excised and preserved in ice-cold Krebs-Henseleit buffer (KHB) supplemented with 25 mM D-glucose (Merck, Darmstadt, Germany), 25 mM NaHCO₃ (Merck), 10 mM HEPES (MP Biomedicals, Aurora, OH, USA), saturated with carbogen (95% O₂/5% CO₂) and adjusted to pH 7.4 (Pham et al., 2015).

The jejunum was cleansed by flushing KHB through the lumen and subsequently divided into 2 cm segments. These segments were filled with 3% (w/v) agarose solution in 0.9% NaCl at 37°C and embedded in an agarose core-embedding unit (Pham et al., 2015).

Preparation of PCIS

PCIS were prepared in ice-cold KHB using the Krumdieck tissue slicer (Alabama Research and Development, USA). The slices, with a wet weight of approximately 3 mg, have an estimated thickness of 300-400 µm. Slices were stored in ice-cold KHB until the start of the experiments (de Graaf et al., 2010).

Pro- and antifibrotic compounds:

TGFβ1 (5 ng/ml; hTGFβ1; Roche Applied Science, Mannheim, Germany) and PDGF-BB (50 ng/ml; Recombinant Human PDGFBB; Peprotech, Bioconnect, Huissen, The Netherlands) were used as profibrotic stimuli.

Different antifibrotic compounds were tested. The TGF β inhibitors *i.e.* valproic acid (1 mM; Sigma Aldrich, Zwijndrecht, Netherlands), tetrandrine (5 μ M; Sigma Aldrich), pirfenidone (2.5 mM; Sigma Aldrich) and LY2109761 (10 nM; Selleck Chemicals, Houston, USA) and the PDGF inhibitors *i.e.* imatinib (10 μ M; Novartis, Basel, Switzerland), sorafenib (4 μ M; LC laboratories, Woburn, USA) and sunitinib (5 μ M; LC laboratories) and the p38 MapK inhibitor SB203580 (5 μ M; Bioconnect, Huissen, The Netherlands).

Incubation of intestinal slices

Slices were incubated in 24-well plates and each slice was cultured individually in 0.5 ml of Williams Medium E with L-glutamine (Invitrogen, Paisley, UK) supplemented with 25 mM glucose, 50 μ g/mL gentamycin (Invitrogen) and 2.5 μ g/mL amphotericin-B (Invitrogen). During incubation at 37°C and 80% O₂/5% CO₂, the plates were horizontally shaken at 90 rpm (amplitude 2 cm). PCIS were incubated up to 48 h during which time slices were exposed to pro- and/or antifibrotic compounds.

Viability

Viability was assessed by measuring the adenosine triphosphate (ATP) content of the PCIS using the ATP bioluminescence kit (Roche diagnostics, Mannheim, Germany), as previously described (de Graaf et al., 2010). ATP values (pmol) were normalized to the total protein content (μ g) of the PCIS estimated by the Lowry protein assay (Bio-rad RC DC Protein Assay, Bio-Rad, Veenendaal, The Netherlands). Values are displayed as relative values compared to the related controls.

Gene expression

After incubation, slices were snap-frozen in liquid nitrogen and stored at -80°C until use. Total RNA of three to six pooled snap-frozen slices was isolated using the Qiagen RNAeasy mini kit (Qiagen, Venlo, The Netherlands). The amount of isolated RNA was measured with the BioTek Synergy HT (BioTek Instruments, Vermont, USA). Afterwards, 1 μ g RNA was reverse transcribed using the Reverse

Transcription System (Promega, Leiden, The Netherlands). The RT-PCR reaction was performed in the Eppendorf mastercycler with the following gradient: 25°C for 10 minutes, 45°C for 60 minutes and 95°C for 5 minutes.

The expression of several fibrosis genes *i.e.* *Colla1*, *αSma*, *Hsp47* and *Fn2* (Table 1); three pathway-specific genes *Pai-1*, *C-myc* and *Ctgf* (Table 1) were determined by SYBR green method. The Real Time PCR reaction was performed in a 7900HT Real Time PCR (Applied Biosystems, Bleiswijk, The Netherlands) with 45 cycles of 10 minutes 95°C, 15 seconds at 95°C and 25 seconds at 60°C followed by a dissociation curve. Ct values were corrected for the Ct values of the housekeeping gene *Gapdh* (ΔCt) and compared with the control ($\Delta\Delta Ct$). Results are presented as fold induction ($2^{-\Delta\Delta Ct}$).

Table 1. Primers

Gene	Forward (5'-3')	Reverse (5'-3')
<i>Gapdh</i>	ACAGTCCATGCCATCACTGC	GATCCACGACGGACACATTG
<i>Colla1</i>	TGACTGGAAGAGCGGAGAGT	ATCCATCGGTCATGCTCTCT
<i>αSma</i>	ACTACTGCCGAGCGTGAGAT	CCAATGAAAGATGGCTGGAA
<i>Hsp47</i>	AGGTCACCAAGGATGTGGAG	CAGCTTCTCCTTCTCGTCGT
<i>Ctgf</i>	CAAAGCAGCTGCAAATACCA	GGCCAAATGTGTCTTCCAGT
<i>Fn2</i>	CGGAGAGAGTGCCCCTACTA	CGATATTGGTGAATCGCAGA
<i>Pai-1</i>	GCCAGATTTATCATCAATGACTGGG	GGAGAGGTGCACATCTTTCTCAAAG
<i>C-myc</i>	GCTGTAGTAATTCCAGCGAGAGACA	CTCTGCACACACGGCTCTTC

Western Blot

Hsp47, fibronectin and PDGF- β -receptor protein expression was determined by Western blot. Stored PCIS were lysed for 1 h on ice in 250 μ l lysis buffer containing 30 mM Tris HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.54% Triton X-100 (Sigma Aldrich, the Netherlands), 1 mM Na₃VO₄, 10 mM NaF, 1% SDS, and 1 Protease inhibitor cocktail tablet (Roche, Mannheim, Germany). The tissue was homogenized on ice and centrifuged for 45 minutes at 4°C at 16,000 x g. Protein concentrations were determined in the supernatant using a Bio-Rad DC protein assay according to the manufacturer's protocol. Lysates were diluted 4 fold in SDS sample

buffer (50 mM Tris HCl pH 6.8, 2% SDS, 10% glycerol, 0.05 M EDTA pH 8.0, 0.6 M β -mercaptoethanol, and 0.01% bromophenol blue) and heated for 10 minutes (70°C). Then, 50 μ g of protein was separated via SDS/PAGE using 7.5% gels and blotted onto an activated polyvinylidene difluoride membrane (Biorad). Afterwards, the membrane was blocked using Tris-buffered saline supplemented with 5% Blocking Grade Powder (Biorad) and 0.1% Tween-20 during 1 h. Subsequently, membranes were incubated with rabbit- α -collagen-1 (1:1000), rabbit- α -heat shock protein 47 (1:2000), mouse- α -fibronectin (1:1000, Sigma Aldrich), rabbit- α -PDGF- β -receptor (1:1000, Cell Signaling, USA) and mouse- α -Gapdh (1:5000, Rockland Immunochemicals, Rockford, USA). Binding of the antibody was determined using Horseradish Peroxidase conjugated secondary goat- α -rabbit and secondary rat- α -mouse (DAKO, Glostrup, Denmark). Protein levels were visualized with an imaging system using the Visiglo Prime HRP Chemiluminescent Substrate Kit (Amresco, Ohio, USA). Results are displayed as relative values compared to the control and normalized with Gapdh.

Statistics

A minimum of three different intestines was used for each experiment, using 3-6 slices for each determination. The results are expressed as mean \pm standard error of the mean (SEM). Statistics were performed using a paired, one-tailed Student's t-test or a one-way ANOVA followed by Dunnett's multiple comparisons test as appropriate. 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 $\Delta\Delta$ Ct values.

RESULTS

After 48 h of incubation, there was no significant difference in the ATP content of PCIS compared to the 0 h time point (Figure 1A). Indicating that the viability and morphological integrity of the slices was maintained, as demonstrated previously (Pham et al., 2015). During culture, gene expression of *Hsp47* and *Fn2*, early markers of fibrosis, were increased significantly compared to 0 h (Figure 1B). Furthermore, *Pai-1* expression dramatically increased while the other pathway related genes did not change (Figure 1C). These results are in line with previous studies using PCIS from various species (Iswandana et al., 2016; Pham et al., 2015).

Exposure of PCIS to TGF β 1 and PDGF-BB did not affect the viability of the slices (Figure 1A). Gene expression of the fibrosis markers, *Coll1A1*, α *Sma*, *Hsp47* and *Fn2* were upregulated at least 2-fold in the presence of TGF β 1 (Figure 1D). Moreover, TGF β 1 significantly increased the expression of all three pathway related genes (*Pai-1*, *C-myc* and *Ctgf*, Figure 1E). In contrast, treatment with PDGF-BB did not affect the expression levels of both fibrosis and pathway markers (Figure 1D, 1E), despite the presence of the PDGF receptor during culture (Figure 1F). Next, we evaluated the efficacy of multiple putative antifibrotics using the above-mentioned markers.

Antifibrotic effect of TGF β related inhibitors

Drugs, mainly acting on the TGF β pathway, were studied for 48 h in the presence or absence of TGF β 1. The selected concentrations of the studied compounds were non-toxic for PCIS as illustrated by the ATP content of the slices following treatment (Figure 2A, 2B). In the absence of TGF β 1, all tested inhibitors, *i.e.* Vpa, Tet and Pir, significantly decreased the gene expression of *Hsp47*. In addition, Tet and Pir also down-regulated *Fn2* expression. Moreover, Pir was the TGF β pathway associated drug that was able to decrease the gene expression of *Coll1a1* (Figure 3A).

Among TGF β specific inhibitors, Ly decreased the expression of all fibrosis related genes but especially reduced the gene expression of *Coll1a1* by 80%, to the level which was even lower than the expression of *Coll1a1* directly after slicing (Figure 3B). Meanwhile, the p38 MapK inhibitor, SB203580, only slightly down-regulated the gene expression of *Hsp47* in slices (Figure 3B).

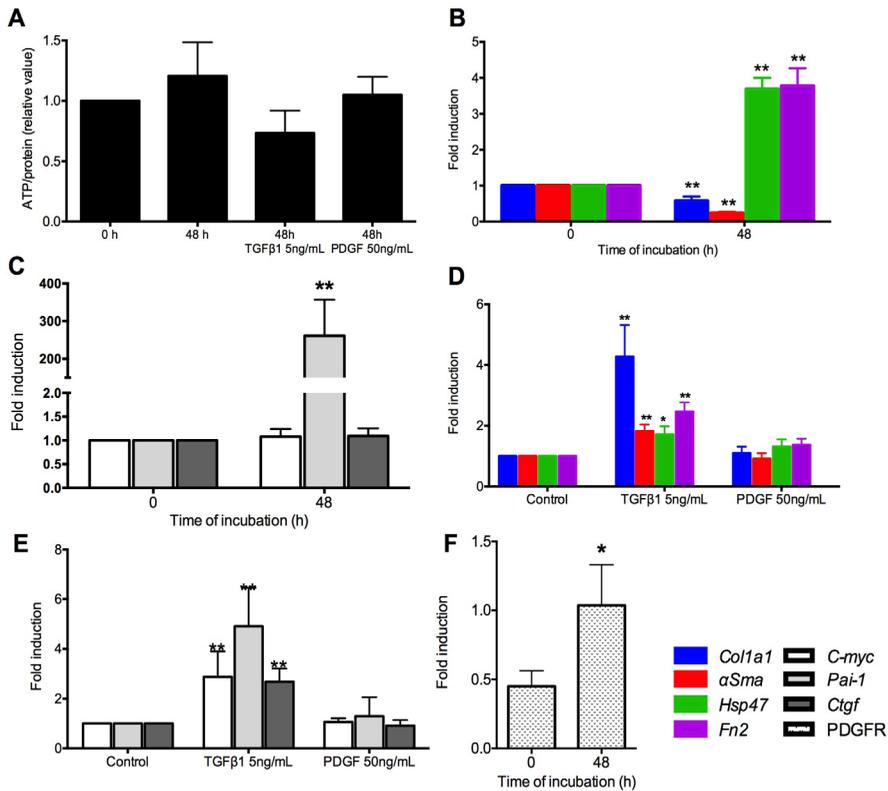


Figure 1. Effect of incubation time, TGFβ1 and PDGF on PCIS. (A) Viability of PCIS determined by ATP content (relative value compare to 0h). Gene expression of (B) *Col1a1*, *Hsp47*, *αSma*, *Fn2*; (C) *Pai-1*, *C-myc* and *Ctgf* following incubation. Gene expression of (D) *Col1a1*, *Hsp47*, *αSma*, *Fn2*; (E) *Pai-1*, *C-myc* and *Ctgf* after treatment with TGFβ1 (5 ng/mL) and with PDGF (50 ng/mL). (F) Protein expression of PDGFR during culture. Data are expressed as mean +/- SEM (n = 3-5). *p < 0.05, **p < 0.01 vs. control.

When investigating the gene expression of pathway related markers *C-myc*, *Pai-1* and *Ctgf*, Vpa down-regulated all these markers significantly; Pir only decreased the expression of *C-myc* and *Pai-1* (Figure 3C). Whereas, Tet had no effect on any of the pathway related markers. Furthermore, Ly, the TGFβ specific inhibitor, significantly decreased *Pai-1* and *Ctgf* gene expression in PCIS. While, SB203580 only decreased *Pai-1* gene expression significantly (Figure 3D).

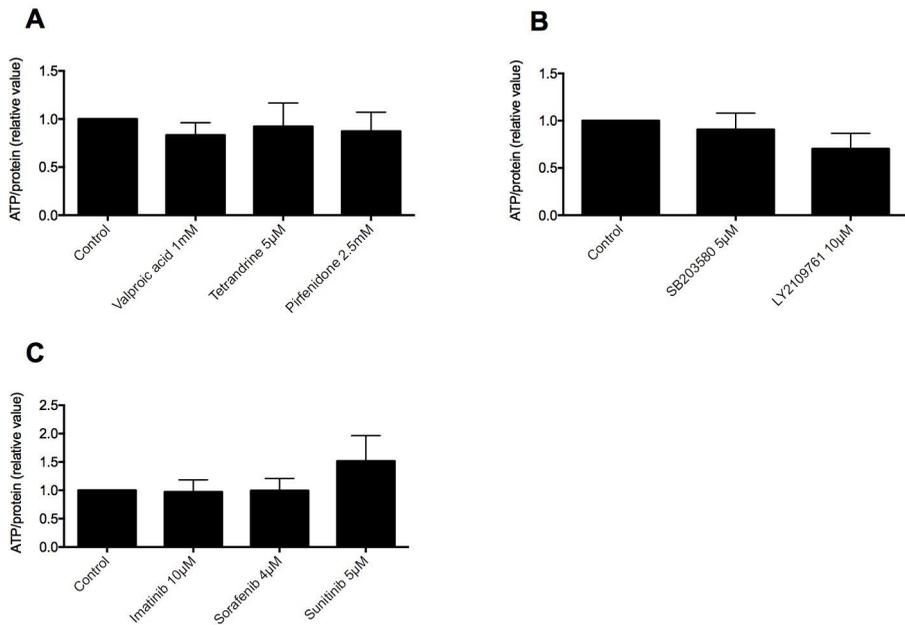


Figure 2. Viability of PCIS determined by ATP content (relative value compare to 0h) following treatment with (A) Vpa, Tet, Pir; (B) SB203580, LY2109761; (C) Ima, Sor, Sun. Data are expressed as mean +/- SEM (n = 3-5).

Next, PCIS were exposed to the putative antifibrotic compounds in the presence of TGF β 1. Under these conditions, Vpa did not change the gene expression of any of the fibrosis markers studied (Figure 3E). However, Tet showed a clear antifibrotic effect, as it significantly reduced the expression of most of the studied genes, with the exception of *Fn2*, compared to PCIS incubated with only TGF β 1 (Figure 3E). In addition, Pir significantly decreased the gene expression of *Colla1*, *Hsp47* and *Fn2* as compared to PCIS incubated with TGF β 1 alone (Figure 3E). Moreover, Ly markedly decreased *Colla1* expression (Figure 3F). In contrast, SB203580 had no effect on any of the fibrosis related genes (Figure 3F).

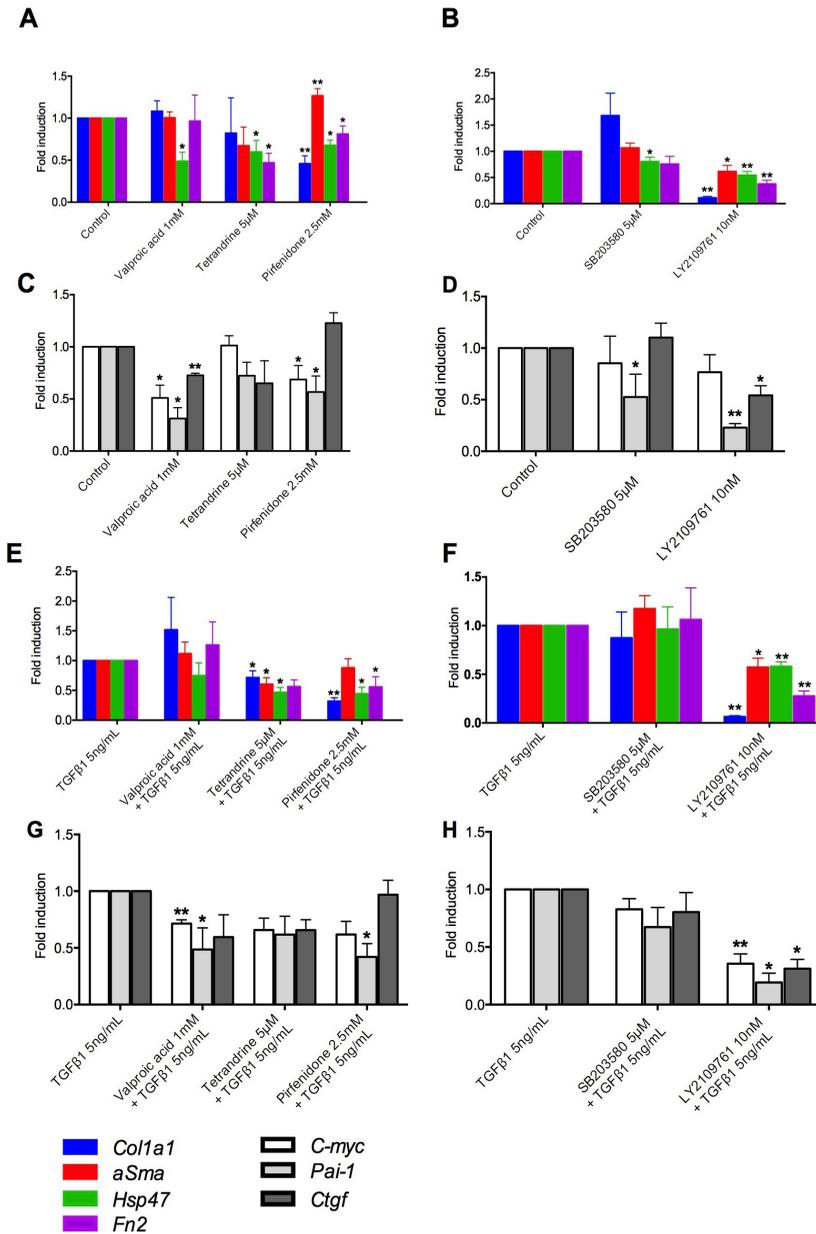


Figure 3. Gene expression of *Col1a1*, *Hsp47*, *aSma* and *Fn2* in PCIS following treatment with (A) Vpa, Tet, Pir; (B) SB203580, LY2109761; (E) Vpa, Tet, Pir in the presence of TGFβ1 and (F) SB203580, LY2109761 in the presence of TGFβ1. Gene expression of *Pai-1*, *C-myc* and *Ctgf* in PCIS following treatment with (C) Vpa, Tet, Pir; (D) SB203580, LY2109761; (G) Vpa, Tet, Pir in the presence of TGFβ1 and (H) SB203580, LY2109761 in the presence of TGFβ1. Data are expressed as mean +/- SEM (n = 3-5). *p < 0.05, **p < 0.01 vs. control.

In the presence of TGF β 1, *Pai-1* gene expression was down-regulated by Vpa, Pir and Ly. Vpa and Ly also significantly decreased *C-myc* gene expression. Gene expression of *Ctgf* was only down-regulated by Ly (Figure 3G, 3H). Pir and Ly were the most effective antifibrotic compounds in both models of the early onset of fibrosis. Therefore, we studied the impact of Ly and Pir, on the protein expression of Hsp47 and fibronectin. Protein expression of both markers was significantly upregulated in PCIS under control conditions when compared to PCIS directly after slicing (Figure 4A). However, these proteins were not regulated in the presence of TGF β 1 or in the presence of Pir and Ly compared to control (Figure 4B, 4C). In the presence of TGF β 1, Ly significantly reduced Hsp47 protein expression (Figure 4D), but Pir increased fibronectin protein levels significantly.

Taken together, Ly showed a significant reduction of the gene and protein expression of the investigated fibrosis markers. Whereas, Pir only showed antifibrotic effects on a gene level.

Antifibrotic effect of PDGF related inhibitors

The impact of the PDGF inhibitors, Ima, Sor and Sun, on the fibrotic response was studied in the presence and absence of PDGF-BB. Viability, as measured by the ATP-content of the slices, showed that all inhibitors were tested at non-toxic concentrations (Figure 2C).

Ima did not influence gene expression of the fibrosis markers as compared to control in the presence and absence of PDGF-BB (Figure 5A, 5B). Sor, by itself, decreased *Hsp47* expression, and in the presence of PDGF-BB both *Hsp47* and α *Sma* levels were reduced (Figure 5A, 5B). Meanwhile, Sun with and without PDGF-BB not only down-regulated the early markers *Hsp47* and *Fn2* but also the gene expression of the main fibrosis marker *Colla1* (Figure 5A, 5B).

While Sor only slightly decreased the gene expression of *Ctgf* in the absence of PDGF-BB (Figure 5C). Sun down-regulated *Pai-1* and *Ctgf* gene expression in both the absence and presence of PDGF-BB (Figure 5C and 5D).

Thus, among PDGF inhibitors, only Sun showed potential antifibrotic effects, both in the presence and absence of PDGF-BB.

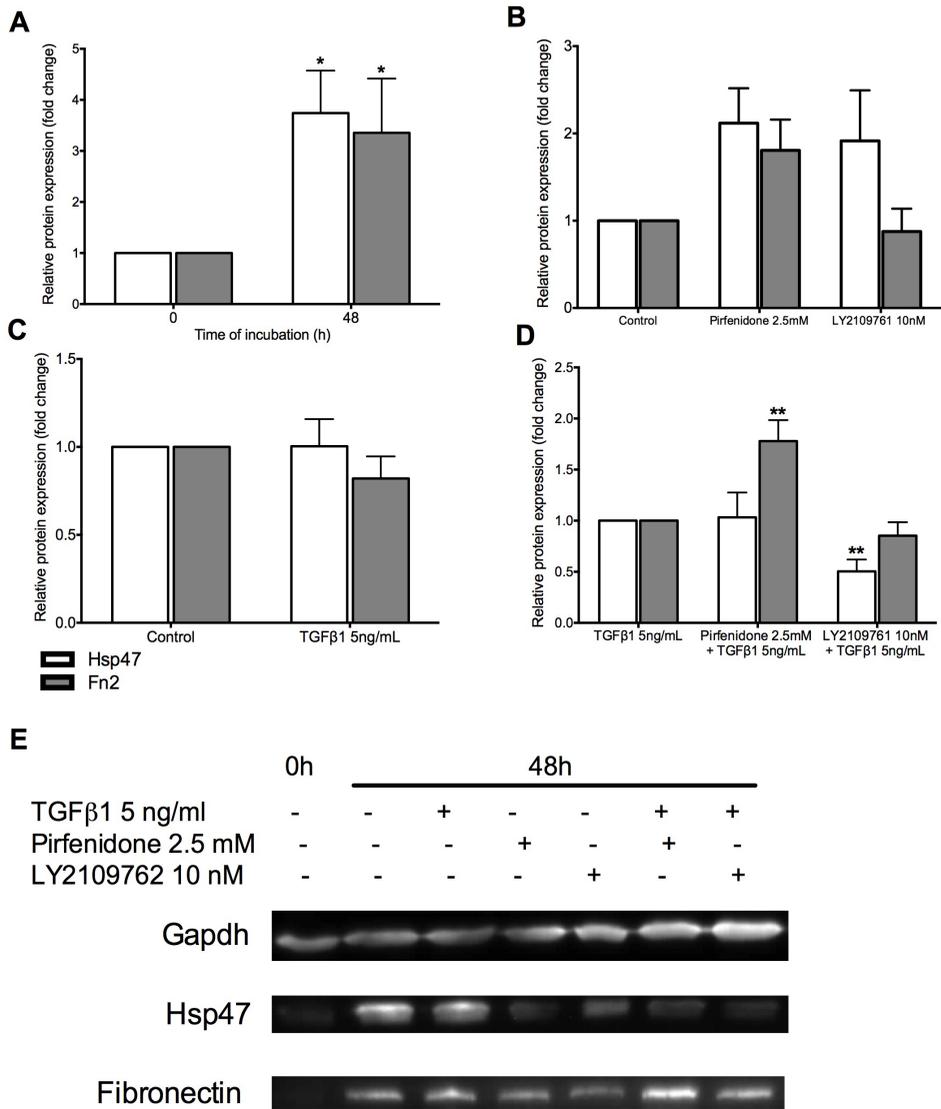


Figure 4. Protein expression of fibrosis markers Hsp47 and Fn2 (A) during culture; (B) following treatment with (B) Pir and LY2109761; (C) TGFβ1; (D) Pir and LY2109761 in the presence of TGFβ1. (E) Representative Western blot. Data are expressed as mean +/- SEM (n = 3-5). *p < 0.05, **p < 0.01 vs. control.

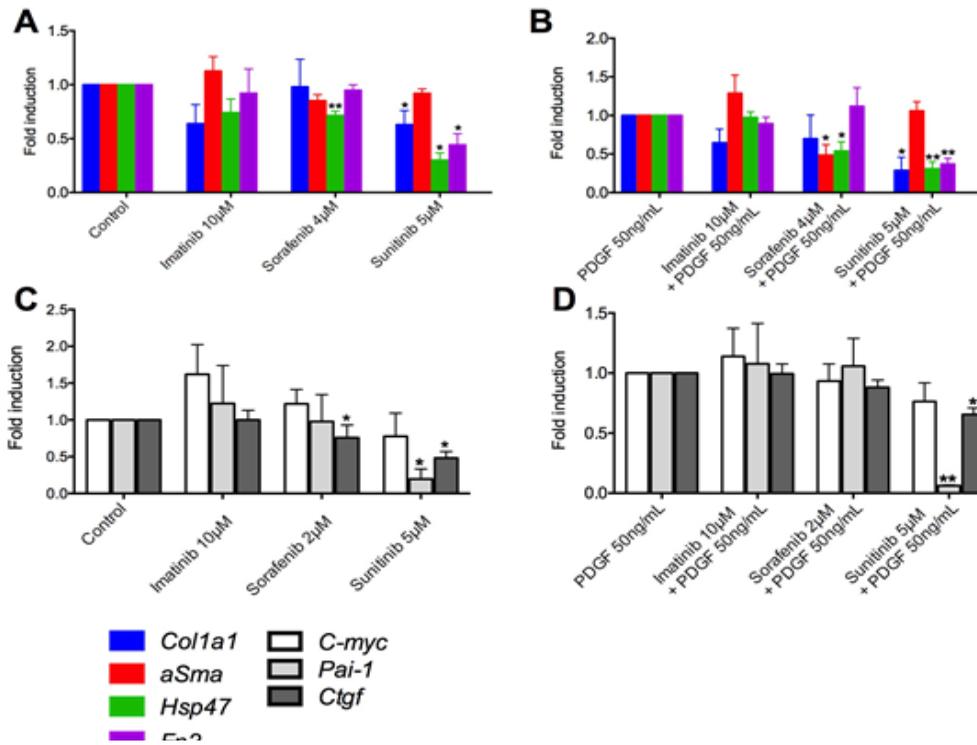


Figure 5. Gene expression of *Coll1a1*, *Hsp47*, *aSma* and *Fn2* in PCIS following treatment with (A) Ima, Sor, Sun; (B) Ima, Sor, Sun in the presence of PDGF. Gene expression of *Pai-1*, *C-myc* and *Ctgf* in PCIS following treatment with (C) Ima, Sor, Sun; (D) Ima, Sor, Sun in the presence of PDGF. Data are expressed as mean \pm SEM (n = 3-5). *p < 0.05, **p < 0.01 vs. control.

DISCUSSION

This is the first study that evaluates potential antifibrotic drugs for the treatment of IF. As previously reported, we have developed rodent PCIS as an *ex vivo* model for the early onset of IF (Iswandana et al., 2016; Pham et al., 2015). Gene expression of fibrosis markers was highly upregulated in PCIS after 48 h of incubation allowing the use of this *ex vivo* model to evaluate and rank the effect of potential antifibrotic drugs. A similar *ex vivo* model has successfully been used to evaluate antifibrotic drugs for liver fibrosis by using precision-cut liver slices (PCLS) (Westra et al., 2014a).

Our results demonstrated that during incubation of PCIS, up to 48 h, the gene expression of several fibrosis markers was increased. To even further induce the onset of fibrosis, PCIS were incubated with TGF β 1 or PDGF-BB. Only TGF β 1 induced fibrosis markers and pathway related genes, which was in line with the study in isolated human intestinal fibroblasts where gene expression of *CTGF* and *COL1A1* is elevated after TGF β 1 stimulation (Beddy et al., 2006; Mulsow et al., 2005). However, a different response to PDGF-BB was observed in PCIS as compared to other *in vitro* models (Westra et al., 2014a). In our hands, incubation of PCIS with PDGF-BB did not have an effect on the expression of the measured fibrosis genes, despite the presence of the PDGF receptor. It might be necessary to use higher concentrations of PDGF-BB, however in PCLS from other species, this resulted in a loss of viability.

Several TGF β pathway related inhibitors were evaluated in this study, including Vpa, Tet and Pir. As reported previously, in an *ex vivo* rat PCLS model, Vpa reduced the gene expression of multiple fibrosis makers (Westra et al., 2014b). In our PCIS model, Vpa did not have an antifibrotic effect. However, it affected the expression of pathway related genes. Indicating that Vpa inhibits the TGF β pathway, but did not alter the early onset of fibrosis. Mannaerts et al., showed that VPA reduced *COL1A1* gene expression in mouse HSC after 64 h of culture (Mannaerts et al., 2010). Therefore, an increased incubation period might be needed to fully unveil the effect of Vpa on the gene expression of fibrosis markers in PCIS. Furthermore, Vpa is a histone deacetylase inhibitor and the effect on the pathway related genes could also be caused by hyper acetylation of histones (Dokmanovic et al., 2007).

Tet blocks the TGF β /Smad pathway by upregulating Smad 7, which inhibits Smad2/3 phosphorylation (Burke et al., 2007; Westra et al., 2014a). In our hands, Tet had no effect on the pathway related genes, but attenuated the levels of several fibrosis markers. This ostensible discrepancy might also be due to timing, as the inhibition of the pathway related genes could have occurred prior to the 48 h sampling time. Therefore, more research is necessary to elucidate the molecular mechanisms involved in the antifibrotic effect of Tet.

Pir decreases gene expression of *Tgfb β* , *Collagen I* and *Hsp47* in both cell cultures and animal fibrosis models from different organs (Hisatomi et al., 2012; Iyer et al., 1999; Schaefer et al., 2011). Pir was the first antifibrotic compound on the market, currently registered for the treatment of idiopathic pulmonary fibrosis (Friedman et al., 2013). The antifibrotic properties for Tet and Pir in the intestine are in line with the results obtained in fibrosis models in other organs and in PCLS (Schaefer et al., 2011; Westra et al., 2014b). Thus, Tet and Pir could be effective for the treatment of IF.

Recently, others inhibitors, albeit no marketed drugs, surfaced that are used to inhibit specific pathways in fibrosis, namely LY2109761 and SB203580. LY2109761 is a TGF β inhibitor that showed promising results in blocking TGF β signaling in cancer and fibrotic diseases (Akhurst and Hata, 2012; Dooley and ten Dijke, 2012; Flechsig et al., 2012; Melisi et al., 2008). SB203580 is a p38 MAP Kinase inhibitor (Otte et al., 2003), which decreased the gene expression of fibrosis markers in precision-cut liver slice (PCLS). In our PCIS model, only LY showed a clear antifibrotic effect. This suggests that the TGF β signaling pathway is instrumental during the development of IF, whereas the p38 Map Kinase pathway does not play a role.

Our results further illustrated that Ly and Pir could dampen the expression of multiple fibrosis markers on both gene and protein level. Further supporting the notion that hampering the TGF β pathway is a promising therapeutic target to treat IF.

We evaluated the antifibrotic activity of the small molecule tyrosine kinase inhibitors: Ima, Sor and Sun. All three drugs are used primarily in cancer therapy (Krause and Van Etten, 2005). However, there is a difference in potency between these compounds. Sun is a type I tyrosine kinase inhibitors, which has a higher affinity to PDGF receptor and thus potentially more effect on the PDGF signaling route than the type II inhibitors *i.e.* Ima and Sor (Gotink and Verheul, 2010; Kufareva

and Abagyan, 2008). Our results showed that Sun had a clear effect in the early onset of IF, compared to Sor and Ima, especially in the presence of PDGF. Sun also significantly down-regulated the pathway related gene expression of *Pai-1* and *Ctgf* suggesting that Sun has an inhibitory effect upstream of the molecular pathogenesis of IF, most likely by blocking the PDGF- α and PDGF- β receptors (Faivre et al., 2007).

Westra et al. used the *ex vivo* rat PCLS model to test Ima, Sor and Sun (Westra et al., 2014b). They demonstrated that Ima was the most effective antifibrotic compounds in both the early and late stages of liver fibrosis in rat PCLS (Westra et al., 2014a; Westra et al., 2014b), while it was not effective in human PCLS (Westra et al., 2016). In addition, our results showed that Ima did not influence IF in murine PCIS. Thus, it is clear that Ima elicits organ- and species-specific effects.

From the result of this study, it can be concluded that although Ima, Sor and Sun all inhibit tyrosine kinase activity, only Sun effectively down-regulated fibrogenesis in the PCIS model.

CONCLUSIONS

This study shows that PCIS could be a valuable tool to evaluate the efficacy of compounds for the treatment of IF. Of the various compounds that we tested, only Tet, Pir, Ly and Sun showed potential antifibrotic efficacy. These candidates should be further investigated to completely unveil their therapeutic aptitude. Future studies using human PCIS will establish whether these potential antifibrotic compounds are also effective in man.

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