Exploring anti-fibrotic drugs
Luangmonkong, Theerut

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In vitro and ex vivo anti-fibrotic effects of LY2109761, a small molecule inhibitor against TGF-β

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Abstract

Background and purpose
Transforming growth factor-beta (TGF-β) is a central profibrotic mediator, and targeting TGF-β is a promising strategy in the development of drugs for the treatment of fibrosis. Therefore, effect of LY2109761, a representative of preclinically effective anti-cancer small molecule inhibitors against TGF-β, on fibrosis was elucidated.

Experimental approach
Gene and protein expression of fibrosis-related markers were elucidated in HepG2 cells, LX-2 cells, human precision-cut liver slices (PCLS) and rat PCLS in the presence and absence of exogenous TGF-β1.

Key results
Effects of LY2109761 on TGF-β signaling and fibrogenesis were observed in vitro and ex vivo. On the TGF-β signaling, inhibition of TGF-β1 expression and SMAD2 phosphorylation were the TGF-β-dependent effect towards anti-fibrotic efficacy of LY2109761. Interestingly, at the high concentration of LY2109761, inhibition of SMAD1 phosphorylation and gene expression of ID1, a bone morphogenetic proteins-related signaling pathway, appeared to be the TGF-β-independent effects. On the fibrosis-related markers, LY2109761 clearly inhibited the expression of collagen type 1.

Conclusion and implications
LY2109761 exhibited anti-fibrotic effects in vitro and ex vivo. Besides anti-fibrotic effects, both TGF-β-dependent and independent pathways were impacted. These results illustrate that small molecule inhibitors, directed against TGF-β, might influence numerous signaling pathways.

Key words: TGF-β; fibrosis; LY2109761; in vitro; ex vivo.

Abbreviations: TGF-β, transforming growth factor beta; SMI, small molecule inhibitors; TβR, TGF-β receptor; Lck, lymphocyte-specific protein tyrosine kinases; MAPK, mitogen-activated protein kinases; MKK, mitogen-activated protein kinase kinases; JNK, c-Jun N-terminal kinases; HSC, hepatic stellate cells; PCLS, precision-cut liver slices; ID1, inhibitor of DNA binding 1; PAI-1, plasminogen activator inhibitor 1; αSMA, alpha smooth muscle actin; HSP47, heat shock protein 47; COL1A1, collagen type I, alpha 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; COL1, collagen type I; BMPs, bone morphogenetic proteins; GDFs, growth differentiation factors; EMT, epithelial-mesenchymal transition; mTOR, mammalian target of rapamycin; PKB, protein kinase B.
Introduction

Since decades, transforming growth factor-beta (TGF-β) has been recognized as a central profibrotic mediator [1, 2]. Consequently, TGF-β and the associated signaling pathways have been important targets during the development of drugs for the treatment of fibrotic diseases [2, 3]; however, none of the identified candidates are eligible for clinical use due to the lack of efficacy and/or severe adverse events [4, 5]. The search for effective anti-fibrotic compounds is greatly hampered by the complexity of the TGF-β signaling pathway and associated networks [6, 7].

Recently, LY2109761, a small molecule inhibitor (SMI) against TGF-β, was developed to target cancer angiogenesis and metastasis in which TGF-β plays an essential role [8]. Nevertheless, due to its poor pharmacokinetic properties, clinical development of LY2109761 was not continued [9]. Selectivity of LY2109761 on diverse kinase enzymes was screened in vitro, and it was demonstrated that LY2109761 was relatively selective for the TGF-β receptor (TβR) [10]. However, its selectivity was inversely correlated with the used concentration. At 20 μM, LY2109761 exhibited weak activity against lymphocyte-specific protein tyrosine kinase (Lck), p38-mitogen-activated protein kinase (p38-MAPK), mitogen-activated protein kinase kinase 6 (M KK6), proto-oncogene tyrosine-protein kinase (FYN) and c-Jun N-terminal kinase 3 (JNK3) [10]. Because of the impact on TGF-β signaling, we postulate that LY2109761 might mitigate the onset of fibrosis.

Currently, the pharmacological activity of putative drugs is preliminary screened using in vitro and ex vivo models. For instance, HepG2, a human hepatocellular carcinoma cell line which constitutionally expresses components of TGF-β signaling, is commonly used to test anti-cancer properties of drugs [11, 12]. In addition, LX-2, a human hepatic stellate cell line that retains key features of primary hepatic stellate cells (HSC), has been extensively used to study fibrogenesis [13]. Nevertheless, these in vitro models fail to capture the intricate cell-cell interactions that underlie the fibrotic process in vivo. Recently, precision-cut liver slices (PCLS) have been proven as an effective ex vivo model for fibrosis; moreover, it was demonstrated that PCLS can be used to test the efficacy of putative anti-fibrotic compounds [14, 15]. Here, we used all these models to investigate the impact of LY2109761 on fibrogenesis.

Methods

Cell cultures

HepG2 (ATCC, Virginia, US) and LX-2 (kindly provided by Prof. Dr. S. L. Friedman, Mount Sinai School of Medicine, New York, US) were cultured in Dulbecco's Modified Eagle's Medium (Invitrogen, Bleiswijk, the Netherlands). The culture medium was supplemented with 10% fetal bovine serum, 50 U/mL penicillin and 50 ng/mL streptomycin.
Precision-cut liver slices (PCLS)

Human PCLS (hPCLS) were prepared from healthy non-fibrotic liver tissue obtained from patients following either partial heptectomy due to metastatic colorectal cancer or from donors, remaining as surgical surplus after reduced-size liver transplantation. The study was approved by the Medical Ethical Committee of the University Medical Center Groningen.

Rat PCLS (rPCLS) were prepared from male, 12-16 weeks old, Wistar rats (Charles River, Sulzfeld, Germany). The study was approved by the Animal Ethical Committee of the University of Groningen.

hPCLS and rPCLS, with an estimated thickness of 250-300 μm, were cultured for 48h in Williams’ medium E with Glutamax (Invitrogen) supplemented with glucose and gentamycin at 37 °C under continuous supply of 80% O₂/5% CO₂ as previously described [16].

Experimental treatment

Stock solutions of LY2109761 (Selleckchem, Munich, Germany) were prepared in dimethyl sulfoxide (DMSO). During experiments, stocks were diluted in culture medium with a final solvent concentration of ≤0.4%. For co-treatment, PCLS were exposed to 5 ng/mL TGF-β1 (Roche Diagnostics, Mannheim, Germany) with 1 μg/mL bovine serum albumin supplementation.

ATP determination

Viability was determined by measuring ATP levels as previously described [17]. Briefly, cell culture lysates and PCLS were transferred to a solution containing 70% ethanol and 2 mM EDTA, snap frozen and stored at -80 °C until analysis. ATP of the cell/tissue was determined by using the ATP bioluminescence kit (Roche Diagnostics). ATP values were corrected for total protein content of each sample estimated by the Bio-Rad DC Protein Assay (Bio-Rad, California, US). Values are expressed as relative values compared to the control group.

Quantitative real-time PCR

Gene expression was assessed by real-time quantitative PCR. Cell culture lysates and PCLS were snap frozen and stored at -80 °C until analysis. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Venlo, the Netherlands) and reverse transcribed using the Reverse Transcription System (Promega, Leiden, the Netherlands) [15, 18]. The mRNA levels of TGF-β1, ID1, PAI-1, αSMA, HSP47 and COL1A1 were detected using specific primer/probe sets (Applied Biosystems, California, US; Table 1) using a 7900HT Real Time PCR apparatus (Applied Biosystems) with 1 cycle of 10 minutes/95 °C followed by 40 cycles of 15 seconds/95 °C and 60 seconds/60 °C. GAPDH was used as reference gene and relative expression levels were calculated as fold change using the 2^ΔΔCT method.
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**Table 1:** Primer/probe sets used in quantitative real-time PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer/probe set</th>
<th>Human</th>
<th>Rat</th>
</tr>
</thead>
<tbody>
<tr>
<td>αSMA (alpha smooth muscle actin)</td>
<td>Hs00426835_g1</td>
<td>Rn01759928_g1</td>
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<tr>
<td>COL1A1 (collagen, type I, alpha 1)</td>
<td>Hs00164004_m1</td>
<td>Rn01463848_m1</td>
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<tr>
<td>GAPDH (glyceraldehyde-3-phosphate dehydrogenase)</td>
<td>Hs02786624_g1</td>
<td>Rn01775763_g1</td>
<td></td>
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<tr>
<td>HSP47 (heat shock protein 47)</td>
<td>Hs01060396_g1</td>
<td>Rn00567777_m1</td>
<td></td>
</tr>
<tr>
<td>ID1 (inhibitor of DNA binding 1)</td>
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<td>Rn00562985_s1</td>
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<tr>
<td>PAI-1 (plasminogen activator inhibitor 1)</td>
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<tr>
<td>TGF-β1 (transforming growth factor beta 1)</td>
<td>Hs00998133_m1</td>
<td>Rn00572010_m1</td>
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</table>

**Western blotting**

Phosphorylated SMAD1 (pSMAD1), pSMAD2, αSMA, HSP47 and COL1 protein levels were assessed by Western blot. Cell culture lysates and PCLS were snap frozen and stored at -80 °C until analysis [15]. Following sample preparation, total protein was separated on 10% sodium dodecyl sulfate polyacrylamide gels and transferred using Trans-Blot Turbo Mini PVDF Transfer Packs (Bio-Rad). Afterwards, the membrane was blocked and incubated with specific antibodies (Table 2). Targeted proteins were visualized with Clarity Western ECL Substrate (Bio-Rad). GAPDH was used as internal control protein.

**Table 2:** Buffer and antibodies used in Western blotting.

**Buffer ingredient**

- **Lysis buffer:** 30 mM Tris-HCl pH 7.4; 150 mM NaCl; 1 µM EDTA; 5.4 mg/mL Triton X-100; 1% SDS; 15 mM sodium orthovanadate; 15 mM sodium fluoride; 1 tablet PhosSTOP™ (Roche Diagnostics)/50 mL lysis buffer.

- **SDS sample buffer:** 50 mM Tris-HCl pH 6.8; 2% SDS; 10% glycerol; 1% beta-mercaptoethanol; 0.0125% bromophenol blue.

- **Blocking buffer:** 50 mM Tris-HCl pH 7.6; 150 mM NaCl; 5% non-fat dry milk (Blocking Grade Powder, Bio-Rad); 0.1% Tween-20.

**Antibody and dilution**

<table>
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<tr>
<th>Antibody and dilution</th>
<th>Manufacturer</th>
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<tr>
<td>Anti-α-smooth muscle actin (αSMA), 1:5000</td>
<td>Sigma, Saint Louis, US</td>
</tr>
<tr>
<td>Anti-collagen type I (COL1), 1:1000</td>
<td>Rockland, Pennsylvania, US</td>
</tr>
<tr>
<td>Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 1:5000</td>
<td>Sigma, Saint Louis, US</td>
</tr>
<tr>
<td>Anti-phospho-SMAD1/5 (Ser463/465), 1:1000</td>
<td>Cell Signaling, Danvers, US</td>
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<tr>
<td>Anti-phospho-SMAD2 (Ser465/467), 1:1000</td>
<td>Cell Signaling, Danvers, US</td>
</tr>
<tr>
<td>Polyclonal goat anti-rabbit immunoglobulins/HRP, 1:2000</td>
<td>Dako, Glostrup, Denmark</td>
</tr>
<tr>
<td>Polyclonal rabbit anti-mouse immunoglobulins/HRP, 1:2000</td>
<td>Dako, Glostrup, Denmark</td>
</tr>
</tbody>
</table>
**Statistics**

Each experiment was performed at least three times. Results are expressed as means ± standard error of the mean (SEM). Statistical analysis was performed via Student’s t-test or ANOVA followed by Dunnett’s post hoc analysis on relative ATP, ΔΔCt and relative signal intensity of the proteins. A p-value <0.05 was considered significant.

**Results**

**HepG2**

We first investigated the impact of LY2109761 on TGF-β signaling in HepG2 cells in the absence and presence of exogenous TGF-β1. Our results demonstrated that none of the treatment modalities impacted the viability of HepG2 cells (Figure 1A-C).

Furthermore, on a gene level (Figure 1D-F), the treatment with exogenous TGF-β1, 5 ng/mL, significantly increased TGF-β1 (2.46-fold when compared to the control) and PAI-1 (2.18-fold) expression. LY2109761, at the highest tested concentration (20 μM), reduced the gene levels of ID1 (82% inhibition when compared to the corresponding control), but increased αSMA (3.06-fold) expression. When HepG2 cells were exposed to both exogenous TGF-β1 and LY2109761, significantly decreased mRNA levels of ID1 (81%) and PAI-1 (63%) were observed.

On a protein level (Figure 1G-L), we did not observe any effects of exogenous TGF-β1. In contrast, treatment with LY2109761 significantly increased αSMA expression both in the absence (4.79-fold) and presence (2.05-fold) of exogenous TGF-β1. In the latter case, pSMAD1 expression was markedly reduced (86%).

**LX-2**

In addition to the impact of LY2109761 on TGF-β signaling, we subsequently studied whether this compound could mitigate fibrogenesis in LX-2 cells. As assessed by the ATP content (Figure 2A-C), treatment with neither 5 ng/mL TGF-β1, LY2109761, nor a combination of both affected the viability of LX-2 cells.

On a gene level (Figure 2D-F), we demonstrated that exogenous TGF-β1 induced a fibrotic response in LX-2 cells as illustrated by an increased expression of TGF-β1 (3.61-fold), PAI-1 (10.54-fold), αSMA (1.49-fold) and COL1A1 (2.53-fold). Additionally, qPCR revealed that LY2109761 lowered TGF-β1 expression (53%), while αSMA levels increased (1.85-fold). Furthermore, our results showed that LY2109761 markedly reduced exogenous TGF-β1-induced fibrogenesis.

These findings were corroborated by Western blotting (Figure 2G-L), showing that exogenous TGF-β1 significantly increased phosphorylation of SMAD1 (2.68-fold) and SMAD2 (6.19-fold). In addition, the co-treatment with LY2109761 significantly inhibited phosphorylation of SMAD1 (up to 84%) and SMAD2 (up to 92%).
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Figure 1: Viability and expression profiling of HepG2 cells treated with LY2109761 (LY) were compared to the corresponding control (n=4). A-C, ATP level/protein; D-F, gene expression; G-I, representative Western blots; J-L, average protein expression of all experimental groups shown as bar graphs after normalization to GAPDH. The concentration of exogenous TGF-β1 was 5 ng/mL. *p<0.05 compared to either control, LY 0 μM or LY 0 μM + TGF-β1.
Figure 2: Viability and expression profiling of LX-2 cells treated with LY2109761 (LY) were compared to the corresponding control (n=4-5). A-C, ATP level/protein; D-F, gene expression; G-I, representative Western blots; J-L, average protein expression of all experimental groups shown as bar graphs after normalization to GAPDH. The concentration of exogenous TGF-β1 was 5 ng/mL. *p<0.05 compared to either control, LY 0 μM or LY 0 μM + TGF-β1.
**Human precision-cut liver slices (hPCLS)**

Next, we investigated the anti-fibrotic efficacy in hPCLS, a multicellular human ex vivo model of liver fibrosis. Our results demonstrated that LY2109761 slightly decreased the viability of hPCLS; however, the effect was not statistically significant. Moreover, 5 ng/mL TGF-β1 treatment did not impact viability of hPCLS (Figure 3A-C).

qPCR revealed that exogenous TGF-β1 could not elicit a fibrotic response in hPCLS (Figure 3D-F), which is in line with previous results [14]. Nevertheless, LY2109761 significantly inhibited the expression of COL1A1 both in the absence (86%) and presence (94%) of exogenous TGF-β1. During co-treatment, significantly reduced mRNA levels of TGF-β1 (up to 75%) and HSP47 (up to 67%) were observed.

On a protein level (Figure 3G-L), LY2109761 treatment resulted in a diminished phosphorylation of both SMAD1 (70%) and SMAD2 (84%), while only SMAD2 phosphorylation was antagonized in the presence of exogenous TGF-β1.

**Rat precision-cut liver slices (rPCLS)**

Because exogenous TGF-β1 (5 ng/mL) could not elicit a fibrotic response in hPCLS, we additionally studied the effects of LY2109761 on fibrogenesis and TGF-β signaling in rPCLS [15]. As shown in Figure 4A-C, none of the experimental treatments altered the viability of rPCLS.

In rPCLS (Figure 4D-F), exogenous TGF-β1 significantly increased the expression of Tgf-β1 (1.86-fold), αSma (4.45-fold), Hsp47 (2.43-fold) and Col1a1 (3.85-fold). Moreover, in the absence of exogenous TGF-β1, LY2109761 at both 2.5 and 20 µM significantly decreased the expression of Id1 (up to 86%), αSma (up to 82%) and Col1a1 (up to 93%). This inhibitory effect persisted in the presence of exogenous TGF-β1 with the treatment of 20 µM LY2109761 only.

Protein expression in rPCLS appeared to be in line with the gene expression (Figure 4G-L); however, exogenous TGF-β1 did not increase Hsp47 expression. Exogenous TGF-β1 significantly increased Smad2 phosphorylation (3.75-fold), and the up-regulation was concentration-dependently antagonized (up to 83%) by LY2109761. Inhibition of Smad2 phosphorylation was also observed (up to 96%) in the absence of exogenous TGF-β1. Although decreased phosphorylation of Smad1 was observed (up to 84% in the absence of exogenous TGF-β1), the effect was however not statistically significant.
Figure 3: Viability and expression profiling of human precision-cut liver slices treated with LY2109761 (LY) were compared to the corresponding control (n=4-8). A-C, ATP level/protein; D-F, gene expression; G-I, representative Western blots; J-L, average protein expression of all experimental groups shown as bar graphs after normalization to GAPDH. The concentration of exogenous TGF-β1 was 5 ng/mL. *p<0.05 compared to either control, LY 0 μM or LY 0 μM + TGF-β1.
**Figure 4:** Viability and expression profiling of rat precision-cut liver slices treated with LY2109761 (LY) were compared to the corresponding control (n=3-4). A-C, ATP level/protein; D-F, gene expression; G-I, representative Western blots; J-L, average protein expression of all experimental groups shown as bar graphs after normalization to GAPDH. The concentration of exogenous TGF-β1 was 5 ng/mL. *p<0.05 compared to either control, LY 0 µM or LY 0 µM + TGF-β1.
Discussion

TGF-β is a member of the TGF-β superfamily which consists of TGF-β, bone morphogenetic proteins (BMPs), growth differentiation factors (GDFs), activin and inhibin [19]. The multifunctional proteins of this superfamily possess different physiologic roles; however, they all elicit their biological activity via similar transmembrane serine/threonine-protein kinase receptors and SMADs [7, 20]. Recently, hampering TGF-β-mediated signaling can be achieved by either depleting TGF-β itself, blocking the binding between TGF-β and the TβR, or by using SMI [2]. Here, we studied the impact of LY2109761, a SMI against TGF-β [8], on the expressions of genes and proteins related to TGF-β pathway activity and fibrogenesis.

Effect on TGF-β signaling

Our results demonstrated that LY2109761 clearly inhibited activation of the TGF-β pathway, as illustrated by a decreased expression of TGF-β1 [3], and reduced phosphorylation of SMAD2, the master transcription factor mediating fibrogenic responses of TGF-β [9], in LX-2, hPCLS and rPCLS. In general, inhibition of TGF-β signaling is associated with decreased PAI-1 expression in vitro [21-23]; however, we only observed this interplay in LX-2 and HepG2 cells, not in hPCLS and rPCLS. The observed effects of LY2109761 in PCLS were in line with a previous study using galunisertib, another SMI against TGF-β which elicited anti-fibrotic effects without inhibiting PAI-1 expression [15]. These results imply that PAI-1 is not a surrogate marker for TGF-β activation, and indeed it is known that this gene can be regulated by multiple mediators such as thrombin, plasmin and pro-inflammatory cytokines during coagulation, fibrinolysis and inflammatory process [24]. Furthermore, another distinctive feature observed in PCLS was the inhibitory effect of LY2109761 on phosphorylation of SMAD2 in the absence of exogenous TGF-β1. This finding emphasizes that the activation of TGF-β signaling is spontaneous in PCLS, and in contrast to the cell lines, the impact of drugs affecting TGF-β pathway can be observed without the additive effect of exogenous TGF-β [15].

In this study, the expression of TGF-β1 and phosphorylation of SMAD2 were not affected by LY2109761 in HepG2 cells. This finding is not consistent with previous studies showing that SMAD2 phosphorylation in HepG2 cells was modulated by other TGF-β inhibitors: galunisertib and D10, a monoclonal antibody against TβR [12, 25]. Nevertheless, drug response inconsistencies have been previously reported in different well-controlled studies using cancer cell lines [26].
Interestingly, LY2109761 also appeared to concentration-dependently inhibit SMAD1 phosphorylation and ID1 expression which are principally regulated via BMP-mediated signaling [7, 19, 27, 28]. Thus, the decrease in SMAD1 phosphorylation and ID1 expression may be considered as a TβR-independent effect of LY2109761 on other members of the TGF-β superfamily including BMPs [7, 19]. Noteworthy, this observed inhibition of BMP-related pathways is in line with results obtained using irradiated primary human pulmonary fibroblasts [29]. However, the effect of LY2109761 was contrary to the effects seen with galunisertib which up-regulated SMAD1 phosphorylation in rPCLS [15]. In addition, galunisertib did not affect the expression of ID1 (Figure 5). Thus, it is clear that individual SMIs against TGF-β particularly at high concentrations may elicit diverse effects beyond inhibition of TGF-β signaling.

Figure 5: Effect of galunisertib (10 µM) on the expression of Id1 in rat precision-cut liver slices after cultured for 72h in the presence and absence of TGF-β1 (1 ng/mL). Data are expressed as means ± SEM (n=3).

Effect on fibrogenesis

In the present study, LY2109761 regulated the expression of αSMA, a marker of myofibroblasts, in PCLS in a similar fashion as galunisertib [15]. However, in HepG2 cells, LY2109761 up-regulated both the gene and protein levels of αSMA irrespective of the presence of exogenous TGF-β1. Up-regulation of αSMA in HepG2 cells was also observed following treatment with everolimus, an inhibitor of mammalian target of rapamycin (mTOR), resulting in epithelial-mesenchymal transition (EMT) due to activation of protein kinase B (PKB) which is a serine/threonine-protein kinase that plays a key role in proliferation [30]. These results suggest that LY2109761 can induce EMT in HepG2 cells via non-selective off-target interference.

The effect of LY2109761 on the gene expression of HSP47, a chaperone protein for collagen maturation, was solely observed in rPCLS. Note that longer incubation periods may be necessary to observe inhibitory effects on protein expression [15, 31]. The fact that the inhibitory effect on the expression of HSP47 was not observed in human-derived cells and tissues: hPCLS, LX-2 and HepG2 cells, might be due to species differences in response to TGF-β and variation in constitutive expression levels of HSP47 [15, 32].
Importantly, treatment with LY2109761 had a remarkable impact on the expression of collagen type I, a principal extracellular matrix component in liver fibrosis [33], in LX-2, hPCLS and rPCLS. However, due to the lack of spontaneous fibrogenesis in LX-2 [13], the anti-fibrotic potency of LY2109761 was only shown in the presence of exogenous TGF-β1. It should be noted that due to technical difficulties, the extraction of extracellular matrix components from tissue slices was not efficient and therefore highly variable [34]; thus, we solely present the protein expression of collagen type I in LX-2 cells.

**Targeting fibrosis using small molecule inhibitors against TGF-β**

As illustrated in our study, LY2109761 may interfere with both TGF-β-dependent and -independent signaling *in vitro* and *ex vivo*. This dual effect may be extremely beneficial for the treatment of fibrosis since numerous proteins of the TGF-β superfamily including BMP-2 and BMP-9 are known to play a negative role in fibrogenesis [35, 36]. However, interfering with the beneficial roles of BMPs particularly with regard to bone healing/regeneration is a concern [37, 38].

Lastly, it is worthwhile to note that LY2109761 was the only representative of SMI s that target TGF-β tested in our study. Therefore, the effect of other SMI on fibrogenesis might be different. Furthermore, our study illustrated that LY2109761 elicited TGF-β-independent effects. Thus, possible off-target effects of SMI should be taken into account during drug development.

**Conclusion**

LY2109761 exhibited anti-fibrotic effects *in vitro* and *ex vivo*. In LX-2 cells, a human HSC cell line, the activity of LY2109761 was observed only in the presence of exogenous TGF-β. In contrast, the anti-fibrotic effect of LY2109761 can be observed in PCLS without TGF-β co-treatment. Moreover, in PCLS, cell-cell and cell-matrix interactions are preserved; thus, it is likely that the observed anti-fibrotic effect of LY2109761 in slices resembles the *in vivo* efficacy of this compound. Furthermore, our results indicate that LY2109761 can also affect BMP-mediated signaling, illustrating that SMI directed against TGF-β might influence numerous signaling pathways.

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