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

Theerut Luangmonkong^{a,b,1}, Su Suriguga^{b,1}, Adhyatmika Adhyatmika^c, Amirah Adlia^c, Dorenda Oosterhuis^b, Chuthamanee Suthisisang^a, Koert P. de Jong^d, Henricus A.M. Mutsaers^{b,e}, Peter Olinga^{b,*}

^a Department of Pharmacology, Faculty of Pharmacy, Mahidol University, Thailand

^b Department of Pharmaceutical Technology and Biopharmacy, University of Groningen, The Netherlands

^c Department of Pharmacokinetics, Toxicology and Targeting, University of Groningen, The Netherlands

^d Department of Hepato-Pancreato-Biliary Surgery and Liver Transplantation, University Medical Center Groningen, University of Groningen, The Netherlands

^e Department of Clinical Medicine, Aarhus University, Denmark

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ABSTRACT

Fibrosis is a pathophysiological state characterized by the excessive formation/deposition of fibrous extracellular matrix. 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, the effect of LY2109761, a small molecule inhibitor against TGF- β with targets beyond TGF- β signaling, on fibrogenesis was elucidated *in vitro* (HepG2 cells and LX-2 cells) and *ex vivo* (human and rat precision-cut liver slices). Our results displayed an anti-fibrotic effect of LY2109761, as it markedly down-regulated gene and protein expression of collagen type 1, as well as gene expression of the inhibitor of metalloproteinases 1. This effect on fibrosis markers was partially mediated by targeting TGF- β signaling, seeing that LY2109761 inhibited TGF- β 1 gene expression and SMAD2 protein phosphorylation. Interestingly, particularly at a high concentration, LY2109761 decreased SMAD1 protein phosphorylation and gene expression of the inhibitor of DNA binding 1, which appeared to be TGF- β -independent effects. In conclusion, LY2109761 exhibited preclinical anti-fibrotic effects *via* both TGF- β -dependent and -independent pathways. These results illustrate that small molecule inhibitors directed against TGF- β could possibly influence numerous signaling pathways and thereby mitigate fibrogenesis.

1. Introduction

Persistent scar formation, or fibrosis, is a pathophysiological state characterized by the excessive formation and deposition of fibrous extracellular matrix (ECM), particularly collagens, and unresolved fibrosis is known as a common pathway to organ dysfunction and failure (Rockey et al., 2015). Since decades, transforming growth factor-beta (TGF- β) has been recognized as a central profibrotic mediator (Pohlers et al., 2009; Dooley and ten Dijke, 2012). Consequently, TGF- β and the

associated signaling pathways have been important targets during the development of drugs for the treatment of fibrotic diseases (Akhurst and Hata, 2012; Dooley and ten Dijke, 2012); however, none of the identified candidates are eligible for clinical use due to the lack of efficacy and/or severe adverse events (Denton et al., 2007; Khaw et al., 2007). The search for effective anti-fibrotic compounds is greatly hampered by the complexity of the TGF- β signaling pathway and associated networks (Shi and Massagué, 2003; Schmierer and Hill, 2007).

Recently, LY2109761, a small molecule inhibitor (SMI) of TGF- β ,

Abbreviations: ECM, extracellular matrix; TGF- β , transforming growth factor-beta; SMIs, small molecule inhibitors; T β R, 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; α SMA, alpha smooth muscle actin; HSP47, heat shock protein 47; COL1A1, collagen type I-alpha 1; COL3A1, collagen type III-alpha1; TIMP1, tissue inhibitor of metalloproteinases 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; COL1, collagen type I; BMPs, bone morphogenetic proteins; GDFs, growth differentiation factors

* Corresponding author at: Department of Pharmaceutical Technology and Biopharmacy, University of Groningen, Antonius Deusinglaan 1, 9713 AV Groningen, The Netherlands

E-mail address: p.olinga@rug.nl (P. Olinga).

¹ Theerut Luangmonkong and Su Suriguga contributed equally to this work.

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was developed to target cancer angiogenesis and metastasis in which TGF- β plays an essential role (Melisi et al., 2008). Even though a clinical application of LY2109761 was not continued due to its poor pharmacokinetic properties (Herbertz et al., 2015), the selectivity on diverse kinase enzymes was screened *in vitro*. It was demonstrated that LY2109761 was relatively selective for the TGF- β receptor (T β R); however, at high dosage of 20 μ M, LY2109761 exhibited weak activity against lymphocyte-specific protein tyrosine kinase (Lck), p38- mitogen-activated protein kinase (p38-MAPK), mitogen-activated protein kinase kinase 6 (MKK6), proto-oncogene tyrosine-protein kinase FYN and c-Jun N-terminal kinase 3 (JNK3) (Li et al., 2008). In addition, a study in irradiated primary human fibroblasts showed that LY2109761 affected multiple mediators of the TGF- β superfamily (Flechsigs et al., 2012). Because of the impact beyond TGF- β signaling, the effects of LY2109761 on the onset of fibrosis are still of interest.

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 (Donato et al., 2015; Serova et al., 2015). 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 (Xu et al., 2005). 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 (Westra et al., 2016; Luangmonkong et al., 2017). Here, we used all these models to investigate the impact of LY2109761 on fibrogenesis.

2. Materials and methods

2.1. 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. The cells were treated for 24 h.

2.2. Precision-cut liver slices (PCLS)

Human PCLS (hPCLS) were prepared from healthy non-fibrotic liver tissue obtained from patients following either partial hepatectomy 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, according to Dutch legislation and the Code of Conduct for dealing responsibly with human tissue in the context of health research (<http://www.federa.org>), refraining the need of written consent for “further use” of coded-anonymous human tissue.

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. The study complies with the ARRIVE guidelines and carry out in accordance with the EU Directive 2010/63/EU for animal experiments.

hPCLS and rPCLS, with an estimated thickness of 250–300 μ m, were cultured for 48 h 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 (Olinga and Schuppan, 2013; Luangmonkong et al., 2017).

2.3. 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 $\leq 0.4\%$. For co-treatment, PCLS were exposed to 5 ng/mL TGF- β 1 (Roche Diagnostics, Mannheim, Germany) with 1 μ g/mL bovine serum albumin supplementation.

2.4. ATP determination

Viability was determined by measuring ATP levels as previously described (de Graaf et al., 2010). 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.

2.5. 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) (Poosti et al., 2016; Luangmonkong et al., 2017). The mRNA levels of TGF- β 1, ID1, PAI-1, α SMA, HSP47, COL1A1, COL3A1 and TIMP1 were detected using specific primer/probe sets (Applied Biosystems, California, US; Supplementary Information Table S1) using a 7900HT Real Time PCR apparatus (Applied Biosystems) with 1 cycle of 10 min/95 °C followed by 40 cycles of 15 s/95 °C and 60 s/60 °C. GAPDH was used as reference gene and relative expression levels were calculated as fold change using the $2^{-\Delta\Delta CT}$ method.

2.6. Western blotting

Phosphorylated SMAD1 (pSMAD1), pSMAD2, α SMA, 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 (Luangmonkong et al., 2017). Following sample preparation, total protein was separated on 10% sodium dodecylsulfate polyacrylamide gels and transferred using Trans-Blot Turbo Mini PVDF Transfer Packs (Bio-Rad). Afterwards, the membrane was blocked and incubated with specific antibodies (Supplementary Information Table S2). Targeted proteins were visualized with Clarity Western ECL Substrate (Bio-Rad). In a preliminary study, the protein level of total SMADs was not impacted by LY2109761; therefore, GAPDH was used as internal control protein in the remainder of the study.

2.7. Statistics

Each experiment was performed at least three times. Results are expressed as means $\pm$ 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, Δ Ct and relative signal intensity of the proteins. A *p*-value $< .05$ was considered significant.

3. Results

3.1. HepG2

We first investigated the impact of LY2109761 on TGF- β signaling in HepG2 cells in the absence and presence of exogenous TGF- β 1. Our

Fig. 1. Gene expression of HepG2 cells treated with LY2109761 (LY), compared to the corresponding control ($n = 4$). (A) The treatment with exogenous TGF-β1 significantly increased *TGF-β1* and *PAI-1*. (B) LY2109761 (20 μM) reduced *ID1* but increased *αSMA*. (C) In the co-treatment, decreased *ID1* and *PAI-1* were observed. The concentration of exogenous TGF-β1 was 5 ng/mL. * $p < .05$ compared to either control, LY 0 μM, or LY 0 μM + TGF-β1.

results demonstrated that none of the treatment modalities impacted the viability of HepG2 cells (Supplementary Information Fig. S1).

Furthermore, on a gene level, 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 (Fig. 1A). LY2109761, in particular at the highest tested concentration (20 μM), reduced the gene levels of *ID1* (82% inhibition when compared to the corresponding control), but increased *αSMA* (2.80-fold) expression (Fig. 1B). When HepG2 cells were exposed to both exogenous TGF-β1 and LY2109761, significant decreased mRNA levels of *ID1* (81%) and *PAI-1* (63% at 2.5

μM) were observed (Fig. 1C). Again, increased *αSMA* (3.07-fold) expression was observed at the high concentration of LY2109761 (Fig. 1C).

On a protein level, we did not observe clear effects of exogenous TGF-β1 (Fig. 2A). 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 (Fig. 2B and C). In the latter case, pSMAD1 expression was markedly reduced (86%; Fig. 2C).

3.2. LX-2

In addition to the impact of LY2109761 on TGF-β signaling, we subsequently studied whether this compound could mitigate the expression of fibrosis-related markers in LX-2 cells. As assessed by the ATP content, treatment with neither 5 ng/mL TGF-β1, LY2109761, nor a combination of both affected the viability of LX-2 cells (Supplementary Information Fig. S2).

On a gene level, we demonstrated that exogenous TGF-β1 induced a fibrotic response in LX-2 cells as illustrated by an increased expression of *TGF-β1* (3.62-fold), *PAI-1* (10.55-fold), *αSMA* (1.50-fold) and *COL1A1* (2.53-fold; Fig. 3A), while the expression of *COL3A1* was decreased. Additionally, qPCR revealed that LY2109761 lowered TGF-β1 expression (53%), while *αSMA* and *TIMP1* levels increased (1.85- and 1.60-fold, respectively), particularly at 20 μM (Fig. 3B). Furthermore, our results showed that LY2109761 markedly reduced exogenous TGF-β1-induced fibrotic response, as it decreased the expression of *TGF-β1* (86%), *PAI-1* (87% at 2.5 μM) and *COL1A1* (67%; Fig. 3C). Interestingly, although neither exogenous TGF-β1 nor LY2109761 affected *ID1* expression, an obvious down-regulation was observed with the co-treatment (Fig. 3C).

These findings were corroborated by Western blotting showing that exogenous TGF-β1 significantly increased phosphorylation of SMAD1 (2.68-fold) and SMAD2 (6.19-fold; Fig. 4A). As a result, the level of those phosphorylated proteins was not clearly detectable without the addition of TGF-β1; thus, the quantification of SMADs phosphorylation, which showed a large variation, was not displayed (Fig. 4B). The co-treatment with LY2109761 significantly inhibited phosphorylation of SMAD1 (84%) and SMAD2 (92% at 2.5 μM; Fig. 4C). Notably, exogenous TGF-β1 markedly increased the expression of *COL1* (4.17-fold; Fig. 4A), which was antagonized by LY2109761 in a concentration-dependent manner (up to 91%; Fig. 4C).

3.3. Human precision-cut liver slices (hPCLS)

Next, we investigated the anti-fibrotic efficacy of LY2109761 in hPCLS, a multicellular human *ex vivo* model of liver fibrosis. Our results demonstrated that neither LY2109761 nor 5 ng/mL TGF-β1 treatment had the effect on the viability of hPCLS (Supplementary Information Fig. S3).

qPCR revealed that, besides significant up-regulation of *TGF-β1* (2.11-fold), exogenous TGF-β1 could not markedly elicit a fibrotic response in hPCLS (Fig. 5A), which was in line with previous results (Westra et al., 2016). Nevertheless, LY2109761 significantly inhibited the expression of *COL1A1* both in the absence (86%) and presence (96%) of exogenous TGF-β1 (Fig. 5B and C). During co-treatment, significant decreased mRNA levels of *TGF-β1* (75% at 2.5 μM), *HSP47* (57%) and *TIMP1* (81% at 2.5 μM) were observed (Fig. 5C).

On a protein level, exogenous TGF-β1 significantly increased phosphorylated SMAD2 (1.54-fold) and phosphorylated SMAD1, although the latter was not statistically significant. It is worthwhile to note that the expression of *αSMA* was slightly decreased (37%) by exogenous TGF-β1 (Fig. 6A). LY2109761 treatment resulted in a diminished phosphorylation of both SMAD1 (70%) and SMAD2 (84%; Fig. 6B), while only SMAD2 phosphorylation was obviously antagonized (95%) in the presence of exogenous TGF-β1 (Fig. 6C).

Fig. 2. Protein expression of HepG2 cells treated with LY2109761 (LY), compared to the corresponding control (n = 4). (A) The treatment with exogenous TGF-β1 did not significantly influence the expression. (B) LY2109761 (20 μM) significantly increased αSMA in the absence exogenous TGF-β1. (C) Increased αSMA and decreased pSMAD1 were observed in the co-treatment. Representative Western blots were shown on the left. Average protein expression of all experimental groups shown as bar graphs after normalization to GAPDH were shown on the right. The concentration of exogenous TGF-β1 was 5 ng/mL. *p < .05 compared to either control, LY 0 μM, or LY 0 μM + TGF-β1.

3.4. Rat precision-cut liver slices (rPCLS)

Because exogenous TGF-β1 (5 ng/mL) could not markedly increase the expression of fibrosis markers in hPCLS, we additionally studied the effects of LY2109761 on fibrogenesis and TGF-β signaling in rPCLS (Luangmonkong et al., 2017). It was shown that none of the experimental treatments altered the viability of rPCLS (Supplementary Information Fig. S4).

In rPCLS, exogenous TGF-β1 significantly increased the expression of *Tgf-β1* (1.86-fold), *αSma* (4.45-fold), *Hsp47* (2.43-fold), *Col1a1* (3.85-fold) and *Timp1* (2.27-fold; Fig. 7A). Moreover, in the absence of exogenous TGF-β1, LY2109761 significantly decreased the expression of *Id1* (86%), *αSma* (82%) and *Col1a1* (93%; Fig. 7B). This inhibitory effect persisted in the presence of exogenous TGF-β1 with the treatment of 20 μM LY2109761 only (*Id1* (65%), *αSma* (83%) and *Col1a1* (94%; Fig. 7C)). In addition, a down-regulation of *Tgf-β1* (57%), *Hsp47* (76%) and *Timp1* (73%) was observed in the co-treatment with 20 μM (Fig. 7C).

Protein expression in rPCLS treated with exogenous TGF-β1 appeared to be partially in line with the gene expression as αSma was

elevated (1.62-fold); however, Hsp47 expression was not increased (Fig. 8A). Exogenous TGF-β1 significantly increased Smad2 phosphorylation (3.75-fold; Fig. 8A), and the up-regulation was concentration-dependently antagonized (up to 83%) by LY2109761 (Fig. 8C). Inhibition of Smad2 phosphorylation was also observed (96%) in the absence of exogenous TGF-β1 (Fig. 8B). Although decreased phosphorylation of Smad1 was observed (84%) in the absence of exogenous TGF-β1, the effect was not statistically significant (Fig. 8B).

4. 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 (Weiss and Attisano, 2013). The multi-functional 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 (Schmierer and Hill, 2007; Gordon and Blobbe, 2008). 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

Fig. 3. Gene expression of LX-2 cells treated with LY2109761 (LY), compared to the corresponding control ($n = 4-5$). (A) Exogenous TGF-β1 increased *TGF-β1*, *PAI-1*, *αSMA* and *COL1A1*, while decreased *COL3A1*. (B) LY2109761 lowered *TGF-β1*, while *αSMA* and *TIMP1* were increased. (C) In the co-treatment, LY2109761 markedly reduced TGF-β1-induced up-regulation of *TGF-β1*, *PAI-1*, *COL1A1* and *ID1*. The concentration of exogenous TGF-β1 was 5 ng/mL. * $p < .05$ compared to either control, LY 0 μM, or LY 0 μM + TGF-β1.

using SMIs (Dooley and ten Dijke, 2012). Here, we studied the impact of LY2109761, a SMI against TGF-β with targets beyond TGF-β signaling (Melisi et al., 2008), on the expressions of genes and proteins related to TGF-β pathway activity and fibrogenesis.

4.1. LY2109761 inhibited TGF-β-dependent and -independent pathways

Our results demonstrated that LY2109761 clearly inhibited activation of the TGF-β pathway, as illustrated by a decreased expression of *TGF-β1* (Akhurst and Hata, 2012), and reduced phosphorylation of SMAD2, the master transcription factor mediating fibrogenic responses of TGF-β (Herbert et al., 2015), in LX-2, hPCLS and rPCLS. In general, inhibition of TGF-β signaling is associated with decreased *PAI-1* expression *in vitro* (Kortlever et al., 2008; Samarakoon and Higgins, 2008; Kawarada et al., 2016); 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 *via* inhibition of SMAD2 phosphorylation, without affecting *PAI-1* expression (Luangmonkong et al., 2017). 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 (Cesari et al., 2010).

One 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, and subsequent fibrogenesis, is spontaneous in PCLS, and in contrast to cell lines, the impact of drugs affecting the TGF-β pathway can be studied in PCLS without the addition of exogenous TGF-β (Luangmonkong et al., 2017).

In this study, the expression of *TGF-β1* and phosphorylation of SMAD2 were not affected by LY2109761 in HepG2 cells. This finding was not consistent with previous studies showing that SMAD2

Fig. 4. Protein expression of LX-2 cells treated with LY2109761 (LY), compared to the corresponding control (n = 4–5). (A) Exogenous TGF-β1 significantly increased pSMAD1, pSMAD2 and COL1. (B) Effect of LY2109761 was not observed in the absence of exogenous TGF-β1 (quantification of pSMAD1 and pSMAD2 was not displayed). (C) The co-treatment apparently inhibited pSMAD1, pSMAD2 and COL1. Representative Western blots were shown on the left. Average protein expression of all experimental groups shown as bar graphs after normalization to GAPDH were shown on the right. The concentration of exogenous TGF-β1 was 5 ng/mL. **p* < .05 compared to either control, LY 0 μM, or LY 0 μM + TGF-β1.

phosphorylation in HepG2 cells was modulated by other TGF-β inhibitors: galunisertib and D10, a monoclonal antibody against TβR (Dituri et al., 2013; Serova et al., 2015). Nevertheless, drug response inconsistencies have been previously reported in different well-controlled studies using cancer cell lines (Haibe-Kains et al., 2013).

Interestingly, LY2109761 also appeared to concentration-dependently inhibit SMAD1 phosphorylation and the expression of inhibitor of DNA binding 1 (*ID1*) which are principally regulated via BMP-mediated signaling (Katagiri et al., 2002; Valdimarsdottir et al., 2002; Schmierer and Hill, 2007; Weiss and Attisano, 2013). Both SMAD1 and *ID1* were not impacted by exogenous TGF-β1; thus, the decrease in SMAD1 phosphorylation and *ID1* expression could be considered as a TβR-independent effect of LY2109761 on other members of the TGF-β superfamily including BMPs (Schmierer and Hill, 2007; Weiss and Attisano, 2013). Noteworthy, this observed inhibition of BMPs-related pathways was in line with results obtained using irradiated primary human fibroblasts (Fleischig et al., 2012). However, the effect of LY2109761 was contrary to the effects seen with galunisertib which up-

regulated SMAD1 phosphorylation in rPCLS (Luangmonkong et al., 2017). In addition, galunisertib did not affect the expression of *ID1* (Supplementary Information Fig. S5). Therefore, it is clear that individual SMIs against TGF-β particularly at high concentrations may elicit diverse effects beyond inhibition of TGF-β signaling.

The inhibition of SMAD1 protein phosphorylation and *ID1* gene expression by LY2109761 might be involved in the observed decrease in fibrogenesis both *in vitro* and *ex vivo*. As shown in a previous study using isolated rat HSCs, *Id1* was identified as a critical mediator in transdifferentiation and in liver fibrogenesis. Moreover, this *in vitro* study displayed that the overexpression of *Id1* was due to TGF-β1/SMAD1 pathway activation (Wiercinska et al., 2006). The reason why exogenous TGF-β1 could not augment SMAD1 phosphorylation and *ID1* expression in our study (except increased phosphorylated SMAD1 in LX-2 cells) is unknown. However, in primary rat HSCs it was shown that the levels of phosphorylated Smad1 and *Id1* peaked at one hour after TGF-β1 exposure and then decreased to control levels (Wiercinska et al., 2006). Thus, timing is critical when studying these proteins.

Fig. 5. Gene expression of human precision-cut liver slices (hPCLS) treated with LY2109761 (LY), compared to the corresponding control (n = 4–8). (A) Besides up-regulation of *TGF-β1*, exogenous *TGF-β1* could not markedly elicit a fibrotic response. (B) LY2109761 significantly decreased *COL1A1* in the absence of exogenous *TGF-β1*. (C) During co-treatment, significant decreased mRNA levels of *TGF-β1*, *HSP47*, *COL1A1* and *TIMP1* were observed. The concentration of exogenous *TGF-β1* was 5 ng/mL. **p* < .05 compared to either control, LY 0 μM, or LY 0 μM + *TGF-β1*.

4.2. In vitro and ex vivo anti-fibrotic effect of LY2109761

In our study, LY2109761 inhibited the expression αSMA, which is a popular marker of myofibroblasts (the major ECM-producing cells in fibrosis), in rPCLS and not in hPCLS. This is in line with a previous study from our group in which we tested the anti-fibrotic efficacy of galunisertib (Luangmonkong et al., 2017). Furthermore, another study demonstrated that αSMA protein expression decreased in PCLS prepared from cirrhotic liver during culture (Guyot et al., 2010), these results again question the validity of αSMA as a fibrosis marker in human tissues, in particular in PCLS.

Another point of interest was the up-regulation αSMA mRNA in LX-2 cells treated with LY2109761 alone. Nevertheless, this change was observed following neither the treatment with exogenous *TGF-β1* nor on protein level. Although the reason why this event was seen needs to be elucidated further, LY2109761 was previously shown to inhibit SMAD6, 7, and 9 expression in human fibroblasts (Flechsigs et al., 2012).

These inhibitory proteins of *TGF-β* signaling, especially SMAD7, could obfuscate the impact of LY2109761 on the expression profile of fibrosis markers, including αSMA (Dooley et al., 2003; Fu et al., 2017). In HepG2 cells, only the highest concentration of LY2109761 increased both the gene and protein levels of αSMA irrespective of the presence of exogenous *TGF-β1*. This finding could also be due to the inhibitory effect of LY2109761 on SMAD7, which is known to be expressed in HepG2 cells (Masola et al., 2015; Serova et al., 2015).

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 (Ishida and Nagata, 2011; Luangmonkong et al., 2017). 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* (Brown et al., 2005; Luangmonkong et al., 2017).

Fig. 6. Protein expression of human precision-cut liver slices (hPCLS) treated with LY2109761 (LY), compared to the corresponding control ($n = 4-8$). (A) Exogenous TGF-β1 significantly augmented pSMAD2 and non-significantly raised pSMAD1; however, it slightly decreased αSMA. (B) LY2109761 diminished pSMAD1 and pSMAD2. (C) Antagonization of pSMAD2 by LY2109761 was obvious in the presence of exogenous TGF-β1. Representative Western blots were shown on the left. Average protein expression of all experimental groups shown as bar graphs after normalization to GAPDH were shown on the right. The concentration of exogenous TGF-β1 was 5 ng/mL. * $p < .05$ compared to either control, LY 0 μM, or LY 0 μM + TGF-β1.

Importantly, treatment with LY2109761 had a remarkable impact on the expression of collagen type I, a principal extracellular matrix component in liver fibrosis (Voss et al., 1980), in LX-2, hPCLS and rPCLS. However, due to the lack of spontaneous fibrogenesis in LX-2 (Xu et al., 2005), 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 ECM components from tissue slices was not efficient and therefore highly variable (Pacak et al., 2011); thus, we solely present the protein expression of collagen type I in LX-2 cells. Compared to collagen type I, the expression of collagen type III was not markedly affected by exogenous TGF-β1 and LY2109761, even though both types of collagen are known to co-express in human liver (Voss et al., 1980; Yamamoto et al., 1984). This finding supports the versatility and sensitivity of collagen type I as a biomarker in the preclinical study of liver fibrosis.

Furthermore, the anti-fibrotic efficacy of LY2109761 was underlined by the decreased gene expression of tissue inhibitor of metalloproteinases 1 (TIMP1), which plays a vital role in the degradation of

ECM (Busk et al., 2014), in both human and rat PCLS. Noteworthy, the up-regulation of TIMP1 in LX-2 cells treated with LY2109761 correlated with the increased expression of αSMA. Therefore, the impact of LY2109761 on inhibitory SMADs may also affect the regulation of TIMP1 in LX-2 cells, as also observed in SMAD7-overexpressing bone mesenchymal stem cells (Wu et al., 2017).

4.3. Targeting fibrosis using small molecule inhibitors against TGF-β

In our study, LY2109761 could 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 (Simone et al., 2012; Munoz-Felix et al., 2016). Therefore, the inhibition of the SMAD1/ID1 pathway, which regulates the activity of BMPs and HSC activation, might be beneficial (Wiercinska et al., 2006). On the other hand, interfering with the beneficial roles of BMPs particularly with regard to

Fig. 7. Gene expression of rat precision-cut liver slices (rPCLS) treated with LY2109761 (LY), compared to the corresponding control ($n = 3-4$). (A) Exogenous TGF- β 1 significantly increased *Tgf-β1*, *αSma*, *Hsp47*, *Col1a1* and *Timp1*. (B) In the absence of exogenous TGF- β 1, LY2109761 significantly decreased *Id1*, *αSma* and *Col1a1*. (C) A down-regulation of *Tgf-β1*, *Id1*, *αSma*, *Hsp47*, *Col1a1* and *Timp1* was observed in the co-treatment with 20 μ M only. The concentration of exogenous TGF- β 1 was 5 ng/mL. * $p < .05$ compared to either control, LY 0 μ M, or LY 0 μ M + TGF- β 1.

bone healing/regeneration is a concern (Duangkumpha et al., 2014; Wang et al., 2014).

Lastly, it is worthwhile to note that LY2109761 was the only representative of SMIs that targets TGF- β tested in our study. Therefore, the effect of other SMIs on fibrogenesis might be different. Furthermore, our study illustrated that LY2109761 elicited TGF- β -independent effects. Thus, possible off-target effects of SMIs, which may augment/deteriorate anti-fibrotic efficacy or even cause adverse events, should be taken into account during drug development.

5. 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, besides TGF- β -dependent effects, our results indicate that LY2109761 can also affect TGF- β -independent pathways. Therefore, SMIs directed against TGF- β might influence numerous signaling pathways thereby mitigating fibrogenesis.

Conflict of interest

No conflict of interest.

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Fig. 8. Protein expression of rat precision-cut liver slices (rPCLS) treated with LY2109761 (LY), compared to the corresponding control (n = 3–4). (A) Exogenous TGF-β1 significantly increased pSmad2 and αSma. (B) LY2109761 lowered pSmad2. (C) In the co-treatment, the decreased pSmad2 was persisted, while αSma was not significantly affected. pSMAD1 tended to be diminished by LY2109761 in a concentration-dependent manner. Representative Western blots were shown on the left. Average protein expression of all experimental groups shown as bar graphs after normalization to GAPDH were shown on the right. The concentration of exogenous TGF-β1 was 5 ng/mL. *p < .05 compared to either control, LY 0 μM, or LY 0 μM + TGF-β1.

Author's contributions

T. Luangmonkong and S. Suriguga: study design; experiments and procedures; acquisition, analysis, and interpretation of data; drafting of the manuscript; statistical analysis. A. Adhyatmika, A. Adlia and D. Oosterhuis: experiments and procedures. C. Suthisang and K. P. de Jong: revision of the manuscript for important intellectual content. H.A.M. Mutsaers: analysis and interpretation of data; drafting and revision of the manuscript for important intellectual content. P. Olinga: analysis and interpretation of data; drafting and revision of the manuscript for important intellectual content; obtained funding; study supervision.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.taap.2018.07.001>.

References

- Akhurst, R.J., Hata, A., 2012. Targeting the TGFβ signalling pathway in disease. *Nat. Rev. Drug Discov.* 11, 790–811.
- Brown, K.E., Broadhurst, K.A., Mathahs, M.M., Brunt, E.M., Schmidt, W.N., 2005. Expression of HSP47, a collagen-specific chaperone, in normal and diseased human liver. *Lab. Invest.* 85, 789–797.
- Busk, T.M., Bendtsen, F., Nielsen, H.J., Jensen, V., Brunner, N., Møller, S., 2014. TIMP-1 in patients with cirrhosis: relation to liver dysfunction, portal hypertension, and hemodynamic changes. *Scand. J. Gastroenterol.* 49, 1103–1110.

- Cesari, M., Pahor, M., Incalzi, R.A., 2010. Plasminogen activator inhibitor-1 (PAI-1): a key factor linking fibrinolysis and age-related subclinical and clinical conditions. *Cardiovasc. Ther.* 28, e72–e91.
- 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.
- Denton, C.P., Merkel, P.A., Furst, D.E., Khanna, D., Emery, P., Hsu, V.M., Silliman, N., Streisand, J., Powell, J., Akesson, A., Coppock, J., Hoogen, F., Herrick, A., Mayes, M.D., Veale, D., Haas, J., Ledbetter, S., Korn, J.H., Black, C.M., Seibold, J.R., 2007. Recombinant human anti-transforming growth factor beta1 antibody therapy in systemic sclerosis: a multicenter, randomized, placebo-controlled phase I/II trial of CAT-192. *Arthritis Rheum.* 56, 323–333.
- Dituri, F., Mazzocca, A., Peidrò, F.J., Papappicco, P., Fabregat, I., De Santis, F., Paradiso, A., Sabbà, C., Giannelli, G., 2013. Differential inhibition of the TGF- β signaling pathway in HCC cells using the small molecule inhibitor LY2157299 and the D10 monoclonal antibody against TGF- β receptor type II. *PLoS One* 8, e67109.
- Donato, M.T., Tolosa, L., Gomez-Lechon, M.J., 2015. Culture and functional characterization of human hepatoma HepG2 cells. *Methods Mol. Biol.* (Clifton, N.J.) 1250, 77–93.
- Dooley, S., ten Dijke, P., 2012. TGF- β in progression of liver disease. *Cell Tissue Res.* 347, 245–256.
- Dooley, S., Hamzavi, J., Breitkopf, K., Wiercinska, E., Said, H.M., Lorenzen, J., Ten Dijke, P., Gressner, A.M., 2003. Smad7 prevents activation of hepatic stellate cells and liver fibrosis in rats. *Gastroenterology* 125, 178–191.
- Duangkumpha, K., Techasen, A., Loilome, W., Namwat, N., Thanan, R., Khuntikeo, N., Yongvanit, P., 2014. BMP-7 blocks the effects of TGF-beta-induced EMT in cholangiocarcinoma. *Tumour Biol.* 35, 9667–9676.
- Flechsigs, P., Dadrich, M., Bickelhaupt, S., Jenne, J., Hauser, K., Timke, C., Peschke, P., Hahn, E.W., Grone, H.J., Yingling, J., Lahn, M., Wirkner, U., Huber, P.E., 2012. LY2109761 attenuates radiation-induced pulmonary murine fibrosis via reversal of TGF-beta and BMP-associated proinflammatory and proangiogenic signals. *Clin. Cancer Res.* 18, 3616–3627.
- Fu, R.Q., Hu, D.P., Hu, Y.B., Hong, L., Sun, Q.F., Ding, J.G., 2017. miR21 promotes alphaSMA and collagen I expression in hepatic stellate cells via the Smad7 signaling pathway. *Mol. Med. Rep.* 16, 4327–4333.
- Gordon, K.J., Blobel, G.C., 2008. Role of transforming growth factor- β superfamily signaling pathways in human disease. *Biochim. Biophys. Acta (BBA) - Mol. Basis Dis.* 1782, 197–228.
- Guyot, C., Lepreux, S., Combe, C., Sarrazay, V., Billet, F., Balabaud, C., Bioulac-Sage, P., Desmouliere, A., 2010. Fibrogenic cell phenotype modifications during remodelling of normal and pathological human liver in cultured slices. *Liver Int.* 30, 1529–1540.
- Haibe-Kains, B., El-Hachem, N., Birkbak, N.J., Jin, A.C., Beck, A.H., Aerts, H.J.W.L., Quackenbush, J., 2013. Inconsistency in large pharmacogenomic studies. *Nature* 504, 389–393.
- Herbertz, S., Sawyer, J.S., Stauber, A.J., Gueorgieva, I., Driscoll, K.E., Estrem, S.T., Cleverly, A.L., Desai, D., Guba, S.C., Benhadji, K.A., Slapak, C.A., Lahn, M.M., 2015. Clinical development of galunisertib (LY2157299 monohydrate), a small molecule inhibitor of transforming growth factor-beta signaling pathway. *Drug Des. Dev. Ther.* 9, 4479–4499.
- Ishida, Y., Nagata, K., 2011. Hsp47 as a collagen-specific molecular chaperone. *Methods Enzymol.* 499, 167–182.
- Katagiri, T., Imada, M., Yanai, T., Suda, T., Takahashi, N., Kamijo, R., 2002. Identification of a BMP-responsive element in Id1, the gene for inhibition of myogenesis. *Genes Cells* 7, 949–960.
- Kawarada, Y., Inoue, Y., Kawasaki, F., Fukaura, K., Sato, K., Tanaka, T., Itoh, Y., Hayashi, H., 2016. TGF-beta induces p53/Smads complex formation in the PAI-1 promoter to activate transcription. *Sci. Rep.* 6, 35483.
- Khaw, P., Grehn, F., Hollo, G., Overton, B., Wilson, R., Vogel, R., Smith, Z., 2007. A phase III study of subconjunctival human anti-transforming growth factor beta(2) monoclonal antibody (CAT-152) to prevent scarring after first-time trabeculectomy. *Ophthalmology* 114, 1822–1830.
- Kortlever, R.M., Nijwening, J.H., Bernards, R., 2008. Transforming growth factor-beta requires its target plasminogen activator inhibitor-1 for cytostatic activity. *J. Biol. Chem.* 283, 24308–24313.
- Li, H.Y., McMillen, W.T., Heap, C.R., McCann, D.J., Yan, L., Campbell, R.M., Mundla, S.R., King, C.H., Dierks, E.A., Anderson, B.D., Britt, K.S., Huss, K.L., Voss, M.D., Wang, Y., Clawson, D.K., Yingling, J.M., Sawyer, J.S., 2008. Optimization of a dihydropyrazole series of transforming growth factor-beta type I receptor kinase domain inhibitors: discovery of an orally bioavailable transforming growth factor-beta receptor type I inhibitor as antitumor agent. *J. Med. Chem.* 51, 2302–2306.
- Luangmonkong, T., Suriguga, S., Bigaeva, E., Boersema, M., Oosterhuis, D., de Jong, K.P., Schuppan, D., Mutsaers, H.A.M., Olinga, P., 2017. Evaluating the antifibrotic potency of galunisertib in a human ex vivo model of liver fibrosis. *Br. J. Pharmacol.* 174, 3107–3117.
- Masola, V., Carraro, A., Zaza, G., Bellin, G., Montin, U., Violi, P., Lupo, A., Tedeschi, U., 2015. Epithelial to mesenchymal transition in the liver field: the double face of Everolimus in vitro. *BMC Gastroenterol.* 15, 118.
- Melisi, D., Ishiyama, S., Scabas, G.M., Fleming, J.B., Xia, Q., Tortora, G., Abbruzzese, J.L., Chiao, P.J., 2008. LY2109761, a novel transforming growth factor beta receptor type I and type II dual inhibitor, as a therapeutic approach to suppressing pancreatic cancer metastasis. *Mol. Cancer Ther.* 7, 829–840.
- Munoz-Felix, J.M., Cuesta, C., Perretta-Tejedor, N., Subileau, M., Lopez-Hernandez, F.J., Lopez-Novoa, J.M., Martinez-Salgado, C., 2016. Identification of bone morphogenetic protein 9 (BMP9) as a novel profibrotic factor in vitro. *Cell. Signal.* 28, 1252–1261.
- Olinga, P., Schuppan, D., 2013. Precision-cut liver slices: a tool to model the liver ex vivo. *J. Hepatol.* 58, 1252–1253.
- Pacak, C.A., Powers, J.M., Cowan, D.B., 2011. Ultrarapid purification of collagen type I for tissue engineering applications. *Tissue Eng. C* 17, 879–885.
- Pohlert, D., Brenmoehl, J., Löffler, I., Müller, C.K., Leipner, C., Schultze-Mosgau, S., Stallmach, A., Kinne, R.W., Wolf, G., 2009. TGF- β and fibrosis in different organs — molecular pathway imprints. *Biochim. Biophys. Acta (BBA) - Mol. Basis Dis.* 1792, 746–756.
- Poosti, F., Bansal, R., Yazdani, S., Prakash, J., Beljaars, L., van den Born, J., de Borst, M.H., van Goor, H., Hillebrands, J.-L., Poelstra, K., 2016. Interferon gamma peptidomimetic targeted to interstitial myofibroblasts attenuates renal fibrosis after unilateral ureteral obstruction in mice. *Oncotarget* 7, 54240–54252.
- Rockey, D.C., Bell, P.D., Hill, J.A., 2015. Fibrosis—a common pathway to organ injury and failure. *N. Engl. J. Med.* 372, 1138–1149.
- Samarakoon, R., Higgins, P.J., 2008. Integration of non-SMAD and SMAD signaling in TGF-beta1-induced plasminogen activator inhibitor type-1 gene expression in vascular smooth muscle cells. *Thromb. Haemost.* 100, 976–983.
- Schmierer, B., Hill, C.S., 2007. TGFbeta-SMAD signal transduction: molecular specificity and functional flexibility. *Nat. Rev. Mol. Cell Biol.* 8, 970–982.
- Serova, M., Tijeras-Raballand, A., Dos Santos, C., Albuquerque, M., Paradis, V., Neuzillet, C., Benhadji, K.A., Raymond, E., Faivre, S., de Gramont, A., 2015. Effects of TGF-beta signalling inhibition with galunisertib (LY2157299) in hepatocellular carcinoma models and in ex vivo whole tumor tissue samples from patients. *Oncotarget* 6, 21614–21627.
- Shi, Y., Massagué, J., 2003. Mechanisms of TGF- β signaling from cell membrane to the nucleus. *Cell* 113, 685–700.
- Simone, S., Cosola, C., Loverre, A., Cariello, M., Sallustio, F., Rascio, F., Gesualdo, L., Schena, F.P., Grandaliano, G., Pertosa, G., 2012. BMP-2 induces a profibrotic phenotype in adult renal progenitor cells through Nox4 activation. *Am. J. Physiol. Ren. Physiol.* 303, F23–F34.
- Valdimarsdottir, G., Goumans, M.J., Rosendahl, A., Brugman, M., Itoh, S., Lebrin, F., Sideras, P., ten Dijke, P., 2002. Stimulation of Id1 expression by bone morphogenetic protein is sufficient and necessary for bone morphogenetic protein-induced activation of endothelial cells. *Circulation* 106, 2263–2270.
- Voss, B., Rauterberg, J., Allam, S., Pott, G., 1980. Distribution of collagen type I and type III and of two collagenous components of basement membranes in the human liver. *Pathol. Res. Pract.* 170, 50–60.
- Wang, R.N., Green, J., Wang, Z., Deng, Y., Qiao, M., Peabody, M., Zhang, Q., Ye, J., Yan, Z., Denduluri, S., Idowu, O., Li, M., Shen, C., Hu, A., Haydon, R.C., Kang, R., Mok, J., Lee, M.J., Luu, H.L., Shi, L.L., 2014. Bone morphogenetic protein (BMP) signaling in development and human diseases. *Genes Diseases* 1, 87–105.
- Weiss, A., Attisano, L., 2013. The TGFbeta superfamily signaling pathway. *Wiley Interdiscip. Rev. Dev. Biol.* 2, 47–63.
- Westra, I.M., Mutsaers, H.A., Luangmonkong, T., Hadi, M., Oosterhuis, D., de Jong, K.P., Groothuis, G.M., Olinga, P., 2016. Human precision-cut liver slices as a model to test antifibrotic drugs in the early onset of liver fibrosis. *Toxicol. In Vitro* 35, 77–85.
- Wiercinska, E., Wickert, L., Denecke, B., Said, H.M., Hamzavi, J., Gressner, A.M., Thorikay, M., ten Dijke, P., Mertens, P.R., Breitkopf, K., Dooley, S., 2006. Id1 is a critical mediator in TGF-beta-induced transdifferentiation of rat hepatic stellate cells. *Hepatology* 43, 1032–1041.
- Wu, S.P., Yang, Z., Li, F.R., Liu, X.D., Chen, H.T., Su, D.N., 2017. Smad7-overexpressing rat BMSCs inhibit the fibrosis of hepatic stellate cells by regulating the TGF- β 1/Smad signaling pathway. *Exp. Ther. Med.* 14, 2568–2576.
- Xu, L., Hui, A.Y., Albanis, E., Arthur, M.J., O'Byrne, S.M., Blaner, W.S., Mukherjee, P., Friedman, S.L., Eng, F.J., 2005. Human hepatic stellate cell lines, LX-1 and LX-2: new tools for analysis of hepatic fibrosis. *Gut* 54, 142–151.
- Yamamoto, M., Sumiyoshi, H., Nakagami, K., Tahara, E., 1984. Distribution of collagen types I, III, and V in fibrotic and neoplastic human liver. *Acta Pathol. Japon.* 34, 77–86.