Exploring anti-fibrotic drugs

Luangmonkong, Theerut

General discussion and perspectives

During the conceptualization of this thesis, I searched “fibrosis” on Google, resulting in more than 26,700,000 hits. Interestingly, this number hardly decreased when the search was restricted with the addition of “treatment”. Even though Google, the most popular search engine in the world, should not be used as a scientific reference, this observation illustrates that a treatment for fibrosis is of great interest to the public. Moreover, the scientific community has shown great interest in fibrosis research, resulting in more than 20,000 publications since 2016. Noticeably, despite this fascination, only two anti-fibrotic drugs, pirfenidone and nintedanib, are currently approved for the treatment of a single disease, idiopathic pulmonary fibrosis (IPF) [1, 2]. The persistence of this unmet clinical need is probably due to the greatest hurdle in fibrosis research, that is, the complexity of the disease process which involves numerous signaling pathways and cell types [3-5]. Therefore, this thesis delineates the use of precision-cut tissue slices as an *ex vivo* model for the exploration of the effectiveness of putative anti-fibrotic drugs.

TGF-β: Potent, but complicated, therapeutic target

Transforming growth factor beta (TGF-β) is one of the key factors driving the fibrotic response in most organs, and numerous studies, including my own work, have shown that targeting this protein and associated signaling pathways can successfully mitigate fibrosis [6]. Additionally, in this thesis, the benefits of mitigating TGF-β for the treatment of liver fibrosis were proven *ex vivo*. Taken together, TGF-β may be a significant therapeutic candidate for liver fibrosis. Nonetheless, mitigating TGF-β and its corresponding signaling pathway emerges with several challenges in clinical practice.

The most obvious challenge is how the optimal therapeutic approach should be. In mammals, three isoforms of TGF-β exist: TGF-β1, TGF-β2 and TGF-β3. These highly homologous proteins are synthesized, processed and regulated in a similar fashion; however, the isoforms are differently expressed in different organs [6-8]. As illustrated in Figure 1, the TGF-β pathway can be targeted at various levels, firstly via the inhibition of transcription/translation by gene silencing and anti-sense approaches in order to halt protein synthesis [9-12]. Prime examples hereof are trabedersen and belagenpumatucel-L, which are TGF-β2 anti-sense oligonucleotides. Trabedersen exhibited promising therapeutic efficacy in patients with glioblastoma [9, 10], and belagenpumatucel-L improved survival of non-small cell lung cancer patients [13]. However, targeted drug delivery is needed to avoid off-target toxicity of these oligonucleotides. In the case of trabedersen, this was achieved by intrathecal administration to directly target the tumor [10]. Following intracellular synthesis, TGF-β is secreted as an inactive protein complex, which remains in the extracellular matrix (ECM). Active TGF-β can be released by various activators such as reactive oxygen species

(ROS), plasmin, thrombospondin-1 and $\alpha_v\beta_6$ integrin [6]. A clinical study of STX-100, a humanized monoclonal antibody against $\alpha_v\beta_6$ integrin, for the treatment of IPF was completed in March, 2017 (clinicaltrials.gov: NCT01371035); however, the primary outcome was not revealed yet. Once active TGF-β is released, it can be sequestered using monoclonal antibodies such as lerdelimumab, metalimumab and fresolimumab, but the effectiveness of these antibodies in human studies was not impressive [14, 15]. Thus, they were not further developed for clinical use. In addition to specific sequestering of TGF-β, small molecule inhibitors such as galunisertib (chapter A1) and LY2109761 (chapter A2) were developed to inhibit kinase activity of the TGF-β receptor. To date, as described in chapter A1, galunisertib is under clinical investigation for the treatment of various cancers. Finally, another possible approach is activating SMAD7, which antagonizes TGF-β signaling. In a rat unilateral ureteral obstruction model, it was shown that SMAD7 gene transfer could inhibit renal fibrosis [16]. However, the feasibility of gene therapy for the treatment of fibroproliferative diseases requires further investigation [17]. Noteworthy, the majority of compounds that affect the TGF-β pathway have been developed for the management of cancers, and it remains to be studied whether these putative drugs also mitigate fibrosis.

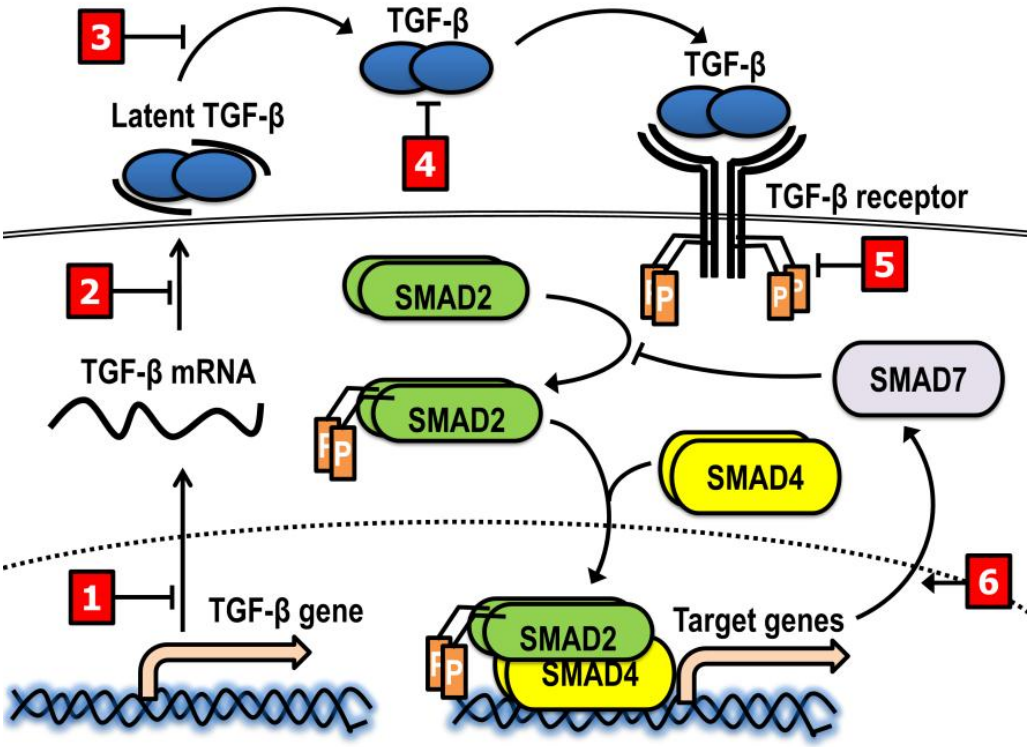

Figure 1: Therapeutic approaches for inhibiting transforming growth factor beta (TGF-β) signaling. (1) Inhibiting TGF-β gene transcription, (2) inhibiting TGF-β mRNA translation, (3) inhibiting TGF-β maturation, (4) sequestering active TGF-β, (5) inhibiting TGF-β receptor kinase activity and (6) up-regulating SMAD7. Only key components are depicted.

Another challenge regarding anti-TGF-β therapies is the therapeutic time window, as seen with several cancers. In normal cells and early carcinomas, TGF-β generally exerts tumor suppressive effects [18, 19]. However, when tumor development progresses, genetic alterations of TGF-β signaling components can result in the loss of tumor protective effects [20]. Consequently, TGF-β signaling switches to promote cancer progression, invasion and metastasis [18-20]. Therefore, it is crucial to inhibit TGF-β signaling during the correct stage of tumor development. In a similar fashion, anti-fibrotic therapies should specifically aim at blocking the pro-fibrogenic activities of TGF-β during fibrogenesis, without affecting its cytostatic effects in normal cells.

Furthermore, therapeutic modalities should take into consideration that TGF-β can also activate a number of SMAD-independent pathways via alternative ligand-receptors complexes or downstream cellular responses; for example, mitogen-activated protein kinases (MAPK), phosphatidylinositol-3-kinase (PI3K)/protein kinase B (AKT) and Rho-like GTPases [21]. Although the SMAD-dependent pathway is regarded as the main intracellular signaling route of TGF-β during fibrogenesis, activation of SMAD-independent pathway can also contribute to the fibrotic process [6]. Therefore, mitigating SMAD-independent signaling might be additionally required to completely inhibit the pro-fibrogenic activity of TGF-β.

Fibrosis treatment: Single pathway modulation is inadequate

Even though TGF-β is seen as the master regulator of fibrogenesis, therapies that solely target this factor might still lack clinical efficacy due to the influence of other signaling routes on disease development. This notion is supported by the fact that antibodies against TGF-β failed to elicit clear beneficial effects in several clinical trials [14, 15]. Thus, the inhibition of multiple deleterious pathways is probably required to increase the effectiveness of anti-fibrotic therapies.

Bone morphogenetic proteins (BMPs), a member of the TGF-β superfamily, may be an attractive therapeutic target for the treatment of fibrosis. Thus far, more than twenty different isoforms of BMPs are identified [22]. As illustrated in Figure 2, BMPs interact with specific cell surface receptors to modulate tissue architecture throughout the body via SMAD1, SMAD5 or SMAD8 [23, 24]. Recently, it became apparent that BMPs are involved in cell proliferation, apoptosis, autophagy, inflammation, angiogenesis and fibrosis [23]. Among various isoforms, BMP-7, BMP-2 and BMP-9 appear to play a role in fibrogenesis. BMP-7 was shown to be an anti-fibrotic mediator in experimental models of chronic kidney disease [25]. In contrast, BMP-2 induced a pro-fibrogenic phenotype in adult renal progenitor cells, thereby contributing to renal damage [26]. Similar to BMP-2, BMP-9 was identified as a pro-fibrogenic factor in promoting ECM expression in mice embryonic fibroblasts [27]. Due to this duality, non-specific inhibition of BMP-related signaling pathways may be deleterious, and specific inhibition of BMP-2 and BMP-9 could be a promising approach. However, till now, no data has been published showing the impact of BMP-2 or BMP-9 inhibition on fibrogenesis. Recently, the fibrogenic role of activated SMAD1, the downstream transcription factor of BMP signaling, was shown in hepatic stellate cells (HSC) isolated from carbon tetrachloride (CCl_4)-treated rats. In these cells, the expression of alpha-smooth muscle actin was positively correlated with the gene and protein expression of SMAD1 [28]. In addition, the herbal

compound Cpd861 inhibited the activation of LX-2 cells, a human HSC cell line, by inhibiting SMAD1 phosphorylation [29]. This was in line with *in vitro* and *in vivo* studies of the inhibitor of differentiation 1 (ID1), a marker of SMAD1 activation, showing that ID1 was a critical mediator in transdifferentiation of HSC in the development of fibrosis [30]. These results, together with the data presented in chapter A2, signify that inhibition of SMAD1 and ID1 could be a promising therapeutic approach for the treatment of fibrosis. Nevertheless, using BMPs as therapeutic target should be approached with caution since BMPs have a crucial role in coordinating tissue architecture and bone healing/regeneration [23, 31].

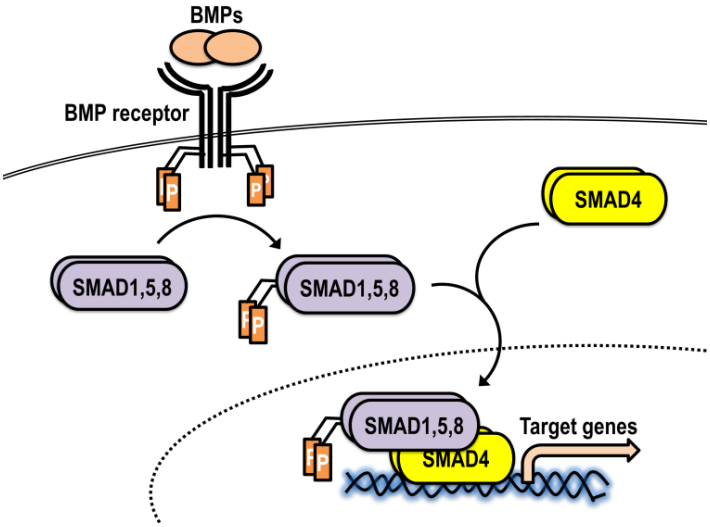

Figure 2: Signaling of bone morphogenetic proteins (BMPs). Only key components are depicted.

Another interesting observation supporting the notion that inhibiting multiple pathways might be essential for the treatment of fibrosis was the lack of a fibrotic response in human liver slices treated with exogenous TGF-β1. It was shown in chapter A3 that only co-treatment with TGF-β1 and platelet-derived growth factor (PDGF) could induce fibrogenesis in human precision-cut liver slices. This finding emphasizes the role of PDGF, which induce proliferation, chemotaxis, migration and survival of activated HSC, in fibrogenesis, and shows that various pathways act as a network in the progression of fibrosis [32]. Thus, simultaneous inhibition of both PDGF and TGF-β is another attractive therapeutic strategy for the management of fibrosis. However, effectiveness of anti-PDGF therapy on fibrosis was only seen preclinically [33, 34], and imatinib, a tyrosine kinase inhibitor disrupting PDGF signaling, was ineffective to improve lung functions and survival in IPF patients [35]. Thus, the clinical benefit of anti-PDGF therapy for the treatment of fibrosis is still questionable. To date, multi-targeted kinase inhibitors affecting various tyrosine kinases including PDGF receptor, such as sunitinib [36], sorafenib [37] and pazopanib [38], are already on the market for the treatment of cancers. Since these inhibitors appear to be more capable of inhibiting the PDGF receptor and other fibrosis-related kinases [39], it is very attractive to elucidate their anti-fibrotic potential in clinical trials.

As shown in chapter A3, targeting of p38–mitogen-activated protein kinase (MAPK) can also mitigate fibrosis. These findings are in line with previous studies demonstrating that this pathway can promote fibrogenesis [40, 41]. Currently, several compounds targeting the p38-MAPK signaling pathway have entered clinical trials for the treatment of inflammatory-related diseases, including rheumatoid arthritis (pamapimod) [42], chronic obstructive pulmonary disease (PH-797804) [43], atherosclerosis (losmapimod) [44] and neuropathic pain (dilmapimod) [45]. However, the efficacy of these compounds was limited, and many are reevaluated for other therapeutic purposes, including for the treatment of fibrosis [46].

Another therapeutic strategy in the treatment of liver fibrosis is the inhibition of oxidative stress, as illustrated in chapter A4. As mentioned earlier, ROS promote the release of mature TGF-β from the ECM reservoir. In addition, oxidative stress appears to be a crucial modulator of TGF-β signaling in various disorders such as obesity, diabetes, non-alcoholic fatty liver disease (NAFLD) and chronic kidney disease [47]. Thus, mitigating oxidative stress would be a good add-on to an anti-TGF-β therapy, and this approach is currently established as a therapeutic strategy in fibrotic disorders [48].

Even though the multi-target approach, which seems to be pivotal for fibrosis treatment, is relatively new, this treatment strategy has been approved for the treatment of various chronic diseases such as cancers and rheumatoid arthritis [49-51]. Thus, such a therapeutic approach for the treatment of fibrosis could be clinically feasible.

Ideal anti-fibrotic drug: Specificity needed

For the development of anti-fibrotic drugs, it should be taken into consideration that wound healing and scar formation are indispensable physiological processes, and direct interference herewith can cause severe adverse side effects [52]. Again, lessons can be gleaned from cancer research. To illustrate, paclitaxel, a drug used for the treatment of various cancers can cause severe adverse events due to off-target effects. To overcome this issue, nanoparticle albumin–bound paclitaxel, nab-paclitaxel, was developed to facilitate drug delivery to tumor cells. In clinical trials, the drug-protein complex showed greater efficacy and a favorable safety profile compared to unmodified paclitaxel [53]. The observed increase in therapeutic outcome of nab-paclitaxel is probably due to enhanced active transport via the gp60/caveolin-1 receptor pathway and increased binding to SPARC (secreted protein acid and rich in cysteine), which is highly expressed in tumor cells [54]. In order to apply a similar strategy for targeted delivery of anti-fibrotic drugs, it is essential to identify the unique attributes of fibrotic cells and tissue. Recently, it was demonstrated that PDGF receptor beta (PDGFRβ) is highly expressed in activated pro-fibrogenic myofibroblasts, and treatment with a construct of PEGylated interferon-gamma (IFN-γ) conjugated to a PDGFRβ-recognizing peptide could attenuate renal fibrosis in an obstructive nephropathy mouse model, while adverse effects were reduced as compared to treatment with unmodified IFN-γ [55]. Since activated myofibroblasts are the ultimate effector cells in organ fibrosis, they are extremely useful targets for selective delivery of anti-fibrotic drugs.

Clinical evaluation: Biomarkers required

Elucidating the anti-fibrotic efficacy of putative drugs in clinical trials is hampered by the lack of clinically relevant biomarkers that can be used to monitor disease development and progression [56]. Ideally, biomarkers should be easy to detect, reliable and non-invasive. Unfortunately, for liver fibrosis, liver biopsy is still the golden standard to stratify patients [57]. However, several non-invasive biomarkers have been developed as illustrated in Table 1. These biomarkers, mostly found in blood, can be divided into simple markers, complex markers, ECM remodeling markers and cytokine markers [58]. In addition, multiple imaging techniques have emerged as a tool to evaluate liver fibrosis (Table 1) [59-61]. Nevertheless, none of these biomarkers and imaging methods prevail the diagnostic potential of liver biopsy [58, 62], and the identification of surrogate biomarkers of fibrosis remains an urgent and unmet clinical need.

Table 1: Examples of currently well-known non-invasive biomarkers and imaging methods for detecting liver fibrosis.

Characteristic	Biomarker/method
Simple/indirect biomarker	▪ Alanine transaminase (ALT) [63-65] ▪ Aspartate transaminase (AST) and AST/ALT ratio [65] ▪ Platelet count (PLT) [66] ▪ Pro-thrombin index (PI) [67]
Complex/indirect biomarker*	▪ ActiTest (age, sex, ALT, gamma-glutamyl transferase (GGT), bilirubin, apolipoprotein A1 (ApoA1), alpha 2-macroglobulin, haptoglobin) [68] ▪ APRI (AST, PLT) [68] ▪ FIB-4 (age, PLT, AST, ALT) [69] ▪ FibroTest (age, sex, GGT, bilirubin, ApoA1, alpha 2-macroglobulin, haptoglobin) [68]
ECM remodeling biomarker	▪ Collagen type IV [63, 70] ▪ Hyaluronic acid [71, 72] ▪ Laminin [63, 72] ▪ Matrix metalloproteinases (MMPs) [72, 73] ▪ Pro-collagen I C peptide (PICP) [70] ▪ Pro-collagen III N peptide (PIIINP) [70, 71, 73] ▪ Tissue inhibitors of metalloproteinases (TIMPs) [71-73] ▪ YKL-40 [71, 74]
Cytokine biomarker	▪ PDGF [72] ▪ TGF-β1 [72, 75]
Imaging method	▪ Computed tomography (CT) [59-61] ▪ Magnetic resonance elastography (MRE) [59-61] ▪ Transient ultrasound elastography (TE) [59-61]

*Complex/indirect biomarkers are calculated from multiple simple markers as shown in parentheses.

Study fibrosis: Choosing the right model

In general, ideal experimental disease models represent the true pathophysiological processes in humans and should be reproducible, reliable, cost effective and easy to handle [3-5]. Concerning fibrosis studies in liver and kidney, various *in vitro* and *in vivo* models have been proposed and applied as illustrated in Table 2. Simplicity and species-specificity are the most prominent advantage of *in vitro* models. For example, results obtained with primary human HSC or renal human fibroblasts, which are the main cells responsible for liver and renal fibrosis progression, can be directly interpreted without concerns of species differences. However, due to the lack of complexity, extrapolation of *in vitro* drug effects to the clinic is still a big challenge [76]. On the other hand, *in vivo* models offer several advantages, among others, the possibility to use genetically modified animals and the crosstalk between the different organs. Nevertheless, dissimilarities between humans and other organisms exist at both pharmacokinetic and pharmacodynamic level [77]; therefore, as discussed in this thesis, variations in drug responses can be expected. In addition, human diseases often do not occur in animals. For instance, the hepatitis C virus cannot infect and cause liver disease in rodents [78]; thus, studying anti-HCV drugs in these animals is irrelevant. Furthermore, the pathology of animal models might not totally recapitulate human diseases. To illustrate, the unilateral ureteral obstruction model is often used to study renal disease. However, this model is based on complete obstruction of the ureter, while in patients with congenital obstructive nephropathy, the ureter is only partially obstructed [79, 80].

Table 2: Examples of *in vitro* and *in vivo* models of liver and kidney fibrosis.

Liver fibrosis	Kidney fibrosis
In vitro	
▪ Primary hepatic stellate cells [81]	▪ Primary renal fibroblasts [82]
▪ Immortal hepatic stellate cell lines [83]	▪ Immortal renal fibroblasts cell lines [84]
▪ Co-cultured of liver-specific cells [85]	▪ Co-cultured of renal-specific cells [86]
In vivo	
▪ Autoimmune liver fibrosis [87]	▪ Nephrotoxic serum nephritis [88]
▪ Alcohol-induced liver disease [89]	▪ Renal ischemia/reperfusion injury [90]
▪ Biliary fibrosis [91]	▪ Subtotal nephrectomy [92]
▪ Non-alcoholic fatty liver disease [93]	▪ Toxin-induced kidney fibrosis [94]
▪ Toxin-induced liver fibrosis [95]	▪ Unilateral ureteric obstruction [80]

Even though the existing *in vitro* and *in vivo* models are not ideal, information obtained with these models may be helpful as long as they are thoroughly validated, and shortcomings are taken into account when interpreting results. Therefore, choosing the appropriate model to address specific research hypothesis is pivotal. However, as discussed in this thesis, more complex human *in vitro* models are necessary to discover new therapeutic targets and to test anti-fibrotic drugs.

Precision-cut tissue slices: Promising research tool

This thesis has provided additional evidence supporting the application of precision-cut tissue slices (PCTS) in studying liver and kidney fibrosis (chapter B2). Furthermore, an additional value of PCTS was demonstrated for the study of NAFLD, as described in chapter B1. Besides the utilization of liver slices for the study of drug-related toxicity [96, 97], PCTS might be applicable for studying other multicellular diseases. For example, precision-cut liver slices were validated to be an effective tool for testing tumor invasiveness [98]. To date, tissue slices can be successfully prepared from intestines [99], heart [100], lung [101] and pancreas [102] for studying a variety of physiological/pathophysiological processes. Therefore, it seems obvious that PCTS will become a powerful research tool in the future. Nevertheless, PCTS still have shortcomings which need to be improved. The main drawback is that tissue slices can only be used for short-term experiments, while many diseases, including fibrosis, develop over several months/years [52, 103]. Recently, by modifying the culture medium, the culture period of liver slices could be extended up to 5 days [103, 104]. This promising development will be very useful for studying liver fibrosis. Furthermore, PCTS cannot recapitulate the *in vivo* crosstalk between organs. This limitation may be surmounted by the development of a tissue/organ-on-a-chip model, which can mimic the interplay between multiple organs in the body [106].

Conclusion

The results presented in this thesis show the benefit of mitigating TGF-β for the treatment of liver fibrosis. Furthermore, it is demonstrated that precision-cut tissue slices may become a promising tool to advance medicinal research, especially in the development of anti-fibrotic drugs. The studies described in this thesis have led to several attractive novel research challenges, which await further investigations, particularly the necessity of inhibiting multiple fibrogenesis-related pathways for the treatment of fibrosis and the need of surrogate biomarkers for clinical trials. These challenges require extensive collaboration between researchers from different disciplines within the fibrosis research field. Nonetheless, I am hopeful that, when Googling “fibrosis” in the near future, many clinically effective drugs will appear on screen.

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