Chapter 1

INTRODUCTION:
Scope of the Present Thesis
The present thesis aims at the development of potential therapeutic approaches through which potent drugs (or cytokines) can be selectively targeted to a key pathogenic cell type in the chronic diseases. Liver cirrhosis, induced by viral infections (e.g. hepatitis B and C), alcohol abuse, metabolic syndrome or genetic disorders, is the major cause of morbidity and mortality worldwide (1). Currently, there are no effective and clinically approved anti-fibrotic therapies available and the treatment is mainly based on the removal of the underlying cause of the disease. Liver transplantation is the only treatment for the patients suffering from advanced liver fibrosis or end stage liver cirrhosis (2, 3). Hepatic stellate cells (HSC) are the key pathogenic cells involved in the progression of liver fibrosis. Quiescent HSC gets activated following release of growth factors and profibrotic cytokines such as transforming growth factor beta (TGFβ), platelet derived growth factor (PDGF) etc. from damaged hepatocytes and infiltrating inflammatory cells. These activated HSC are then transformed into proliferative and contractile myofibroblast-like cells that produce large amounts of extracellular matrix proteins leading to a distortion of the architecture and function of the liver (4, 5). Among the potent anti-fibrotic therapeutic cytokines, Interferon gamma (IFNγ) is shown to be highly efficacious in vitro and in vivo in liver fibrosis models (6, 7), but it failed in clinical trials due to lack of efficacy and unwanted systemic effects (8-10).

![Figure 1. Schematic synthesis of PEGylated IFNγ](image)

Interferon gamma is a homodimeric Th1 proinflammatory cytokine produced by activated inflammatory cells and has been documented to be highly effective in immunodeficiency diseases, chronic inflammatory diseases, fibrosis, tumors and atypical mycobacterial infections (11-14). Encouragingly, several clinical studies have explored the potential role of systemic IFNγ in renal, pulmonary and liver fibrosis (10, 15, 16). Despite the potent biological therapeutic activities of IFNγ, its clinical application is limited to the treatment of chronic granulomatous disease and malignant osteopetrosis due to rapid renal clearance and systemic side effects. When administered intravenously (a tolerated dose with minimal side effects), rapid plasma clearance was observed, while higher doses or frequent doses induced severe adverse effects. Therefore, in this thesis we moved step-by-step forward to design a clinically relevant targeted drug, which is highly effective, specific, and devoid of systemic adverse effects to treat a slowly progressive liver disease and can possibly be applied to treat other chronic fibrotic diseases. Aiming at improving the therapeutic efficacy of interferon gamma (Chapter 2, Figure 1), we first PEGylated IFNγ with different sized linear PEG molecules (5, 10 and 20KDa) and assessed the biological activity in vitro in mouse macrophages and fibroblasts. Thereafter, we compared
the pharmacokinetic profile of PEGylated IFNγ constructs with unmodified IFNγ in mice with CCl₄-induced liver injury. Subsequently, we investigated the liver accumulation, therapeutic efficacy and adverse effects in vivo in CCl₄-induced early liver fibrogenesis mouse model. We found that PEGylation significantly improved the pharmacokinetics, liver uptake and anti-fibrotic effects of IFNγ. However, following further investigation, we found that PEGylation of IFNγ also enhanced its pro-inflammatory effects and induced severe systemic inflammation in mice.

Figure 2. Targeted delivery of IFNγ to hepatic stellate cells (HSC) using PDGFβ receptor recognizing cyclic peptide. PDGFβR is highly expressed on activated HSC during liver fibrogenesis. The redirected IFNγ binds to PDGFβR and inhibits HSC activities, which leads to a reversal of liver fibrosis. In contrast, unmodified IFNγ binds to the ubiquitously expressed IFNγR e.g. on macrophages and induces adverse effects.

The contradicting results achieved in chapter 2, showing increased therapeutic efficacy associated with severe systemic inflammation, steered us to deliver IFNγ to the specific pathogenic cells (hepatic stellate cells, HSC) in the liver (Figure 2). In chapter 3, different targeted IFNγ constructs were synthesized by chemical modification of IFNγ using a PEG linker and PPB. In this two-pronged approach, PEG was incorporated in the molecule in order to enhance plasma half-life, and PPB, a cyclic peptide against Platelet derived growth factor beta receptor (PDGFβR) was used to provide target specificity. PDGFβR is known to be highly and strongly expressed on activated hepatic stellate cells (HSC) or portal fibroblasts (collectively known as myofibroblasts-like cells), the key cells involved in the pathogenesis of liver fibrosis (17, 18). Expression of PDGFβR is very weak in other normal organs and normal cells implicating its appropriateness as the target receptor. The PDGFβR expression was analyzed in different fibrotic livers (from mice and humans) and in normal mouse organs. The synthesized HSC targeted IFNγ constructs were characterized for in vitro biological activity in mouse macrophages. Thereafter, the constructs were analyzed for PDGFβR-specific binding and effects in mouse 3T3 fibroblasts, human HSC and primary rat HSC. The effectivity of the constructs was further examined in vivo in an early fibrosis model associated with HSC activation in mice for proof-of-concept. The targeted IFNγ construct with most significant beneficial effects in
vitro and in in vivo was further investigated extensively in an established and advanced liver cirrhosis model in mice. The results were very promising (*as highlighted in nature reviews gastroenterology and hepatology), as this novel HSC-targeted IFNγ, in contrast to systemic IFNγ, strongly inhibited liver fibrogenesis and was found to be devoid of IFN-related adverse effects.

Subsequently, we explored a cell-specific drug-delivery carrier (PPB-HSA) for the delivery of IFNγ to activated hepatic stellate cells (Chapter 4). A PDGFβR-specific drug delivery carrier (PPB-HSA) was earlier developed by modification of albumin with PDGFβR-recognizing cyclic peptides (17, 19). In this study, IFNγ was conjugated to PPB-HSA via bi-functional PEG linkers to synthesize PPB-HSA-PEG-IFNγ (Figure 3). The PPB-HSA-PEG-IFNγ construct was analyzed in vitro and in vivo in the acute liver fibrogenesis mouse model. In vitro, biologically active PPB-HSA-PEG-IFNγ induced significant anti-fibrotic effects in vitro in human hepatic stellate cells and mouse fibroblasts and in vivo in acute CCl4-induced liver fibrosis model in mice without inducing off-target effects.

The targeted drug-delivery carrier modified IFNγ construct (Figure 3) was further analyzed for anti-tumor properties in a subcutaneous melanoma B16 mouse model (Chapter 5). Tumor stromal cells such as fibroblasts and pericytes potentially induce tumor growth by supporting angiogenesis and other tumor-inducing processes (20, 21). In vitro, we studied the direct effects of PDGFβR-targeted IFNγ on fibroblasts activation and investigated the fibroblast-mediated paracrine effects on mouse endothelial and tumor cells. Moreover the targeted construct was examined in vivo in B16 tumor melanoma bearing mice. We observed that targeted IFNγ significantly reduced the tumor growth by inactivating stromal fibroblasts and pericytes. Moreover, induction of MHC-II expression in multiple organs by unmodified IFNγ but not by targeted IFNγ signified high tumor-specificity and lower side effects in other organs due to the cell-specific therapy.
The therapeutic cell-specific approaches described in chapter 3, 4 and 5 provided highly encouraging results, but to progress to clinics these potential pharmaceuticals have some disadvantages such as complicated chemical synthesis (which makes IFNγ susceptible to loss of function), a heterogeneous population of targeted IFNγ molecules (due to multiple coupling of peptides) and the presence of a IFNγ receptor binding site. Therefore, to resolve these issues, we have synthesized an IFNγ peptidomimetic (mimIFNγ) that contains the nuclear localization sequence (signaling domain) of IFNγ but lacks the extracellular receptor recognition site (22, 23) (Chapter 6). We conjugated mimIFNγ to one molecule of PDGFβR-binding bicyclic peptide (BiPPB, to achieve appropriate PDGFβR binding) at the N-terminal to have chemically well-defined homogenous compounds (Figure 4). The synthesized targeted peptidomimetic of IFNγ (mimγ-BiPPB) was extensively investigated in acute and established (8 weeks) liver fibrosis models in mice. In contrast to untargeted IFNγ, mimIFNγ-BiPPB induced potent anti-fibrotic effects and did not induce any off-target effects. We further evaluated mimγ-BiPPB, in subcutaneous C26 colon carcinoma tumor-bearing mice where it exhibited strong reduction in angiogenesis and tumor size whereas native IFNγ had no effect.
To facilitate the pharmaceutical production, IFNγ and mimIFNγ fused to BiPPB was produced in *E. coli* using recombinant techniques (Chapter 7, Figure 5). The purified HSC-targeted IFNγ and mimIFNγ fusion proteins were evaluated for PDGFβR-specific binding and effects in TGFβ-activated human HSC. Furthermore, the fusion proteins were analyzed for therapeutic efficacy in the acute and early fibrogenesis mouse model. *In vivo*, targeted IFNγ and mimIFNγ significantly inhibited liver fibrogenesis. IFNγ also induced reduction in fibrosis but also exhibited a significant reduction in platelet counts, which was not observed with the targeted proteins.

To summarize, the objective of the studies described in this thesis is to develop a cell-specific therapy for the treatment of liver fibrosis and fibrosis-related chronic diseases. We explored delivery of IFNγ and mimic IFNγ to PDGFβR-expressing key pathogenic cells in liver fibrosis and/or tumors using different targeting approaches. This study is an endeavor to apply a potent cytokine for therapeutical purposes. The thesis describes the whole spectrum of drug targeting research as it covers different coupling strategies to couple IFNγ or mimIFNγ to different targeting peptides, different characterization methods to characterize drug-peptide conjugates; *in vitro* analysis in different cell types, *in vivo* distribution and uptake in specific target cells and finally *in vivo* pharmacological evaluations in different acute and chronic disease models in mice. Therefore, this thesis broadly and extensively explores a novel targeted cytokine therapy and based on the very significant effects obtained with our novel constructs so far, it provides a step forward towards the development of effective and novel therapy for the treatment of fibrotic diseases.

REFERENCES


