# Interferon gamma peptidomimetic targeted to hepatic stellate cells ameliorates acute and chronic liver fibrosis *in vivo*

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## ARTICLE INFO

## ABSTRACT

Hepatic stellate cells play a crucial role in the pathogenesis of hepatic fibrosis. Thus, pharmacological inhibition of pro-fibrotic activities of these cells might lead to an effective therapy for this disease. Among the potent anti-fibrotics, interferon gamma (IFNγ), a proinflammatory cytokine, is highly efficacious but it failed in clinical trials due to the poor efficacy and multiple adverse effects attributed to the ubiquitous IFNγ receptor (IFNγR) expression. To resolve these drawbacks, we chemically synthesized a chimeric molecule containing (a) IFNγ signaling peptide (IFNγ peptidomimetic, mimγ) that retains the agonistic activities of IFNγ but lacks an extracellular receptor recognition sequence for IFNγR; coupled *via* heterobifunctional PEG linker to (b) bicyclic platelet derived growth factor beta receptor (PDGFβR)-binding peptide (BiPPB) to induce internalization into the stellate cells that express PDGFβR. The synthesized targeted IFNγ peptidomimetic (mimγ-BiPPB) was extensively investigated for its anti-fibrotic and adverse effects in acute and chronic CCl4-induced liver fibrosis models in mice. Treatment with mimγ-BiPPB, after the onset of disease, markedly inhibited both early and established hepatic fibrosis as reflected by a reduced intrahepatic α-SMA, desmin and collagen-I mRNA expression and protein levels. While untargeted mimγ and BiPPB had no effect, and native IFNγ only induced a moderate reduction. Additionally, no off-target effects, *e.g.* systemic inflammation, were found with mimγ-BiPPB, which were substantially observed in mice treated with native IFNγ. The present study highlights the beneficial effects of a novel BiPPB mediated cell-specific targeting of IFNγ peptidomimetic to the disease-inducing cells and therefore represents a highly potential therapeutic approach to treat fibrotic diseases.

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## 1. Introduction

Hepatic fibrosis is characterized by an extensive accumulation of abnormal extracellular matrix (ECM) proteins, which ultimately leads to liver dysfunction. It represents a growing cause of morbidity and mortality worldwide [1–4], which warrants the search of new effective anti-fibrotics [5,6]. During liver injury, damaged hepatocytes, inflammatory cells and non-parenchymal cells release growth factors and profibrogenic cytokines such as PDGF and TGFβ, which in turn activate and differentiate quiescent hepatic stellate cells (HSCs) into proliferative, contractile and ECM-producing myofibroblasts (MFs) [7,8]. Therefore, therapeutic approaches to silence these activated HSCs or MFs would be highly interesting to inhibit or reverse liver fibrosis [9]. An emerging concept is the direct delivery of anti-fibrotics to target cell-types using receptor-specific carriers to increase local drug concentrations while preventing deleterious effects on non-target cells or other organs [9].

Interferon gamma (IFNγ) is recognized as a potent anti-fibrotic cytokine and has shown to be effective in preclinical studies for immunodeficiency disorders, chronic inflammatory diseases and tumors [10–13]. It has also been tested in clinical trials for hepatic and pulmonary fibrosis but due to rapid renal clearance and systemic side effects, its clinical application is limited [14–16]. Adverse effects are elicited by the widespread expression of IFNγ receptors on nearly all cell types. Previously, we have attempted to resolve these drawbacks by (i) PEGylation [17] or (ii) cell-specific targeting to the target cells (HSCs or tumor stromal cells *e.g.* cancer-associated fibroblasts and pericytes) [18–20]. PEGylation led to the increased *in vivo* stability and effectivity of IFNγ [17] but it enhanced its adverse effects. Interestingly, targeting of IFNγ led to increased efficacy and reduced side effects in mice with CCl4-induced liver fibrosis [18,19] or with subcutaneous B16 melanoma tumors [20]. So, the re-direction of IFNγ to myofibroblasts in fibrotic tissue or stromal cells in tumors appears to be a successful approach.

Therefore to pursue this targeting strategy further, we have now created a small chimeric molecule containing the IFNγ-signaling moiety, that is, nuclear translocation sequence (NLS) representing the activity domain of IFNγ (IFNγ97–132aa, mimγ) which lacks extracellular IFNγ-

receptor binding sequence [21,22], and chemically conjugated it with only two cyclic platelet derived growth factor beta receptor (PDGFβR) binding peptides (bicyclic PPB, BiPPB) to be specifically taken up by the PDGFβR-expressing disease-inducing cells. We used PDGFβ receptors as target receptor, since they are abundantly expressed on the key pathogenic cells (activated HSCs in liver fibrosis, interstitial fibroblasts in kidney fibrosis and stromal cells in various cancers) relative to normal cells and tissues [23,24].

The synthesized chimeric molecule has many advantages e.g. (a) lack of IFNγR-binding sequence avoids IFNγ interaction with the ubiquitously expressed IFNγR; (b) bicyclic PDGFβR-binding peptide (BiPPB) provides target specificity and dimeric ligand-receptor interaction; (c) its small size (~9 kDa) with minimum structural complexity makes it more feasible for clinical administration e.g. insulin (7 kDa). In vivo stability, cell-selectivity, minimal size and complexity, and lack of species specificity are the main benefits of this compound. The objective of this study therefore is to evaluate the biological effects of this novel chimeric peptide in acute and advanced models of liver fibrosis.

## 2. Materials and methods

### 2.1. Synthesis of targeted and non-targeted IFNγ peptidomimetics

All the reactions were performed in low protein binding tubes (LoBind tubes, Eppendorf, Hamburg, Germany). Bicyclic PDGFβR-recognizing peptide (2223 Da, *Cys-Ser-Arg-Asn-Leu-Ile-Asp-Cys*Gly-Gly-Gly-Asp-Gly-Gly*Cys-Ser-Arg-Asn-Leu-Ile-Asp-Cys*) was custom-made by Genosphere (Paris, France) and was characterized for disulfide dimeric bicyclic SS bridge formation (as depicted in Fig. 2A). Peptidomimetic IFNγ (4689 Da, AKFEVNNPQVQRQAFNELIRVVHQLL PESSLRKRKRSR)-ATA, peptidomimetic IFNγ modified with single molecule of S-acetyl thioacetate (SATA) at the N-terminal was custom-made by Ansynth Service B.V. (Roosendaal, The Netherlands).

To synthesize the targeted IFNγ peptidomimetic construct as depicted in Fig. 2A, BiPPB (0.112 μmol) was reacted with 0.337 μmol of Maleimide-PEG-succinimidyl carboxy methyl ester (Mal-PEG-SCM, 2KDa, Creative PEGworks, Winston-Salem, NC) for 3 h. Thereafter, excess of Mal-PEG-SCM was blocked with lysine (0.337 μmol) for 1 h. Subsequently, the prepared BiPPB-PEG-Mal was reacted overnight with mimIFNγ-ATA in the presence of deacetylating reagent (0.1 M hydroxylamine, 25 mM EDTA in PBS, pH 7.2). Finally, the prepared mimIFNγ-PEG-BiPPB (mimγ-BiPPB) conjugate (8.9 kDa) was extensively dialyzed against PBS using a 7 kDa dialysis membrane (Thermo Scientific, Rockford, IL). The mimIFNγ-PEG-BiPPB construct was prepared under mild conditions to maintain the dimeric BiPPB ring structures for appropriate receptor interaction.

For synthesis of untargeted mimetic IFNγ-PEG (Fig. 2B), 0.107 μmol mimetic IFNγ-ATA (4689 Da) was reacted with 0.321 μmol of poly(ethylene glycol)-succinimidyl α-methylbutanoate (mPEG-SMB, 2 kDa, Nektar Therapeutics) for 2 h and subsequently the prepared construct was dialyzed extensively against PBS using a 7 kDa dialysis membrane.

### 2.2. Synthesis of FITC-coupled PPB and FITC-coupled BiPPB

1 μmol PPB or 0.45 μmol BiPPB (prepared in 0.1 M sodium bicarbonate buffer pH 9.0) was reacted with 3.16 μmol or 1.35 μmol of fluorescein isothiocyanate (FITC, prepared in DMSO) respectively for 2 h. The prepared constructs (PPB-FITC and BiPPB-FITC) were extensively dialyzed against PBS using a 500 Da dialysis membrane (Thermo Scientific).

### 2.3. Cells

Primary rat hepatic stellate cells (HSCs) were harvested from Wistar rats as described previously [25]. Freshly harvested cells were cultured in DMEM supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin and 1% L-glutamine. Freshly-isolated hepatic human myofibroblasts were kindly provided by Dr. Lotersztajn (Inserm, Créteil, France). Cells were cultured in DMEM containing 10% serum (5% fetal calf serum and 5% human serum).

### 2.4. Cell binding experiments

Cells were plated and incubated with 10 μg/ml of FITC-coupled PPB or FITC-coupled BiPPB. To block PDGFβR-mediated binding, anti-PDGFβR IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added 1 h before FITC-labeled constructs. After 2 h, cells were fixed, and nuclei were counterstained with DAPI and examined under a fluorescence microscope. For human myofibroblasts, phalloidin-rhodamine (Sigma) was used to stain the cytoskeleton as a counterstain. The cell binding studies were performed at least three times independently.

### 2.5. Induction of acute liver fibrogenesis and advanced liver fibrosis in mice

All animals (male mice, 20–22 g, Harlan, Zeist, Netherlands) received ad libitum diet and 12/12 h light/dark cycle. All experimental protocols were approved by the Animal Ethics Committee of the University of Groningen.

Acute liver fibrosis was induced in C57BL/6 mice by a single intraperitoneal injection of carbon tetrachloride (CCl₄; 1 ml/kg prepared in olive oil) at day 0. At day 1 and day 2, mice (n = 5 per group) received intravenous injections of IFNγ (5 μg IFNγ/mouse), mimγ-PEG (5 μg mimγ/mouse), mimγ-BiPPB (equivalent to 5 μg mimγ/mouse and 15 μg mimγ/mouse) or PBS alone. At day 3, all mice were sacrificed; blood and different organs were collected for subsequent analyses.

For induction of advanced liver fibrosis, BALB/c mice were treated with olive oil or increasing CCl₄ doses twice weekly for 8 weeks as described previously [19]. In weeks 7 and 8, mice (n = 6 per group) were treated intravenously with PBS, IFNγ (5 μg IFNγ/mouse), mimγ-PEG (5 μg mimγ/mouse), BiPPB or mimγ-BiPPB (equivalent to 5 μg mimγ/mouse) thrice per week. All mice were sacrificed; blood and different organs were collected for the subsequent measurements. Plasma TNF-α and IL-6 levels were analyzed using a cytometric bead array (BDPharmingen, San Diego, CA, USA) as per manufacturer's instructions. All the in vivo analyses were performed at least three times independently.

### 2.6. Immunohistochemistry

Livers were cut using a cryostat (Leica CM 3050, Leica Microsystems, Nussloch, Germany) at 4 μm of thickness, dried and stored at −20 °C until the stainings. The liver sections were immunohistochemically stained according to standard procedures as described earlier [17] using antibodies mentioned in Supplementary Table 1. For quantitative analysis, 10–15 random microscopic fields at 100× magnification per liver section from each mouse were captured. The stained area in the digital photomicrographs was quantified using Cell-D imaging software (Olympus) and was represented as %positive area per field.

### 2.7. Quantitative real time PCR

Total RNA from liver tissues was isolated using RNeasy mini kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. The RNA concentration was quantitated by a UV spectrophotometer (NanoDrop Technologies, Wilmington, DE). Total RNA (1.6 μg) was reverse transcribed in a volume of 50 μl using cDNA synthesis kit (Promega). All the primers were purchased from Sigma-Genosys (Haverhill, UK). The sequences of primers used in the study are enlisted in Supplementary Table 2. 20 ng of cDNA was used for quantitative real time PCR analysis. The reactions were performed using SYBR green PCR master mix (Applied Biosystems) according to the manufacturer's instructions and were analyzed by ABI7900HT sequence detection system

(Applied Biosystems). Finally, the threshold cycles (Ct) were calculated and relative gene expression was normalized with GAPDH (for mouse) as a housekeeping gene. GAPDH remained unchanged with the different treatments in acute and chronic models of liver fibrosis (Supplementary Fig. 1A and B).

## 2.8. Western blot analysis

Liver tissues were homogenized in cold RIPA buffer (50 mM Tris–HCl, 150 mM NaCl, 0.1% SDS, 0.1% Igepal in 0.5% sodium deoxycholate with 1 tablet of protease inhibitor cocktail and 1 tablet of phosphatase inhibitor (Roche Diagnostics, Mannheim, Germany) in 10 ml) on ice with a tissue homogenizer for 4 min and the lysates were centrifuged at 12,000 rpm for 1 h at 4 °C. The supernatants were stored at −70 °C until use. The protein concentrations in the supernatants were quantified using Bradford quick start protein assay reagent (BioRad Laboratories, Hercules, CA) according to manufacturer's instructions with bovine serum albumin (BSA) as the standard. 20 μg protein from each sample was applied on the SDS-PAGE gels (10%) and then blotted to PVDF membranes. The membranes were developed according to the standard protocols using primary and secondary antibodies as mentioned in Supplementary Table 3. The protein bands were visualized using ECL detection reagent (Perkin-Elmer) and photographed using G-Box (Syngene, Cambridge, UK).

## 2.9. Statistical analyses

All the data are presented as mean ± standard deviation (SD). The graphs and statistical analyses were performed using GraphPad Prism version 5.02 (GraphPad Prism Software, Inc., La Jolla, CA, USA). Differences between groups were assessed by one-way analysis of variance (one-way ANOVA) with Bonferroni post-test. The differences were considered significant at $p < 0.05$. The number of animals was determined prior to the animal studies by power analysis (power 90%; alpha 0.05; beta 0.15 and coefficient of variation 15%) using one way ANOVA.

## 3. Results

### 3.1. Design of the PDGFβR-targeted IFNγ peptidomimetic construct

We first designed a bicyclic PDGFβR-recognizing peptide (CSRNLIDC-GGGDGG-CSRNLIDC with C"C disulfide bonds, BiPPB), in which two cyclic peptides are attached via a spacer of 6 amino acids (GGGDGG). This spacer allows the appropriate binding of two peptides to the dimeric PDGF receptor. As illustrated in Fig. 1, FITC-labeled BiPPB showed PDGFβR-specific binding to primary activated rat HSCs and human myofibroblasts. This binding was almost completely blocked by a monoclonal PDGFβR-specific antibody. Consistent with previous findings [26], FITC-labeled monocyclic PPB did not show any binding to these cells suggesting that dimeric interaction is essential for PDGFβR binding.
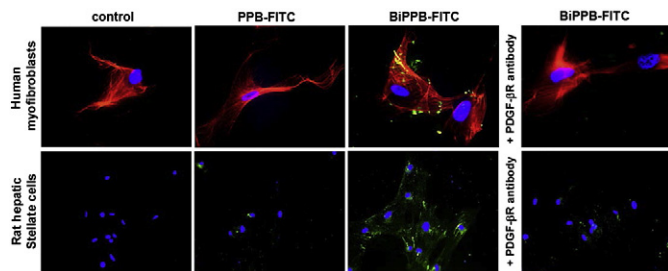
**Fig. 1.** In vitro binding of the PDGFβR-recognizing bicyclic peptide. Representative fluorescent photomicrographs (200x) showing binding of FITC-BiPPB versus FITC-PPB to primary culture activated rat HSCs and human myofibroblasts and blocking of PDGFβR-specific binding by anti-PDGFβR antibody.
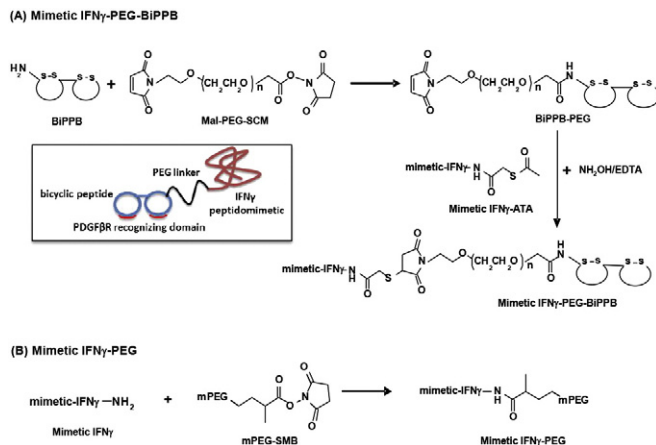
**Fig. 2.** Synthesis of targeted and untargeted IFNγ peptidomimetic constructs. Reaction scheme for the synthesis of targeted mimIFNγ-PEG-BiPPB (mimγ-BiPPB) conjugate (A) and untargeted mimIFNγ-PEG (mimγ-PEG) conjugate (B). BiPPB, PDGFβR-recognizing bicyclic peptide; MAL-PEG-SCM, Maleimide-PEG-succinimidyl carboxy methyl ester; mimIFNγ, mimetic interferon gamma. The inset depicts the schematic representation of the chemically synthesized IFNγ peptidomimetic construct.

Thereafter, mimIFNγ-PEG-BiPPB (mimγ-BiPPB, Fig. 2, inset) was synthesized by coupling of IFNγ peptidomimetic to BiPPB through sequential chemical reactions as depicted in Fig. 2A. A hetero-bifunctional PEG linker (2 kDa) was introduced to provide in vitro and in vivo stabilities. As a control, untargeted mimγ (mimγ-PEG) was also synthesized (Fig. 2B).

### 3.2. Targeted IFNγ peptidomimetic inhibits early fibrogenesis in vivo

To investigate the in vivo efficacy, we first explored the anti-fibrotic effects of the targeted mimIFNγ (mimγ-BiPPB) at two doses (equivalent to 5 μg mimγ/mouse and 15 μg mimγ/mouse) in an acute liver injury model in mice. As shown in Fig. 3 and Supplementary Figs. 2 and 3, a single dose of CCl4 induced early liver fibrogenesis as characterized by increased collagen-I expression, alpha smooth muscle actin expression (HSC activation marker) and desmin expression (HSC proliferation). Treatment with mimγ-BiPPB at both doses caused a significant
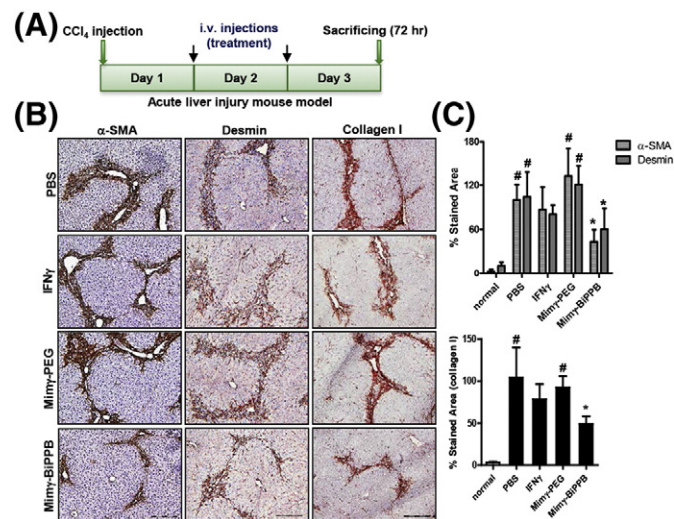
**Fig. 3.** Effects of targeted IFNγ peptidomimetic in acute liver fibrogenesis in mice. (A) Regimen of liver injury model. (B) Representative pictures (100×) and (C) quantitative analysis of α-SMA, desmin and collagen I-stained liver sections from olive oil-treated normal mice and CCl4-treated mice (acute model) that received PBS, IFNγ, mimγ-PEG or mimγ-BiPPB. For quantitative analysis, the groups were normalized to vehicle group (PBS-treated CCl4 mice). Bars represent mean ± SD of 5 mice per group. #p < 0.05, when compared with control mice (PBS-treated); *p < 0.05 when compared with CCl4 mice (PBS-treated).
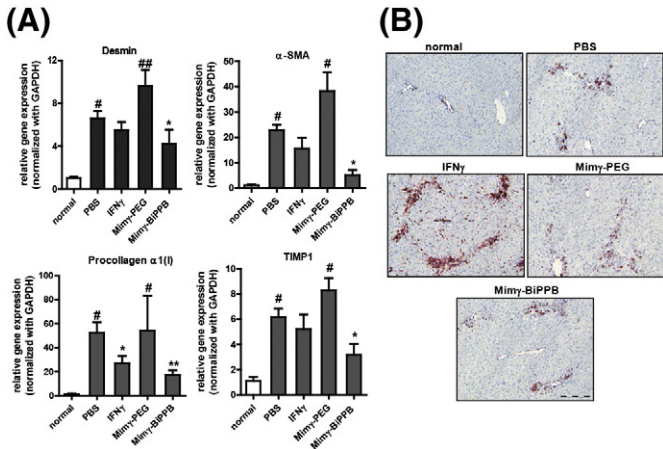
**Fig. 4.** Effects of targeted IFNγ peptidomimetic in acute liver fibrogenesis in mice. (A) Quantitative RT-PCR analysis of desmin, α-SMA, procollagen-α1(I) and TIMP-1 (normalized with GAPDH) in normal and CCl4-animals treated with different treatments. (B) Representative photomicrographs (100×) of MHC-II stained liver sections from normal animals and treated CCl4-animals. Bars represent mean ± SD of 5 mice per group. #p < 0.05, ##p < 0.01 when compared with control mice (PBS-treated); *p < 0.05, **p < 0.01 when compared with CCl4 mice (PBS-treated).

inhibition of HSC activation and proliferation (50% reduction, p < 0.05) (Fig. 3 and Supplementary Fig. 2) which in-effect ameliorated liver injury as further shown by substantial reduction in the expression of collagen I (50% reduction, p < 0.05). These reductions were paralleled by pronounced reductions in the respective mRNA expression levels (Fig. 4A and Supplementary Fig. 3). Apart from collagen expression and deposition, the balance between collagen-degrading matrix metalloproteinases-13 (MMP-13) and their major endogenous inhibitor, tissue inhibitor of metalloproteinases-1 (TIMP-1), is also an important determinant of fibrogenesis *versus* fibrolysis [27,28]. In this study, we found that TIMP-1 expression was significantly inhibited by mimγ-BiPPB treatment (50% reduction, p < 0.05) (Fig. 4A) shifting the intra-hepatic process towards fibrolysis. IFNγ exhibited moderate effects and mimγ-PEG did not induce any reduction. Since the reduction is significantly indifferent between 5 μg and 15 μg of mimγ-BiPPB (Supplementary Figs. 2 and 3), we used 5 μg mimγ-BiPPB dose in the subsequent experiments. No significant differences in the body weights, ALT/AST levels and blood counts were observed with the administered doses in the acute liver fibrogenesis model.

IFNγ is a proinflammatory cytokine known to induce MHC-II expression on macrophages, their main target cells [29]. We found that native IFNγ induced significant MHC-II expression in the livers, clearly showing its off-target effects, while mimγ-BiPPB did not induce MHC-II expression levels (Fig. 4B). In parallel experiments using murine macrophages (RAW cells) that lack PDGFβR, IFNγ induced MHC-II expression and nitric oxide release, while our construct did not induce these effects, corroborating with the *in vivo* findings. Additionally, native IFNγ induced significant infiltration of inflammatory cells (CD68+ macrophages, neutrophils and 33D1+ dendritic cells) in livers and other organs. In contrast, mimγ-BiPPB administration did not induce these inflammatory responses as observed using immunohistochemistry (data not shown).

### 3.3. PDGFβR-targeted IFNγ peptidomimetic inhibits progressive liver fibrosis in vivo

The post-treatment schedule (Fig. 5A) in established liver fibrosis as performed here represents the clinical regimen more closely than the pre-treatment schedule. CCl4 administered mice (PBS treated) developed extensive bridging fibrosis resembling human fibrosis/cirrhosis with substantial collagen deposition and increased HSC marker (α-SMA and desmin) expression (Fig. 5B–D). We found
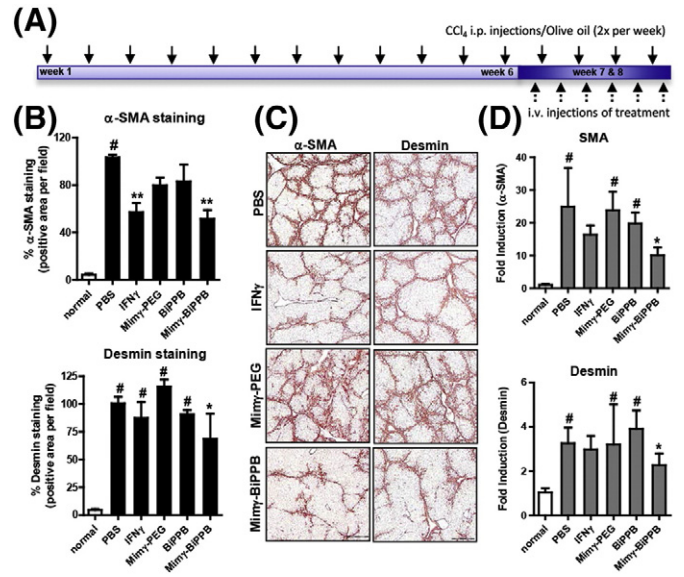


**Fig. 5.** Reduction of HSC markers by targeted IFNγ peptidomimetic in advanced liver fibrosis in mice. (A) Treatment regimen of CCl4-induced chronic liver fibrosis in mice. After 6 weeks of CCl4 injections, animals received intravenous injections of either IFNγ, mimγ-PEG, BiPPB, mimγ-BiPPB or PBS alone for 2 weeks while continuing CCl4 injections. As controls (normal), olive oil was administered for 8 weeks and treated with PBS. (B) Quantitative analysis and (C) representative photomicrographs (40×) of α-SMA and desmin-stained liver sections of CCl4-animals treated with different treatments. For quantitative analysis, the groups were normalized to the vehicle group (PBS-treated CCl4 mice). (D) Quantitative RT-PCR analysis of α-SMA and desmin (normalized with GAPDH) in olive oil-treated mice (normal) and treated CCl4 animals. Bars represent mean ± SD of 6 mice per group. #p < 0.05, when compared with control (olive oil) mice (PBS-treated); *p < 0.05, **p < 0.01 when compared with CCl4 mice (PBS-treated).

mimγ-BiPPB administration significantly inhibited α-SMA expression (>50%, p < 0.01) accompanied by significant decrease in desmin-positive cells (≅35%, p < 0.05) indicating specific attenuation of HSC activation and proliferation (Fig. 5B–D).

The substantial reduction was also observed in bridging of fibrotic septa and collagen-I expression (>50%, p < 0.05) following mimγ-BiPPB treatment (Fig. 6A–D). The reductions in fibrotic parameters as
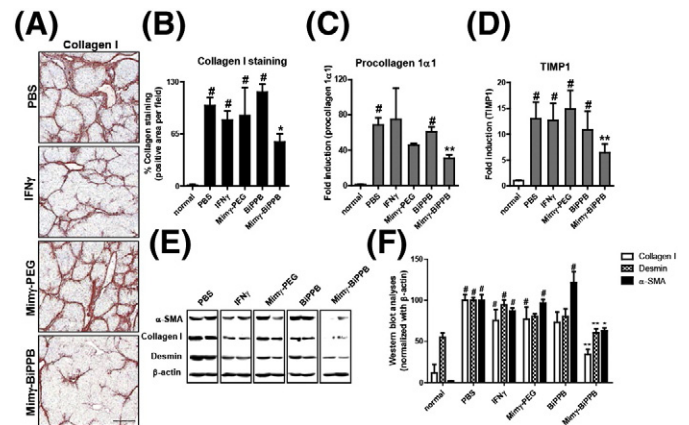


**Fig. 6.** Reduction of bridging and collagen expression with targeted IFNγ peptidomimetic advanced liver fibrosis in mice. (A) Representative pictures (40×) and (B) quantitative analysis of collagen I-stained liver sections from control mice (olive oil treated) and CCl4-treated mice (chronic fibrosis) that received either PBS, IFNγ, mimγ-PEG, BiPPB or mimγ-BiPPB. For quantitative analysis, the groups were normalized to vehicle group (PBS treated-CCl4 mice). Quantitative real-time PCR analysis of procollagen-α1(I) (C) and TIMP-1 (D), normalized with GAPDH, in normal mice (olive oil treated) and treated CCl4-animals. Representative picture (E) and quantitative analysis (F) of the Western blots for α-SMA, desmin, collagen I and β-actin performed on liver homogenates from CCl4-animals treated with different treatments. Bars represent mean ± SD of 6 mice per group. #p < 0.05, when compared with control (olive oil) mice (PBS-treated); *p < 0.05, **p < 0.01 when compared with CCl4 mice (PBS-treated).
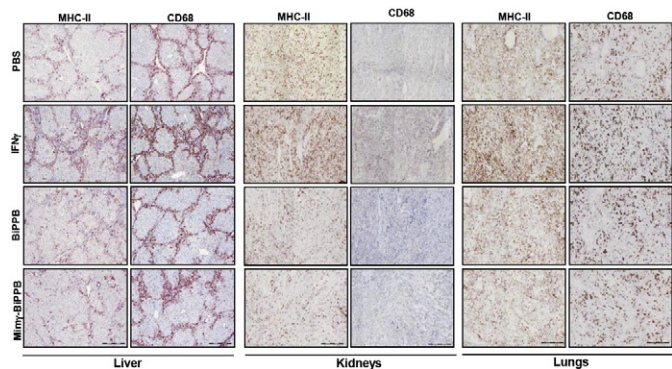
**Fig. 7.** Targeting of IFNγ peptidomimetic prevents IFNγ-related adverse effects. Analysis of adverse effects after 2 weeks of treatment with either PBS, IFNγ, mimγ-PEG, BiPPB or mimγ-BiPPB. Representative photographs (40×) of MHC-II and CD68-stained sections of liver, kidneys and lungs from the fibrotic animals that received different treatments.

observed in stainings and real-time PCR analysis were confirmed by Western blots, which further revealed a profound decline in the fibrotic parameters after treatment with targeted mimIFNγ (Fig. 6E and F). Furthermore, a significant inhibition of TIMP-1 expression (MMP-13 remained unchanged) was also found in mimγ-BiPPB treated mice leading to an increase in the MMP13/TIMP1 ratio, reflecting increased fibrolysis (Fig. 6D). Untargeted IFNγ induced only a slight decrease in fibrotic parameters while mimγ-PEG did not show any effect (Figs. 5B–D and 6A–F). The targeting peptide (BiPPB) may also block the PDGFβR thereby contributing to anti-fibrotic effects. However, equivalent dose of BiPPB did not induce any biological effect (Figs. 5C–D and 6B–F). These data strongly suggest that delivery of mimγ using BiPPB can lead to therapeutic effects in the target cells in vivo. No significant differences in the body weights, ALT/AST levels and blood counts were observed with the administered doses in the chronic liver fibrosis model.

### 3.4. Reduction of IFNγ-related side effects with PDGFβR-targeted mimIFNγ conjugate

Furthermore, we explored whether targeted mimIFNγ could diminish IFNγ-related side effects. We therefore examined inflammation-related effects of IFNγ such as systemic inflammation (release of pro-inflammatory cytokines e.g. IL-1β), central nervous system (CNS) inflammation (MHC-II expression in brain) [30] and off-target effects in the different organs (CD68 and MHC-II expression). IFNγ induced macrophage infiltration and activation in livers, kidneys and lungs as evaluated by CD68 and MHC-II staining (Fig. 7). Furthermore, IFNγ treatment induced a significant increase in macrophage activation in the brains (p < 0.001) as assessed by quantitative PCR (Fig. 8A) and
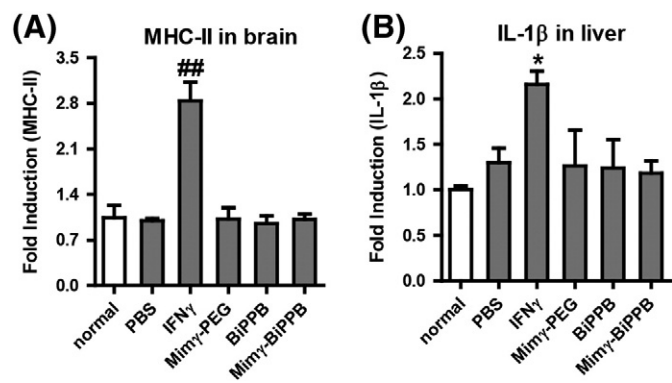


**Fig. 8.** Targeting of IFNγ peptidomimetic prevents IFNγ-related adverse effects. Analysis of adverse effects after 2 weeks of treatment with either PBS, IFNγ, mimγ-PEG, BiPPB or mimγ-BiPPB. (A) Quantitative RT-PCR for MHC-II in the brains. (B) Quantitative RT-PCR for IL-1β in the livers. Bars represent mean ± SD of n = 6 mice per group. *p < 0.05, compared to PBS-treated CCl₄ group.

increased IL-1β expression levels in the livers (Fig. 8B). These effects were completely absent in animals treated with mimγ-BiPPB (Figs. 7 and 8). Since most of IFNγ-based clinical trials failed due to systemic pro-inflammatory effects, the reductions in these off-target effects are therefore highly important for its future clinical application.

## 4. Discussion

This is the first study demonstrating the anti-fibrotic effects of mimetic peptide or signaling moiety of interferon gamma. IFNγ modification which prevented its interaction with its ubiquitously expressed receptor while facilitating its internalization into the target receptor, leading to the biological effects in the target cells, represents an important step towards the therapeutic application and redirection of cytokines. We showed for the first time that the delivery of IFNγ peptidomimetic to fibrotic livers, using a novel bicyclic peptide to PDGFβR-expressing disease-inducing cells, displayed an improved therapeutic efficacy as compared to IFNγ in acute and advanced liver fibrosis mouse models. Furthermore, delivery of this bioactive chimeric peptide largely limited the adverse effects, which were elicited by untargeted IFNγ. Therefore, this novel targeted chimeric cytokine-based therapy provides new opportunities for the treatment of chronic diseases associated with increased PDGFβR expression on the disease-inducing cells.

No pharmacotherapy is available for many of the chronic diseases associated with fibroblasts activation e.g. hepatic fibrosis, while it affects millions of people worldwide. Despite an increasing number of pre-clinical drugs, none of them has reached the clinic [5,6]. IFNγ has been extensively investigated as a potent anti-fibrotic cytokine in in vitro and in vivo models in several fibrotic diseases like pulmonary fibrosis, renal fibrosis and liver fibrosis [11–13,15,31–33]. But its clinical application is limited due to lack of efficacy in patients and severe systemic side effects, both attributed to widespread cellular receptor expression, leading to interaction with multiple cell types thereby eliciting adverse effects and reducing the effective therapeutic dose required in the target cells e.g. stellate cells. A promising approach therefore is to deliver it to specific cells thereby improving its distribution profile and circumventing its adverse and counterbalancing effects on other cells.

In the present study, we have used IFNγ mimetic peptide that lacks extracellular IFNγ receptor recognition sequence therefore preventing its interaction with its widely expressed receptor and it is also documented to be even more potent than IFNγ in inhibiting viral replication [34]. Furthermore, IFNγ mimetic peptide is known to be more stable in vitro and in vivo relative to IFNγ, suggesting the IFNγ (95–133) peptide's potential as an IFNγ drug [35]. While knowing the potential functions of mimIFNγ, its anti-fibrotic activity has not been explored yet. Obviously this is due to the lack of receptor binding site within this molecule preventing its uptake by different cell types and introduction of lipophilic group palmitate for membrane penetration induces non-specific uptake by multiple cell types in the body therefore no significant improvement in adverse effects above IFNγ. The conjugation of BiPPB to this molecule therefore provides target cell-specificity for PDGFβR-expressing cells.

It is known that IFNγ has a receptor binding sequence and a nuclear signaling sequence (NLS) which mediates its biological effects. IFNγ-mediated effects occur through intracellular uptake of the nuclear signaling sequence, which subsequently binds to its intracellular target through JAK–STAT pathway and modulates IFNγ-responsive genes [22]. Upon delivery to the target cells, the mimγ-BiPPB construct is taken up via the target receptor (PDGFβR) and internalized mimIFNγ is apparently able to bind to its intracellular IFNγR-recognizing sequence and therefore can be biologically active but this needs to be further explored. As it has been shown partially, mimIFNγ which is internalized, either through a receptor-mediated uptake mechanism or with the help of a lipophilic compound, binds to the intracellular recognition site of the IFNγ receptor via its NLS region and causes activation of STAT1 signaling and thereby IFNγ-related biological effects [36,37].

We have utilized the platelet-derived growth factor receptor (PDGFβR), which is overexpressed on the key pathogenic cells in pathological conditions like atherosclerosis, fibrosis and cancer. Earlier, we have developed a PDGFβR-recognizing monocyclic peptide to target the PDGFβR [19,38]. However, PDGFBB is a dimeric protein that binds to the homo/hetero-dimeric PDGFβR. Therefore two molecules of PPB are required in the close vicinity to achieve PDGFβR binding. In previous studies, we coupled multiple PPB molecules to core proteins [38] to achieve multivalent interaction with the target receptor. This leads to a complex molecule with a relatively large size and may cause heterogeneity of proteins. To resolve this, we have now developed a novel targeting bicyclic peptide against PDGFβR (BiPPB), where two molecules of PPB are linked via 6 amino acid spacer arm that allows a fitted interaction with the dimeric PDGFβR. Our studies show that FITC-labeled BiPPB alone binds specifically to PDGFβR-expressing rat and human HSCs/MFs whereas FITC-labeled monomeric PPB does not show binding, confirming the requirement of bivalent interaction with PDGFβR. We subsequently coupled a bicyclic peptide to the N-terminus of IFNγ peptidomimetic (mimIFNγ) using a PEG linker to prolong the plasma half-life and stability. Furthermore BiPPB is hydrophobic peptide; therefore PEG linker will provide hydrophilicity and conformational flexibility for appropriate interaction with PDGFβR as also documented earlier [19]. In our previous study, we have shown that though both IFNγ-PPB and IFNγ-PEG-PPB were active in vitro only IFNγ-PEG-PPB induced potent anti-fibrotic effects as compared to IFNγ-PPB in vivo demonstrating the beneficial effect of 2KDa PEG linker [19].

Our group and others have shown the tissue bio-distribution of PPB-modified human serum albumin (HSA), IFNγ and liposomes using radiolabeled and imaging studies demonstrating high distribution of PPB-targeted proteins or liposomes in the fibrotic livers and to PDGFβR-expressing activated hepatic stellate cells [19,26]. Therefore, on the same grounds, mimIFNγ-PEG-BiPPB construct (9 kDa) was synthesized to overcome the hurdles pharmacokinetics (non-globular structure due to linear PEG linker to reduce renal clearance) and to provide specific organ and cellular distribution.

Hepatic stellate cells (HSCs) play a key role in fibrosis and several studies have shown the selective inhibition of HSC using siRNA, gliotoxin, losartan, or some kinase inhibitors that reduce progression of fibrosis significantly [39–42]. The discovery of small peptidic molecules derived from cytokines or other proteins with full agonistic activity and stability opens the possibility for muscular or subcutaneous administration that have provided an impetus for others to design peptidic or non-peptidic mimetics as therapeutics [43–45].

The novel HSC-targeted mimIFNγ induced significant anti-fibrotic effects in both acute and chronic liver injury models in mice indicating the potential application of this compound for the treatment of both early- and late-stage liver fibrosis in patients. The degree of intrahepatic bridges was strongly reduced after treatment with mimγ-biPPB, associated with inhibition of HSC activation and proliferation as reflected by reduced expression of α-SMA and desmin, respectively. In addition, inhibition of TIMP-1 (inhibitor for ECM-degrading metalloproteinases) expression after treatment with targeted mimIFNγ favors fibrinolysis thereby indicating reversal of fibrosis. Also, we started treatment in week 6 (after onset of CCl$_4$ injections) i.e. when fibrosis was established and after 2-week treatment with our construct, collagen levels at week 8 were lower than week 6 (before the onset of treatment) further indicating reversal of fibrosis.

The major problems with interferon-based therapies are the side effects or off-target effects that led to failures of clinical trials or restricted diverse use of IFNγ [14,15,27,32]. IFNγ is a pro-inflammatory cytokine influencing many inflammatory cells, of which macrophages are known to be the main target cell [29]. Of great importance, following targeting, adverse effects or off-target effects in livers and brains were completely abrogated. Also in our study, unmodified IFNγ (at low effective dose) induced significant systemic

pro-inflammatory effects while it induced only a moderate therapeutic effect.

In conclusion, by combining an IFNγ peptidomimetic with a bicyclic peptide, the so-called newly designed chimeric targeted cytokine with a small size, homogenous structure and potent IFNγ agonistic activity has highly favorable characteristics for the long-term administration to chronic patients. It may therefore provide new opportunities to treat liver fibrosis or other chronic diseases associated with (myo-)fibroblast activation.

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