Journal of Controlled Release 179 (2014) 18–24

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

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

Ruchi Bansal [a,b,*], Jai Prakash [a,b], Marieke De Ruiter [a], Klaas Poelstra [a]

[a] Department of Pharmacokinetics, Toxicology and Targeting, University of Groningen, The Netherlands
[b] Department of Targeted Therapeutics, MIRA Institute for Biomedical Technology and Technical Medicine, University of Twente, Enschede, The Netherlands

ARTICLE INFO

Article history:
Received 26 September 2013
Accepted 23 January 2014
Available online 31 January 2014

Keywords:
IFNγ peptidomimetic
PDGFβR-recognizing bicyclic peptide
Targeted delivery
Stellate cells
Fibrosis

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 CCl_4-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.

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 CCl_4-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 ($IFN\gamma_{97-132aa}$, mimγ) which lacks extracellular IFNγ-

Abbreviations: α-SMA, alpha smooth muscle actin; CCl_4, carbon tetrachloride; HSCs, hepatic stellate cells; IFNγ, interferon gamma; mimIFNγ, interferon gamma peptidomimetic; MHC-II, major histocompatibility complex-II; TIMP-1, tissue inhibitor of matrix metalloproteinase-1; PDGFβR, platelet derived growth factor beta receptor; PEG, polyethylene glycol; BiPPB, bicyclic PDGFβ receptor recognizing peptide.

* Corresponding author at: Department of Targeted Therapeutics, MIRA Institute for Biomedical Technology and Technical Medicine, University of Twente, Drienerlolaan 5, 7522 NB, Enschede, The Netherlands. Tel.: +315 34893115.
E-mail addresses: R.Bansal@utwente.nl, R.Bansal@hotmail.com (R. Bansal).

0168-3659/$ – see front matter © 2014 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jconrel.2014.01.022

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 (CCl_4; 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 CCl_4 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