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Specific hepatic delivery of procollagen $\alpha 1(I)$ siRNA in lipid-like nanoparticles resolves liver fibrosis

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Abstract

Fibrosis accompanies the wound-healing response to chronic liver injury and is characterized by excessive hepatic collagen accumulation dominated by collagen type I that often progresses to cirrhosis. Here we present ample in-vivo evidence of an up to 90% suppression of procollagen $\alpha 1(I)$ expression, a reduction of septa formation and a 40–60% decrease of collagen deposition in mice with progressive and advanced liver fibrosis, that received cationic lipid nanoparticles loaded with small interfering RNA to the procollagen $\alpha 1(I)$ gene (LNP-siColl1a1). After intravenous injection up to ninety percent of LNP-siColl1a1 were retained in the liver of fibrotic mice and accumulated in nonparenchymal > parenchymal cells for prolonged periods, significantly ameliorating progression and accelerating regression of fibrosis.

Conclusion—The data reported in the present study extensively show that LNP-siColl1a1 specifically reduce total hepatic collagen content without detectable side effects, potentially qualifying as a therapy for fibrotic liver diseases.

Keywords

collagen; fibrosis; myofibroblast; nanoparticle; siRNA

Introduction

Hepatic fibrosis is characterized by excess accumulation of extracellular matrix (ECM) that follows chronic liver injury¹. Ongoing inflammation often results in massive fibrosis and

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Conflict of interest

The authors declare that they have no conflict of interest. Alfica Sehgal is an employee of Alnylam Pharmaceuticals.

finally cirrhosis which is accompanied by architectural distortion of the hepatic vasculature and sets the stage for hepatic decompensation, primary liver cancer and death². The ECM of fibrotic liver, a result of ongoing inflammation, is dominated by fibrillar collagen type I but also numerous other components that fulfil a plethora of functions beyond mere maintenance of organ cohesion².

Currently there is an urgent need for specific and effective antifibrotic therapies. In rodents, liver fibrosis progression can be inhibited by several approaches most of which lack specificity for activated hepatic stellate cells (HSCs) and myofibroblasts, the major fibrogenic effector cells. Importantly, the production of procollagen type I, the major scar protein which is almost exclusively produced by these two type of cells, remained inaccessible for pharmacological treatment in vivo.

Small interfering RNA (siRNA) is known to be a powerful tool for post-transcriptional gene silencing in vitro and increasingly in vivo³. The systemic delivery of naked siRNA has many obstacles that dramatically reduce their gene silencing efficacy⁴, i.e. degradation by serum/tissue nucleases, opsonization by plasma proteins/circulating cells and inefficient endocytosis. Lipid nanoparticles (LNP), have attracted much interest as safe therapeutic vehicles, especially for liver delivery of siRNA, given a high hepatic retention of some intravenously administered LNP⁵. However, in these studies specific delivery of LNP and their siRNA cargo to certain liver cell types has not been demonstrated, except for hepatocytes⁵, myeloid cells⁶ and non-activated HSC⁷. In particular, effective delivery of siRNA relevant to fibrosis and to the fibrogenic effector cells has not been demonstrated. Here, we encapsulated a procollagen $\alpha 1(I)$ (Col1a1) siRNA duplex (siCol1a1) that was validated for high knockdown efficacy in vitro, into cationic C12-200 LNP (LNP-siCol1a1). These LNP had been optimized for hepatic delivery and showed physicochemical properties that permit in vivo delivery of siRNAs to the liver without stability and safety concerns⁸.

In 2008 Sato and co-workers demonstrated the feasibility to address resting HSC and liver fibrosis in vivo by using vitamin A loaded liposomes carrying siRNA against the global collagen chaperone glycoprotein 46⁽⁷⁾ (gp46). However, while effective in suppressing fibrosis, gp46 targets many collagens and molecules unrelated to fibrosis⁹, and vitamin A is taken up by other cells than resting HSC, including hepatocytes¹⁰.

Here we show that LNP-siCol1a1 address nonparenchymal liver cells in vivo, including activated HSC, specifically block procollagen $\alpha 1(I)$ expression, inhibiting liver fibrosis progression and inducing fibrosis regression in models of parenchymal and biliary liver fibrosis. This treatment has no noticeable side effects, opening the opportunity for a highly targeted antifibrotic treatment using C12-200 LNP loaded with siRNAs against *Col1a1* mRNA which may be extended towards other genes relevant in fibrosis.

Materials and Methods

Induction of parenchymal liver fibrosis

8 week old female Balb/c, FVB and C57BL/6 mice (body weight ~20 g; Charles River, Germany) were housed at 25°C at 40–60% humidity, with 12 h light-dark cycles. Carbon

tetrachloride CCL4) diluted in mineral oil was given by oral gavage 3 times a week for up to 6 weeks to 8 weeks old female C57BL/6 mice, using an escalating dose protocol (first dose 0.875 ml/kg; 1.75 ml/kg from week 1–3; 2.5 ml/kg from week 4 onwards). Mice injected with mineral oil alone served as nonfibrotic controls. For fibrosis regression studies, mice were followed for 4 weeks after discontinuation of a 8 week CCL4 treatment. Animal experiments were performed in accordance with institutional and German legal guidelines for animal protection.

Treatment with LNP-siCol1a1

Eight week old female Mdr2^{−/−} and CCL4 treated mice received weekly or biweekly intravenous injections via the tail vein of PBS, LNP-siLuc (negative control) or LNP-siCol1a1 at siRNA concentrations ranging between 100 and 800 µg/kg BW in 100 µl PBS over 2 or 4 weeks as indicated in Results. Mdr2^{−/−} mice were generated and characterized as previously described¹¹.

LNP formulation methods and chemical compositions

These were as previously described⁴. Chemically-modified siRNAs were synthesized at Alnylam (lower case, 2'-*O*-methyl-modified nucleotides; s, phosphorothioate linkage; dT, deoxythymidine): mouse siCol1a1, sense – GucuAGAcAuGuucAGcuudTsdT, antisense- AAGCUGAAcAUGUCuAGACdTsdT, siLuc, sense- cuuAcGcuGAGuAcuucGAdTsdT, antisense- UCGAAGuACUcAGCGuAAGdTsdT. It was ruled out that siLuc matches a target sequence of *Col1a1* mRNA. In brief, a set of siRNA were screened using RNAimax (Invitrogen) in appropriate cell lines for mRNA knock-down and the lead candidate with lowest IC50 was formulated in C12–200 based LNP at a weight ratio of 1:7 (siRNA vs. lipid components).

Labeling of LNP-siCol1a1 with DiR or Dil

See Supporting Materials and Methods.

In vivo and ex vivo distribution of DiR labeled LNP-siCol1a1

Normal and fibrotic mice were intravenously injected via the tail vein with 3 mg/kg of DiR-labeled LNP-siCol1a1 suspended in 100 µl of PBS. Moreover, mice injected with PBS alone or free DiR in PBS at 50 µg/kg served as background controls. Thirty minutes, 4 h, 1, 2, 3, 5, 7 and 9 d after injection, mice were anesthetized using isoflurane and subjected to in vivo fluorescent imaging by a Xenogen Spectrum system (Caliper, Hopkinton, MA), using 745 and 800 nm of excitation and emission wavelengths, respectively, and 3 sec of imaging integration time. At day 9, mice were sacrificed by cervical dislocation, and liver, lungs, spleen and kidneys removed for ex vivo imaging using the same system and settings as above. Total fluorescence signals were analyzed by Living Image In Vivo Imaging software (PerkinElmer, Hopkinton, MA) and represented as total Radiant Efficiency.

Kinetics of Dil labeled LNP-siCol1a1 uptake in liver

See Supporting Materials and Methods.

Confirmation of the liver specific uptake of DiI labeled LNP-siCol1a1

Eight week old female *Mdr2*^{-/-} mice were given a single dose of 100 µl of PBS containing 3 mg/kg of DiI labeled LNP-siCol1a1. 24 h later, mice were sacrificed by cervical dislocation and the liver, spleen, lungs and kidney were collected and immediately embedded in OTC media. 7 µm cryo-sections of each organ were stained with DAPI (at 1:100 in PBS; Sigma Aldrich) without fixation for 3 min, mounted with Fluoroshield (DAKO, Glostrup, Denmark) and observed and photographed using a LSM 710 NLO confocal laser microscope (Carl Zeiss) to examine the fluorescence signal of DiI labeled LNP-siCol1a1. Results were calculated as described above and are shown as percentage of area occupied by DiI labelled LNP-siCol1a1 in 10 randomly selected fields of each specimen.

In vivo and ex vivo distribution of free DiR

See Supporting Materials and Methods.

Circulation half-life of LNP-siCol1a1

See Supporting Materials and Methods.

Colocalization of DiI labeled LNP-siCol1a1 with liver cells

Mice received intravenous DI-labeled LNP-siCol1a1 suspended in 100 µl of PBS (3 mg/kg BW). 24 h (in case of vimentin colocalization 30 min, 4 h, 1 and 2 d) after injection the animals were sacrificed by cervical dislocation and the liver was removed. Liver specimens were immediately embedded in OTC medium and cryo-sectioned at 7 µm thickness. Mounted sections were imaged for DiI-LNP-derived red fluorescence by setting an identifiable point as zero and recording x,y coordinates of every position using a LSM 710 NLO confocal laser microscope (Carl Zeiss). Sections were then fixed with 4% formaldehyde for 10 min, washed with PBS and blocked with 5% goat serum (Invitrogen) for 1 h at RT, followed by overnight incubation with cell specific antibodies in 1% BSA at 4°C: 1) rat anti-CD31 (1:500, BD Bioscience); rat anti-CD68 (Serotec, Oxfordshire, UK) at 1:1000 2); 3) rat anti-F4/80 (1:1000, Acris, Herford, Germany); 4) rabbit anti-desmin (1:25, Abcam, Cambridge, UK); rabbit anti-HNF4α (1:50, Santa Cruz Biotechnology, CA) and 5) mouse anti-α-SMA-FITC (1:250, Sigma-Aldrich). Secondary antibodies (1 h at RT) were goat Alexa Fluor 488 anti-rat or rabbit IgG (1:1000 for anti-CD31, 1:2000 for anti-CD68, 1:50 for anti-desmin, 1:2000 for F4/80 and 1:250 for HNF-4α; Molecular Probes, Eugene, OR). Other sections were blocked with 10% goat serum for 20 min at RT and stained with rabbit anti-vimentin-Alexa Fluor 488 (1:200, Cell Signalling) in 10% goat serum using the Mouse on Mouse Fluorescein Kit (Vector Labs, Burlingame, CA) where indicated (excluding the incubation steps with M.O.M. Biotinylated Anti-Mouse IgG Reagent and Fluorescein, since anti-vimentin antibody were directly conjugated with a fluorochrome (AlexaFluor 488). After washing with PBS, nuclei were stained with DAPI (diluted in PBS at 1:100) for 5 min and sections mounted using Fluoroshield (DAKO). Overlay of fluorescence images from the same slide was performed by introducing the recorded x,y coordinates in Zen software. Then, all channels of each field were post-merged and shifted with ZEN software to coincide with the landmarks of DiI-LNP red fluorescence. The

colocalization rate was quantified by manually counting the number of colocalizations (intense and/or light yellow fluorescence and/or immediately adjacent red-green fluorescence) and the total number of, e.g., CD68+ cells in 10 merged images from each specimen. Finally, to calculate the percentage of CD68+ cells that co-localize with DiI-LNP, the number of yellow cells was normalized taking the total number of CD68+ cells as 100%.

Immunofluorescent co-staining

See Supporting Materials and Methods.

ABC immunohistology and quantification

See Supporting Materials and Methods.

Quantification (pro-) collagen type III deposition

See Supporting Materials and Methods.

Quantitative RT-PCR

See Supporting Materials and Methods.

Determination of interstitial collagenase and gelatinase activities

See Supporting Materials and Methods.

Serum chemistry

See Supporting Materials and Methods.

Ethics statement

This study was approved by the Animal Protection and Care Committee of the State of Rhineland-Palatinate (approval No. 23177-07G12-1-007).

Statistics

Statistical analyses were performed using Microsoft Excel ver. 2013. Data are expressed as means $\pm$ SD. All experiments were repeated independently at least 2 to 3 times and assessed in duplicate or triplicate. The statistical significances of differences between treated and control groups of animals were evaluated by the independent variables t-test using STATISTICA ver. 2010 (StatSoft, Tulsa, OK). Significance was defined as $p < 0.05$.

Results

LNP-siCol1a1 inhibit the expression of Col1a1 mRNA in human LX2 cells

Transfection of human LX2 cells with LNP-siCol1a1 revealed a significant dose-dependent knockdown of *Col1a1* up to 80%, and of *Acta2* and *Pdgfrb* mRNAs that represent major transcripts of stellate cell activation. Moreover, the knockdown reduced LX-2 cell spreading at 24 h compared to LNP-siLuc (negative control)-treated cells (Supporting Fig. 1A–C and data not shown). These results suggest that LNP-siCol1a1 does not only inhibit *Col1a1* mRNA expression, but also attenuates HSC activation.

Near infrared and fluorescent labeling of LNP-siCol1a1

For in vivo imaging and quantification of cellular uptake, LNP-siCol1a1 were labeled with the highly lipophilic DiR (infrared) or DiI (visible spectrum) dyes. Assuming an original particle size of 80 nm⁸, 91% and 89% of DiI and DiR were firmly incorporated into the particles respectively, as assessed by fluorescent light-scattering (Supporting Fig. 2A, B). Furthermore, LNP-siCol1a1 consisted of monomers (80–85%) rather than small (7–9%), intermediate (3–6%) and large aggregates (1–2%) (Supporting Fig. 2C, D).

LNP-siCol1a1 accumulate preferentially in the liver

To examine the biodistribution of LNP-siCol1a1, DiR labeled LNP-siCol1a1 (termed DiR-LNP and dosed at 3 mg/kg) were injected intravenously into *Mdr2*^{-/-} mice with spontaneous biliary fibrosis and into mice with mild parenchymal liver fibrosis after a 2 week treatment with CCl₄ and their respective age matched normal controls (FVB and Balb/c mice, the latter having received injections of mineral oil instead of CCl₄) (Fig.1A). After 30 min, 4 h, 1, 2, 3, 5, 7 and 9 d NIR fluorescent emission was measured by in vivo imaging (Fig. 1B). At 30 min, most of the fluorescent signal was identified in the liver of all mice, especially in those with fibrosis (Fig.1B). Fluorescence intensity increased from 30 min on, reaching a peak at day 1 (Fig.1C). These results were confirmed when liver sections of groups of mice were subjected to fluorescence microscopy 30 min, 4 h, 1 and 2 d after a single i.v. injection of DiI-LNP (containing siCol1a1 RNA) at 3 mg/kg ($p=0.05$ for *Mdr2*^{-/-} vs. nonfibrotic FVB controls at 30 min; $p<0.01$ at 4 h, 1 and 2 d) (Supporting Fig.3A,B).

Notably, mice showed detectable signal in liver until day 9, with significant retention in fibrotic vs. nonfibrotic mice ($p<0.01$ and 0.001, for CCl₄-treated and *Mdr2*^{-/-} mice, respectively) (Fig.1C). At day 9, liver, lungs, spleen and kidneys were removed and fluorescence emission was measured by ex vivo imaging (Fig.1D). Fluorescence in livers of *Mdr2*^{-/-} and CCl₄ treated mice was much higher than in spleen and lungs (Fig.1E) and higher than in control livers ($p<0.05$) (Fig.1E). The signal in kidney was low in *Mdr2*^{-/-} and absent in CCl₄-treated and control mice, further confirming prominent sequestration of the LNP in the liver (Fig.1E). Similar results were obtained for early liver uptake when the organs of *Mdr2*^{-/-} mice were examined ex vivo (Supporting Fig.4), and liver sections by fluorescence confocal microscopy at 30 min post-injection of DiI-LNP (loaded with siCol1a1 RNA) at 3 mg/kg ($p<0.05$, vs. spleen; $p=0.01$, vs. lung; $p<0.001$, vs. kidney) (Supporting Fig.5A,B). There was no tissue-associated auto-fluorescence that would skew the tracking of the LNP (Supporting Fig. 6).

DiI-LNP injected intravenously into normal Balb/c mice were detectable in serum by spectrofluorometry for at least 3 days, with 77.82% and 7.28% of the injected dose at 30 min and 3 d, respectively (Fig.1F), indicating a long circulating half-life of 19.3 h. When 50 µg/kg of free DiR dye was injected into normal FVB and Balb/c mice, the fluorescence in the liver at 30 min, 4 h, 1 and 2 d post injection was almost undetectable, with an equally weak signal in other organs, confirming the high liver specificity of the LNP-siCol1a1 (Supporting Figs.7,8).

LNP-siCol1a1 are predominantly retained by nonparenchymal cells and cells involved in fibrogenesis

DiI-labeling was used to study the colocalization of LNP-siCol1a1 (named as DiI-LNP, dosed at 3 mg/kg) relative to the total of each major cell subset in liver sections from fibrotic vs. normal mice. 24 h after intravenous injection in fibrotic mice and their nonfibrotic controls (the preferred time point for evaluation of dynamic fibrosis related parameters) animals were sacrificed and liver sections stained for HNF4 α (a unique marker of hepatocytes), desmin (a specific marker of HSC), CD31 (a marker of endothelial cells), CD68 and F4/80 (markers of macrophages/Kupffer cells) and α -SMA (a marker of activated myofibroblasts, a subset of HSC) (Fig.2A; Supporting Fig.9). Co-localization of desmin-positive HSC and α -SMA positive myofibroblasts with DiI-LNP was high in CCL4 fibrotic mice (33% and 7%, respectively) compared to only 1% for both cell subsets in the nonfibrotic controls (Fig.2B). These values reached 29% and 11%, respectively, in *Mdr2*^{-/-} biliary fibrotic mice, again with much lower uptake in the nonfibrotic controls (Fig.2B). CD68/F4/80-positive macrophages/Kupffer cells showed the highest colocalization with DiI-LNP, 37%/42% and 34%/28% in CCL4 and biliary fibrotic mice, respectively (Fig.2B). Desmin-positive HSC ranged second in both biliary and parenchymal fibrosis, while colocalization with hepatocytes and endothelial cells was clearly lower (Fig.2B). In biliary fibrotic mice, α -SMA positive portal fibroblasts significantly colocalized with DiI-LNP (Fig.2C; Supporting Fig.9C). Together, these results indicate an intrinsic target selectivity of our siRNA-loaded LNP for cells implicated as direct or immediate upstream effectors of liver fibrogenesis, i.e., HSC and Kupffer cells.

To reveal for how long cellular in vivo colocalization/uptake of DiI-LNP was maintained, we stained liver sections from CCL4-treated and *Mdr2*^{-/-} mice 30 min, 4, 24 and 48 h after injection of DiI-LNP for vimentin (a marker of mesenchymal cells, mainly fibroblasts, HSC and endothelial cells) (Supporting Fig.10A). Uptake increased from 30 min to 4 h and 24 h, and persisted until 48 h in both fibrosis models (Supporting Fig.10B). These results indicate along persistence of LNP-siCol1a1 in cells implicated in fibrogenesis in fibrotic mice.

Long-term treatment with LNP-siCol1a1 accelerates the regression of advanced parenchymal liver fibrosis

Mice were treated orally with CCL₄ for 8 weeks which induces advanced fibrosis and incipient cirrhosis, followed by 4 weeks of recovery with injection of 4 doses of LNP-siCol1a1, LNP-siLuc (both loaded either with 100 and 200 μ g/kg of siRNA), or PBS. The area occupied by collagen (Sirius red) and activated HSC (α -SMA), as quantified by computerized image analysis were significantly and dose-dependently decreased (50%) in the mice injected with LNP-siCol1a1 compared to those given LNP-siLuc or PBS ($p < 0.01$) (Fig.3A,B). This was accompanied by a significant and dose-dependent suppression of hepatic *Col1a1* ($p < 0.001$), *Timp1* (tissue inhibitor of metalloproteinases 1) ($p < 0.01$) and *Tgfb1* transcripts, all major effectors of fibrogenesis, with a downward trend for *Acta2* (the gene coding the α -SMA protein) transcripts (Fig.3C; Supporting Fig.11A–C). By contrast, *Mmp3* mRNA (a matrix degrading enzyme held as central mediator of fibrolysis) was dose-dependently increased in LNP-siCol1a1 treated specimens relative to LNP-siLuc or PBS treated fibrotic controls ($p < 0.05$ and $p < 0.01$, at 100 and 200 μ g/kg, respectively) (Supporting

Fig.11D). LNP-siColl1a1 but not the control treatments significantly suppressed *Mmp9* mRNA and gelatinolytic activities in the fibrotic livers (Supporting Fig.11E; Fig.3D). Total liver collagen, as determined biochemically via hydroxyproline decreased significantly by 25% in LNP-siColl1a1 treated animals vs. controls (Fig.3E).

Serum alanine aminotransferase (ALT), bilirubin and creatinine levels, surrogates of liver inflammation, and liver and kidney function remained unchanged in all treatment groups, indicating the absence of proinflammatory or toxic properties of the siRNA-loaded LNP in the fibrotic mice (Supporting Fig.12A–C).

Together, these results suggest us that LNP-siColl1a1 are able to promote regression of advanced parenchymal liver fibrosis without inducing apparent side effects.

LNP-siColl1a1 treatment ameliorates the progression of aggressive CCl₄-induced liver fibrosis

To assess the potency of these procollagen $\alpha 1(I)$ specific LNP to inhibit highly accelerated fibrogenesis, mice received optimized regimen of CCl₄ gavage for 5 weeks and were treated with PBS, LNP-siColl1a1 or LNP-siLuc at 200 and 400 $\mu\text{g/kg}$ of siRNAs for four times during the last 2 weeks of CCl₄ exposure. 24 h after the last injection, computerized image analysis demonstrated significant reductions of the Sirius red and α -SMA positive areas only for mice injected with LNP-siColl1a1 (Fig.4A,B). This was accompanied by a highly significant and dose-dependent silencing of *Colla1* mRNA ($p < 0.001$), and a significant reduction of *Acta2* transcripts, and liver hydroxyproline relative to controls ($p < 0.05$), while interstitial collagenases activity remained unchanged (Fig.4C–E; Supporting Fig.13A). Interestingly, a significant increase of *Mmp9* mRNA levels was detected in mice treated at 400 $\mu\text{g/kg}$ of LNP-siColl1a1 ($p < 0.01$, at 400 $\mu\text{g/kg}$) (Supporting Fig.13B). This data was consistent with gelatinases activity that exhibited a significant up-regulation in both siRNA-treatment groups ($p < 0.05$ and 0.01 , respectively) (Supporting Fig.13C).

Since CCl₄ treatment also damages the small intestine¹², a major collagen-containing organ apart from kidneys and lungs¹³, we analysed duodenal *Colla1* expression in mice with CCl₄-induced fibrosis who were treated with LNP-siColl1a1. No changes on *Colla1* expression were found compared to LNP-siLuc treated controls, confirming the absence of effects on extrahepatic wound-healing (Supporting Fig.14).

Collagen type III represents the second most abundant fibrillar collagen. Its regulation during specific inhibition of procollagen type I expression in the liver remains unknown. Interestingly, computerized image analysis demonstrated a significant increase of (pro-) collagen type III-positive fibrils and of *Col3a1* mRNA in LNP-siColl1a1 RNA treated mice with progressive CCl₄-induced fibrosis compared to controls (Fig.5A–C). This upregulation did not abolish the overall collagen reduction in these mice (Fig.4), further supporting the highly antifibrotic potency of LNP-siColl1a1.

Collectively, these results suggest the suitability of LNP-siColl1a1 as a potential therapy to ameliorate the progression of intermediate to advanced parenchymal liver fibrosis in mice.

Treatment with LNP-siCol1a1 mitigates progression of advanced biliary fibrosis in *Mdr2*^{-/-} mice

To elucidate a possible antifibrotic effect of LNP-siCol1a1 in advanced biliary fibrosis, 8 week old *Mdr2*^{-/-} mice, which develop spontaneous biliary fibrosis due to deficiency of the biliary phospholipid transporter, received LNP-siCol1a1 or LNP-siLuc once weekly for 4 weeks equivalent to 100 and 200 µg/kg of siRNA. 24 h after the last injection, animals were sacrificed. Collagen-stained area, total hydroxyproline and fibrogenic *Col1a1* and *Timp1* transcript levels were significantly reduced in mice injected with LNP-siCol1a1 compared to those given LNP-siLuc (controls), while putatively fibrolytic *Mmp3* mRNA was upregulated (Supporting Fig.15A–F). As in the CCl₄-fibrosis models, serum ALT, bilirubin and creatinine remained in the range of the untreated fibrotic *Mdr2*^{-/-} controls (Supplementary Fig.16A–C).

To assess the effect of short-term, higher dose treatment, 8 week old *Mdr2*^{-/-} mice were injected with LNP-siCol1a1 or LNP-siLuc at 400 and 800 µg/kg of siRNA 4 times for 2 weeks, followed by sacrifice 24 h after the last dose. Computerized image analysis showed a significant diminution of Sirius red-stained collagen in mice that received the LNP-siCol1a1 compared to the LNP-siLuc controls ($p < 0.01$) (Fig.6A,B). *Col1a1* mRNA was markedly and dose dependently suppressed 10–20 fold vs. the controls ($p < 0.001$) (Fig.6C). Equally, in LNP-siLuc treated mice liver hydroxyproline and transcripts for *Timp1*, *Mmp2*, *Acta2* and *Tgfb2* (a TGFβ isoform mainly expressed in biliary fibrosis), all parameters of HSC (or myofibroblast) activation and fibrogenesis, were significantly suppressed (Fig.6D; Supporting Fig.17A–D), while *Mmp8* mRNA (an interstitial collagen-degrading metalloproteinase) was upregulated, in line with increased interstitial collagenases activity (Fig.6E,F), suggesting that inhibition of procollagen type I synthesis increases interstitial (fibril degrading) collagenase expression and activity.

As before, serum levels of ALT, bilirubin and creatinine levels remained unchanged in the siRNA-LNP treated mice (data not shown).

LNP-siCol1a1 do not trigger innate immunity in parenchymal or biliary liver fibrosis

Innate immune activation was studied in advanced CCl₄-induced parenchymal liver fibrosis after a 4 week treatment with intermediate doses of LNP-siCol1a1 (100 and 200 µg/kg of siRNA) in the regression phase (experiment of Figure 3). Liver sections were stained for CD68 (Kupffer cells/macrophages) and TLR4 (major innate immune receptor), and transcript levels of *Cd68* and *Itgam* (myeloid innate immune cells) were determined. There was no upregulation of all these innate immune parameters (Fig.7A–D), including a significant downregulation of the also adaptive immune *Ifng* transcript (Fig.7E), in mouse livers treated with LNP-siCol1a1 when compared to those given LNP-siLuc, or to fibrotic mice that received PBS alone.

Similar results were obtained for the livers of biliary fibrotic mice that were treated with high doses of LNP-siCol1a1 or LNP-siLuc (400 and 800 µg/kg of siRNA) for 2 weeks (experiment of Fig.6). Apart from no change or downregulation of TLR4 and CD68, and *Cd68* expression, *Itgam* and *Il8* (a myeloid neutrophil attractant cytokine) mRNA levels

were even significantly decreased in mice treated with LNP-siCol1a1 vs. LNP-siLuc (Supporting Fig.18A–G) and PBS only treated fibrotic controls.

Together, these results indicate an attenuated rather than activated innate immune and adaptive response after treatment with our LNP-siCol1a1 in vivo, likely due to a reduction of myeloid cell numbers and/or suppression of myeloid cell activation.

Discussion

Drugs that specifically and effectively inhibit liver fibrosis are urgently needed. While several agents that may indirectly affect excess ECM production are considered for early clinical trials¹¹, specific inhibition of the production of procollagen $\alpha 1(I)$, which is the major structural component of scar tissue, has remained elusive in vivo. Here we describe lipid-like nanoparticles loaded with optimized siRNA against procollagen $\alpha 1(I)$ transcripts (LNP-siCol1a1) that after intravenous injection 1) are almost exclusively sequestered by the fibrotic liver, 2) are predominantly associated with or engulfed by non-parenchymal cells (Kupffer cells > HSC), and 3) induce an up to 90%, dose-dependent and long-lasting knockdown of *Col1a1* mRNA that encodes the key structural protein in fibrosis.

This knockdown was highly significant in 3 models of liver fibrosis progression, as well as in a model of advanced fibrosis regression, reflecting a broad range of human liver diseases, where antifibrotic efficacy is desired for inhibition of progression and induction of regression. LNP-siCol1a1 treatment achieved this goal, since it lead to a significant histological improvement, with a reduction of septa formation and a 40–60% decrease in collagen deposition, as determined by Sirius red morphometry, in all models tested. Moreover, treatment lead to a significant decrease of total liver collagen, as measured via hydroxyproline content, a quantitative parameter which tends to underestimate changes in septal collagen relative to the functionally less relevant periportal collagen. Interestingly, suppression of collagen type I deposition also lead to a decrease in other major profibrogenic transcripts, such as *Acta2*, *Timp1*, *Tgf β 1*, or *Tgf β 2*, and to an upregulation of the genes encoding putatively fibrolytic matrix metalloproteinases, such as *Mmp3* and *Mmp8*, which was accompanied by enhanced interstitial collagenase activities in the liver. This suggests that specific inhibition of the excess production and deposition of the major fibril forming collagen type I can favourably affect the fibrogenic activation of HSC themselves (as major producers of α -SMA, TIMP1 and TGF β 1), as is suggested by our data on *Acta2* and *Pdgfrb* transcripts in human LX2 cells. Moreover, this may also affect inflammatory cells, such as Kupffer cells which are major producers of putatively fibrolytic MMP3, MMP8 and MMP9⁽¹⁴⁾ and of activated bile ductular cells (which are the only producers of TGF β 2^[14]). This is well in line with prior data showing that an ECM rich in collagen type I leads to HSC activation, with cell spreading and stretching, via engagement of certain collagen binding integrins, activation of focal adhesion kinase and the formation of α -SMA containing stress fibers, resulting in excess ECM production, and in models¹⁵. Our therapeutic intervention would therefore help to interrupt the vicious circle of excess collagen fibril deposition and further HSC activation.

Since activated HSC that loose α -SMA were reported to undergo apoptosis¹⁶, the reduced numbers of α -SMA positive cells in our study may implicate HSC apoptosis due to the specific inhibition of procollagen $\alpha 1(I)$ expression.

To date it has been unknown if therapeutic suppression of procollagen type I synthesis (and collagen type I deposition in the ECM) would affect the expression and deposition of (pro-) collagen type III, the second most abundant fibril forming collagen². Interestingly, both stainable (pro-) collagen type III and procollagen $\alpha 1(III)$ mRNA expression were moderately increased in fibrotic livers of mice treated with LNP-siColl1a1, suggesting a hitherto unknown negative regulation of procollagen $\alpha 1(III)$ expression and deposition by collagen type I and its modest compensatory upregulation during suppressed procollagen $\alpha 1(I)$ production. Thus it can be anticipated that a combination of LNP-siColl1a1 RNA with siCol3a1 LNP would lead to a potentiated antifibrotic effect.

Systemically administered particles are usually rapidly engulfed by the reticuloendothelial system (RES) of liver and spleen^{17,18}. In mice, the pores of the hepatic and splenic sinusoidal endothelium have size exclusions of approx. 280 and 5000 nm, respectively¹⁹. Several studies demonstrated that particles <200 nm are able to escape from splenic clearance to be re-directed to the liver¹⁸. The small size (80 nm) of our LNP⁸, their neutral to mild cationic charge after loading of siRNA⁸ and the absence of larger aggregates (Supporting Fig. 2C,D) favour their preferential distribution to the liver rather than the spleen. These properties, together with the phagocytic activity of HSC²⁰, may also explain their relatively high uptake by these cells, as small neutral lipophilic particles (<100 nm) are able to travel through the liver parenchyma and access mesenchymal cells²¹, whereas larger particles are rapidly engulfed by Kupffer cells²². We hypothesize that some monomers and small aggregates of our LNP are phagocytised by Kupffer cells and HSC, while large aggregates (as observed by fluorescence microscopy) can undergo partial membrane fusion with several neighbouring cells.

A prior study has shown that the C12-200 LNP protect the siRNA cargo from nuclease attack in serum for at least 24 h⁸. Since liver uptake was almost quantitative 30–60 min after injection and persisted thereafter, any unaccounted extrahepatic effects of the particles or siRNA in other organs are unlikely.

Fluorescent (DiI)-labeled LNP were detectable in serum for at least 3 days, which was achieved by PEGylation and addition of cholesterol to the lipid nanoparticle formulation⁸. Interestingly, the siRNA-loaded LNP were highly enriched in the fibrotic vs. normal liver, despite the apparent obstacles for nanoparticle delivery to scar tissue. A reason for their unexpectedly high delivery to the fibrotic tissue may be their above-mentioned, apparently optimal properties for extravasation and retention in the diseased tissue, similar to the EPR effect that is observed for nanoparticulate therapeutics for cancer²³. This would explain the long retention of our LNP in liver up to 9 days (Fig. 1B–E). The lower lipophilicity of DiI (used in the colocalization and confirmatory liver uptake studies) compared to DiR, may explain its shorter apparent liver retention (Supporting Fig. 4A,B) compared to the NIR imaging studies (Fig. 1A,B).

Notably, compared to a prior study that showed significant antifibrotic efficiency of vitamin A loaded liposomes carrying siRNA against the collagen chaperone gp46 in models of hepatic fibrosis⁷, our approach is more specific. Thus gp46 targets many collagens, including collagens of basement membranes that may rather protect from than promote fibrogenesis, such as the basement membrane collagens type IV, XV or XVIII⁽⁹⁾. Moreover, gp46 is involved in other processes apart from serving as a chaperone²⁴.

Our nanoparticulate formulation⁸ combined with the high specificity and stability of the employed siCol1a1⁽²⁵⁾ enabled an unprecedented in vivo delivery and knockdown efficiency to the fibrogenic effector cells, at doses that were previously deemed to be insufficient to induce therapeutic effects in vivo²⁶. Based on prior studies we hypothesize that this potentiated antifibrotic effect can be explained by escape of the LNP from lysosomal attack in the cytosol of the target cells⁵ and efficient release of their cargo by lipases²⁷.

While LNP-siCol1a1 treatment increased the expression and activity of interstitial collagenases in general, it decreased *Mmp9* expression and gelatinases activity in the regression model, but increased *Mmp9* mRNA and gelatinases activity when given during progression. Gelatinases (MMP2 and MMP9) mainly degrade denatured interstitial collagens and native collagen type IV of basement membranes. Here MMP2 is mainly synthesized by HSC, whereas MMP9 mainly derives from macrophages. The divergence of *Mmp9* expression and gelatinolytic activities is best explained by an indirect effect of the LNP-siCol1a1 treatment on the different macrophage populations that mediate ECM remodelling during fibrosis progression vs. regression, with e.g. *Mmp9* expression both mediating early progression or late regression²⁸.

Activation of innate immunity, mainly in myeloid cells and via toll-like receptors 7 (TLR7) and TLR8 has been a major obstacle in the development of safe siRNA-therapeutics²⁹. TLR7 and TLR8 induce several proinflammatory cytokines and promote adaptive (T cell mediated) immune responses³⁰. We used optimized and 2'-O-methyl, phosphorothioate and deoxythymidine linked and shortened siRNA sequences (<23 nucleotides) that are devoid of TLR7 and TLR8 activation⁴. By employing these siRNAs we found no sign of innate or adaptive immune activation, including routine serological or histological parameters of liver inflammation, or enhanced expression of *Tlr4*, *Il8* or *Ifng* genes.

Taken together, we demonstrate a highly targeted and efficient therapeutic in vivo knockdown of procollagen $\alpha 1(I)$ synthesis that results in inhibition of fibrosis progression, promotion of fibrosis regression and favourable matrix remodeling in several models of liver fibrosis, in the absence of detectable side effects. While efficacy has been demonstrated also for human HSC and a major target molecule in fibrosis, our nanoparticulate platform can be easily broadened to include other and multiple therapeutic targets expressed by HSC, likely generating therapeutic synergy. The absence of detectable side effects may permit rapid translation of our technology into clinical application for the benefit of patients with fibrotic liver disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of abbreviations

Acta2	actin- α 2
ALT	alanine aminotransferase
CCL4	carbon tetrachloride
Col1a1	procollagen α 1(I)
Col3a1	procollagen α 1(III)
ECM	extracellular matrix
Gapdh	glyceraldehyde 3-phosphate dehydrogenase
HSC	hepatic stellate cell
gp46	glycoprotein
hsp47	heat shock protein 47
IL8	interleukine 8
Ifng	interferon gamma
Itgam	integrin α m
LNP	lipid nanoparticle
Mmp2/3/8/9	metalloproteinase 2, 3, 8 and 9
siRNA	short interfering RNA
Timp1	tissue inhibitor of metalloproteinase 1
Tlr4	toll-like receptor 4
Tgfb1/2	tissue growth factor beta 1 and 2
α-SMA	α smooth muscle actin

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Figure 1. Biodistribution of DiR-labeled LNP-siCol1a1 in nonfibrotic and fibrotic mice
 (A) Schedule of mineral oil and CCl₄ oral treatments given to Balb/c mice 3 times a week for 2 weeks. 3 days later, mice received a single i.v. injection of DiR-LNP at 3 mg/kg. (B) In vivo NIR fluorescent pictures of normal (nonfibrotic) FVB controls, FVB biliary fibrotic *Mdr2*^{-/-} mice, and of Balb/c mice gavaged with mineral oil (nonfibrotic controls) or CCl₄ (fibrotic mice) at 30 min, 4 h, 1, 2, 3, 5, 7 and 9 d post injection. (C) Fluorescence intensity in livers expressed as total Radiant Efficiency units ([p/s]/[μW/cm²]). Results represent means ± SD (n=3). *p<0.05; **p<0.01; ***p<0.001 vs. the respective nonfibrotic controls. (D) Ex vivo NIR fluorescent images of lungs, kidneys, liver and spleen harvested from nonfibrotic and fibrotic mice 9 d post-injection. (E) Fluorescence intensity in organs expressed as total Radiant Efficiency (means ± SD, n=3). *p<0.05; **p<0.01; ***p<0.001, vs. the respective nonfibrotic controls. (F) Serum levels of DiI-LNP measured spectrofluorimetrically in untreated Balb/c mice 30 min, 4 h, 1 and 2 d after injection of DiI-LNP or PBS at 3 mg/kg. PBS-injected mice served as negative controls. Time point 0 corresponds to the total injected dose of DiI-LNP and PBS (means ± SD, n=3). *P<0.05; **p<0.01; ***p<0.001 relative to PBS. Independent variables t-test.

Figure 2. Colocalization of DiI-labeled LNP-siColla1 with liver cell-subsets in normal and fibrotic mice

Mineral oil or CCl₄ were given orally to Balb/c mice 6 times during two weeks as before and 3 d later mice received a single i.v. injection of DiI-LNP at 3 mg/kg. Livers were harvested 24 h post injection and sections were stained for nuclei with DAPI (blue) and co-localization of DiI-LNP aggregates (red) with cell specific markers (green) was identified and quantified as yellow fluorescence. Percentage of co-localization was calculated from 10 representative microscopic fields at 250x magnification from 3 mice per group as the number of merged yellow fluorescence signals relative to the total number of the respective cell type (sum of yellow and green cells). Endothelial cells, macrophages, activated HSC, hepatocytes and activated myofibroblasts were identified by antibodies to CD31, CD68 or F4/80, desmin, HNF-4α and α-SMA, respectively. Bars= 200 μm. (A) Representative micrographs showing colocalization of the respective cell subsets with DiI-LNP. (B) Percentage of the total population of CD31, CD68, F4/80, desmin, HNF4α and α-SMA-positive cells colocalized with DiI-LNP in 10 merged pictures of each specimen (means ± SD, n=3). *p<0.05; **p<0.01; ***p<0.001 vs. the corresponding nonfibrotic controls. (C) Colocalization (yellow fluorescence) of portal fibroblasts that stained negative for desmin (pink) and positive for α-SMA (green) with DiI-LNP at 1 d post-injection. Yellow arrows mark portal fibroblasts, and white arrows indicate either desmin or α-SMA and desmin-positive cells (activated HSC). Bars= 100 μm. (D) Percentage of DiI-LNP colocalized with α-SMA positive and desmin-negative cells (means ± SD, 10 images/specimen and n=3). *p<0.05, vs. controls. Independent variables t-test.

Figure 3. LNP-siColl1a1 promote regression of advanced parenchymal liver fibrosis

Advanced parenchymal liver fibrosis was induced in C57BL/6 mice by 3 oral gavages of CCl₄ a week for 8 weeks. 24 h after the last dose of CCl₄, mice received i.v. injections of PBS, LNP-siColl1a1, or LNP-siLuc at 100 and 200 µg/kg of siRNAs once weekly for 4 weeks. Livers and serum were collected 24 h after the last injection. (A,B) Liver sections stained for collagen (Sirius red) and for a subset of activated HSC with antibodies to α-SMA. Bars, 200 µm. Percentage of Sirius red and α-SMA positive area in 5 randomly selected fields from each specimen, as assessed by computerized image analysis (means ± SD; n=5/group, n=3 per sample). *p < 0.01 vs. LNP-siLuc. (C) Hepatic expression of Col1a1 mRNA quantified by real time RT-PCR and normalized to Gapdh mRNA as housekeeping gene (means ± SD, n=5/group). ***p < 0.001 vs. LNP-siColl1a1. (D) Hepatic gelatinases activity (means ± SD of n=5 per group). *p < 0.01; **p < 0.001 vs. LNP-siLuc. (E) Total collagen measured as hydroxyproline (means ± SD, n=5 per group). *p < 0.05 vs. LNP-siColl1a1. Independent variables t-test.

Figure 4. LNP-siCol1a1 inhibit progression of CCl4-induced parenchymal liver fibrosis
CCl4 was given to C57BL/6 mice 3 times a week for 5 weeks to induce mild parenchymal liver fibrosis. During the last two weeks, mice received i.v. injections of PBS, LNP-siCol1a1 or LNP-siLuc at siRNA at doses of 200 and 400 µg/kg biweekly for 2 weeks. Liver specimens were harvested at 24 h post-injection. (A,B) Micrographs of Sirius red- and α-SMA stained liver sections. Bars, 200 µm. Quantification of Sirius red and α-SMA-positive areas in 5 randomly selected fields of each specimen (means ± SD, n=10). **p<0.01, vs. LNP-siLuc. (C,D). Expression of Col1a1 and Acta2 transcripts as assessed qRT-PCR, relative to Gapdh mRNA (means ± SD, n=10 per group). *p<0.05; ***p<0.001, vs. LNP-siLuc. (E) Liver hydroxyproline content (means ± SD, n=10/group). *p<0.05, vs. LNP-siCol1a1. Independent variables t-test.

Figure 5. LNP-siCol1a1 upregulate (pro-)collagen type III in mice with CCL4-provoked parenchymal liver fibrosis

Experiment in Fig. 4. (A) Immunofluorescent images of (pro-)collagen type III staining in liver sections. Bars, 200 µm. (B) Percentage of area occupied by (pro-)collagen type III positive fibrils (means $\pm$ SD, n=10/group). *p<0.05; **p<0.01, vs. LNP-siLuc. (C) Expression of Col3a1 mRNA assessed by qRT-PCR and normalized to Gapdh mRNA ratio (means $\pm$ SD, n=10/group). *p<0.05, vs. LNP-siCol1a1. Independent variables t-test.

Figure 6. LNP-siCol1a1 RNA mitigate biliary fibrosis progression in Mdr2^{-/-} mice

LNP-siCol1a1 or LNP-siLuc were intravenously injected into Mdr2^{-/-} mice (8 weeks old) twice weekly for 2 weeks, at siRNA doses of 400 and 800 µg/kg. Liver specimens were harvested 24 h after the last injection. (A) Photomicrographs of Sirius red stained collagen in liver sections. Bars, 100 µm. (B) Percentage of Sirius red stained area in 5 randomly selected fields of each specimen (means ± SD, n=8). *p<0.05; **p<0.01, vs. LNP-siLuc. (C,E) Expression of Col1a1 and Mmp8 transcripts as measured qRT-PCR, relative to Gapdh mRNA (means ± SD, n=8). *p<0.05; **p<0.01; ***p<0.001, vs. LNP-siLuc. (D) Content of hydroxyproline in total livers (means ± SD, n=8). **p<0.01, vs. LNP-siLuc. (F) Interstitial collagenases activity (means ± SD, n=8). *P<0.05, relative to LNP-siLuc. Independent variables t-test.

Figure 7. LNP-siCol1a1 do not activate innate immunity in fibrotic mice

(A,B) Immunostaining for CD68 and TLR4 in liver sections of mice with advanced CCl₄-induced parenchymal liver fibrosis in the regression phase after 4 injections of LNP-siCol1a1 or LNP-siLuc, at siRNA doses of 100 and 200 µg/kg over 4 weeks (experiment in Fig. 3). Bars, 200 µm. Number of CD68-positive macrophages and TLR4-positive area obtained by image analysis. Results are from 5 randomly selected fields and represent means $\pm$ SD (n=5/group, n=3 per sample). *p<0.01, LNP-Col1a1 vs. LNP-siLuc RNA. (C,D,E) Expression of Cd68, Itgam and Ifng mRNAs as assessed by qRT-PCR, normalized to Gapdh mRNA (means $\pm$ SD; n=5). *p<0.05; **p<0.01; ***p<0.001 vs. LNP-siLuc RNA. *p<0.05 vs. PBS for Ifng. Independent variables t-test.