Figure 2. Silencing of quiescent stellate cells

While all CLDs can lead to hepatic fibrosis, therapeutic interventions

Figure 7. Col1a1 knockdown is mediated by RNAi.

Figure 8. Delivery to hepatocytes under conditions of liver injury
Abstract

Approximately 28,000 Americans die every year from chronic liver diseases (CLD) including chronic hepatitis, alcoholism and non-alcoholic fatty liver. While all CLDs can lead to hepatic fibrosis, therapeutic interventions generally target the etiological agent of the disease and do not directly address the underlying fibrosis. Hepatic stellate cells (HSCs) play a key role in the cause and progression of liver fibrosis. An insult/injury to the liver activates the quiescent stellate cells which then synthesize extracellular matrix (ECM) as part of a wound healing response. However, repeated liver injury leads to further proliferation of stellate cells, increased matrix deposition, and ultimately, liver fibrosis or cirrhosis. Therefore, reducing the production of ECM by stellate cells would be predicted to inhibit the progression of fibrotic disease.

Here we describe lipid nanoparticle (LNP) mediated siRNA delivery to quiescent and activated stellate cells in three mouse models of liver injury: induction by carbon tetrachloride, thioacetamide or bile-duct ligation. siRNAs formulated in LNPs comprising the cationic lipid, C12-200 (PNAS, 2010: 107(5)-1864) were administered via intravenous injection. Silencing of the HSC-specific targets, collagen1a1 (col1a1) or rhoN demonstrated effective siRNA delivery to stellate cells. We achieved durable target knock-down in a dose dependent manner with an IC₅₀ of approximately 0.1 mg/kg for col1a1 and 0.1-0.03 mg/kg for rhoN. Delivery to activated HSCs was further confirmed using siRNAs directed against a second HSC target, smooth muscle actin (Acta2). We propose that siRNA-mediated inhibition of col1a1, or other ECM components, warrants further exploration as a novel therapeutic approach for liver fibrosis.

Figure 1. Stellate cells

A Stellate cells play key role in liver fibrosis

Adapted from J. Clinical Investigation 2005 (115:2: 209)
Stellate cells play key role in liver fibrosis


Hepatic stellate cells (HSC):
- Ito cells, fat storing cells, interstitial cells, lipocytes
- Described by Kupffer (1876) as liver sternzellen (star shaped)
- Present in Space of Disse
- 5-10% of total resident liver cells
- Major storage sites for retinoids (vit A) in normal livers

Injury to liver induces a wound healing response
- Chemokine signaling
- Activation of stellate cells
  - Activated HSCs become highly proliferative
  - Synthesize ‘fibrotic’ matrix rich in Type I Collagen
- Scarring of tissue
- Chronic insult leads to fibrosis and eventually cirrhosis

Targeting stellate cells
- Stellate cells are the major players in fibrosis hence targeting them can open different avenues for treatment
- Collagen1 (Col1) is the principal collagen in fibrotic liver and is expressed by stellate cells in the liver
- Decrease in Collagen1a1 (Col1a1) after treatment with siRNA indicates delivery to activated stellate cells
- Col1a1 is a heterotrimer: 2 chains of pro-α1(I) & 1 chain of pro-α2(I)
  - Col1a1 forms homotrimers in absence of Col1a2
  - Col1a2 degrades intra-cellularly in absence of Col1a1
- Decrease in Collagen1 should
  - Destabilize the fibrillar architecture
  - Provide negative feedback to the HSCs and lead to HSC apoptosis/clearing
  - Promote recovery of normal liver function
siRNA delivery to stellate cells. We achieved durable target knock-down in a dose dependent manner with an

Liposome Mediated Delivery of siRNA to Hepatic Stellate Cells

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- Stellate cells are the major players in fibrosis hence targeting them can open different avenues for treatment

Hepatic stellate cells (HSC):
- Present in Space of Disse
- Major storage sites for retinoids (vit A) in normal livers
- Play key role in liver fibrosis

A. Dose dependent silencing of RhoN mediated by LNP-RhoN siRNA. Animals (n=5) livers after treatment (n=15). C. Representative liver sections stained with Sirius red.

B. Percent Survival

C. Levels of ALT and AST measured in serum of treated mice (n=15) B. Average of Col1a1 mRNA measured in livers after treatment (n=15). C. Representative liver sections stained with Sirius red.

Figure 2. Silencing of quiescent stellate cells. A. Dose dependent silencing of RhoN mediated by LNP-RhoN siRNA. Animals (n=5) were IV dosed with 1.0, 0.3, 0.1, 0.03 mg/kg LNP-RhoN or 1mpk LNP-Luc or PBS and sacrificed 48 hours after the dose. B. Animals (n=5) were dosed with 0.5 mg/Kg LNP-RhoN or LNP-Luc and sacrificed on the indicated Day.

RhoN is a small GTPase, and its expression in normal liver is limited to stellate cells. Silencing of RhoN in whole livers signifies delivery to quiescent stellate cells.

Figure 3. CCl4 induced liver injury

A. Liver damage 24 hours after single CCl4 dose

MO CCl4

Relative FVII mRNA/GAPDH

B. Induction of Col1a1 message by CCl4

MO CCl4

Relative Col1a1 mRNA/GAPDH

C

Figure 3. CCl4 induced liver injury. Two doses of CCl4 were administered 7 days apart, and animals were sacrificed 24 (in A) or 48 hours (B, C) after the treatment. A. Levels of ALT and AST measured in serum of treated mice (n=15) B. Average of Col1a1 mRNA measured in livers after treatment (n=15). C. Representative liver sections stained with Sirius red.
Figure 4. Knockdown in activated stellate cells

Day: 1 9 10 12

CCI4 gavage (solvent mineral oil) IV dose Sacrifice

Figure 4. Target knockdown in activated stellate cells. A. Dose dependent Col1a1 mRNA knockdown with LNP formulated siRNA in acute injury model. The mRNA levels were measured using Taqman-QPCR in total livers. The graph represents normalized signal for Col1a1 mRNA, using GAPDH as internal control. Luc: Luciferase, siRNA control (n=10 per CCI4 group) B. αSMA mRNA knock-down with LNP-SMA.
Alfica Sehgal, Mohammad Zafari, Boris Klebanov, Greg Hinkle, Satya Kuchimanchi, Sarfraz Shaikh, Martin Maier, Jonathan O’Shea, Lauren Speciner, Akin Akinc, Daniel Anderson,* Robert Langer,* Tatiana Novobrantseva, Victor Kotelianski, David Bumcrot*

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Delivery to activated HSCs was further confirmed using siRNAs directed against a second HSC target, smooth muscle actin (Acta2). We propose that siRNA delivery to stellate cells would be detrimental to the HSCs and lead to HSC apoptosis/clearing.

Collagen1 (Col1) is the principal collagen in fibrotic liver and is expressed by stellate cells in the liver. A. LNP mediated delivery to stellate cells in a chronic injury model. Liver injury was induced using CCl4. Animals were injected with 1,0.3, 0.1, 0.03 mg/Kg LNP-RhoN or 1 mg/Kg LNP-Luc or PBS and sacrificed 48 hours after the dose. B. Animals (n=5) sacrificed on Day 14 and livers were processed for western blot. C. Collagen1 protein measured by western blot (top). Quantification of Col1 normalized to b-actin is shown in the graph below. The western blot was scanned using LiCor Odyssey.

Figure 5. Delivery to stellate cells in different injury models

Figure 6. LNP delivery to stellate cells in chronic injury model

RT-qPCR was used to measure Col1a1 mRNA, using GAPDH as internal control. Luc: Luciferase, siRNA control (n=10 per CCl4 group) B. Average of Col1a1 mRNA measured in livers after treatment (n=15). C. Representative liver sections stained with Sirius red. (B, C) after the treatment. A. Levels of ALT and AST measured in serum of treated mice (n=15) B. Average of Col1a1 mRNA measured in livers after treatment (n=15).

A. Relative Col1a1 mRNA/GAPDH level quantified by RT-qPCR. Luc: Luciferase, siRNA control (n=10 per CCl4 group) B. Collagen1 protein measured by western blot (top). Quantification of Col1 normalized to b-actin is shown in the graph below. The western blot was scanned using LiCor Odyssey.

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Figure 5. Delivery to activated stellate cells in different injury models. A. LNP mediated delivery to activated stellate cells under different models of liver injury. Mice were given 3 weekly doses of TAA and col1a1 expression was measured in livers 2 days after siRNA dose. (n=10) B. LNP mediated delivery in severe acute injury model of bile-duct ligation (BDL). BDL ligation was done on Day 0. Animals were dosed with siRNA on days 4 and 7 and sacrificed on day 8 (n=5). C. BDL mice were IV dosed on days 5, 7 and 11 with PBS, LNP-Col1a1 or LNP-Luc (n=5). The number of surviving animals were counted each day and all remaining animals were sacrificed on Day 12. Animals treated with LNP-Col1a1 could sustain the injury longer than other two groups.
IC50 of approximately 0.1 mg/kg for col1a1 and 0.1-0.03 mg/kg for rhoN. Delivery to activated HSCs was further siRNA delivery to stellate cells. We achieved durable target knock-down in a dose dependent manner with an intravenous injection. Silencing of the HSC-specific targets, collagen1a1 (col1a1) or rhoN demonstrated effective and ultimately, liver fibrosis or cirrhosis. Therefore, reducing the production of ECM by stellate cells would be activates the quiescent stellate cells which then synthesize extracellular matrix (ECM) as part of a wound healing.

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Liposome Mediated Delivery of siRNA to Hepatic Stellate Cells

- Provide negative feedback to the HSCs and lead to HSC apoptosis/clearing
- Stellate cells are the major players in fibrosis hence targeting them can open different avenues for treatment
- Ito cells, fat storing cells, interstitial cells, lipocytes

Figure 2. Silencing of quiescent stellate cells. Figure 4. Target knockdown in activated stellate cells. Figure 6. LNP mediated delivery to stellate cells in a chronic injury model.

Robust delivery to stellate cells under conditions of chronic liver injury
- Multi-insult, multi-dose model
- ~50-fold induction of Col1a1 mRNA by CCl4
- ~95% knock-down of Col1a1 mRNA
- ~90% reduction in collagen I protein

Figure 6. LNP mediated delivery to stellate cells in a chronic injury model. A. The experimental plan is depicted in the line diagram. Col1a1 mRNA knockdown with LNP- siRNA in multi-insult, multi-dose model. The mRNA levels were measured using Taqman-QPCR in total livers. The graph represents normalized signal for Col1a1 mRNA, using GAPDH as internal control. Luc: Luciferase, siRNA control (n=10) B. Collagen1 protein measured by western blot (top). Quantification of Col1 normalized to b-actin is shown in the graph below. The western blot was scanned using LiCor Odyssey.
Figure 7. Col1a1 knockdown is mediated by RNAi. 10 animals were given CCl4 by oral gavage. A. Scheme for 5’RACE assay. Total liver mRNA was processed using Generacer kit (Invitrogen) for cDNA synthesis and 5’RACE to analyze the cleaved col1a1. The PCR products were cloned into TA vectors and sequenced. B. Animals were injected with LNP-Col1a1 and sacrificed after 24 hours. C. Agarose gel showing DNA bands for nested PCR. 90% of the clones from LNP-Col1a1 group showed expected cleavage product.
Liposome Mediated Delivery of siRNA to Hepatic Stellate Cells

Abstract

Alnylam Pharmaceuticals, Cambridge, MA 02142; *Koch Institute for Integrative Cancer Research at MIT, Cambridge, MA

This manuscript describes the delivery of siRNA to hepatic stellate cells (HSCs) using liposomes.

- Promote recovery of normal liver function
- Provide negative feedback to the HSCs and lead to HSC apoptosis/clearing

Hepatic stellate cells (HSCs) play a key role in the cause and progression of liver fibrosis. An insult/injury to the liver generally targets the etiological agent of the disease and does not directly address the underlying fibrosis. Hepatic alcoholism and non-alcoholic fatty liver disease (NAFLD) are major drivers of fibrosis, which is a serious consequence of both conditions.

**Figure 1. Delivery to activated stellate cells in different injury models**

- **A** shows a cartoon depiction of TGF-β SMAD signaling pathway in liver fibrosis. Different signaling pathways lead to production of extracellular matrix components including Collagen1.

- **B** demonstrates relative SMAD2 mRNA levels after treating the animals with siRNA against Col1a1, SMAD2/3 or Luc.

- **C** illustrates relative FVII mRNA levels after treating the animals with LNP-FVII or LNP-Luc.

**Figure 8. Delivery to hepatocytes under conditions of liver injury.** A set of siRNA targeting both SMAD2 and SMAD3 were screened in NIH 3T3 cells. The lead candidate was formulated for in vivo experiment. Liver injury was induced using CCl4. Animals were injected with siRNA against SMAD2/3, Col1a1 or Luc and samples processed for Taqman-QPCR. A. SMAD2/3 mRNA levels normalized to GAPDH. B. Col1a1 mRNA levels after treating the animals with siRNA against Col1a1, SMAD2/3 or Luc are shown, normalized to GAPDH. C. Delivery to hepatocytes was assessed by measuring knock-down of FVII after CCl4 treatment. Similar levels of knock-down were observed in animals treated with CCl4 or the vehicle, mineral oil. (Luc: siRNA against luciferase; as a control, FVII: siRNA against Factor VII, a protein specifically synthesized by hepatocytes in the liver.)

**Summary**

- Potent and reproducible LNP mediated delivery to quiescent and activated stellate cells
  - Tested LNP-siRNA delivery to activated stellate cells under different models of liver injury
    - Acute and chronic injury model for pericentral injury: CCl4
    - Bile-duct ligation injury model for portal fibrosis
    - Thioacetamide induced injury in pericentral and periportal areas
  - Delivery confirmed with silencing of multiple markers
- Robust knockdown of Col1a1 in activated stellate cells
  - Maximum knockdown ~90%, ED50 around 0.1 mg/kg
- Delivery to hepatocytes in chronically injured liver
- Supports further development of Col1a1 targeting as a therapeutic approach

**Col1a1** is a heterotrimer: 2 chains of pro-Collagen1 and 1 chain of pro-Collagen2, which are expressed by stellate cells in the liver. **Collagen1** is the principal collagen in fibrotic liver and is expressed by stellate cells in the liver, including Ito cells, fat-storing cells, interstitial cells, lipocytes. **Hepatic stellate cells (HSC):** Major storage sites for retinoids (vitamin A) in normal livers are also associated with liver fibrosis. **Chemokine signaling** leads to activation of stellate cells. **Activation of stellate cells** results in proliferation of stellate cells, increased matrix deposition, increased production of collagen, and activation of other stellate cells. **Col1a1** mRNA knockdown with LNP-siRNA in multi-insult, multi-dose model. The mRNA levels were measured using Taqman-QPCR in total livers. The graph represents normalized signal for Col1a1 mRNA, using GAPDH as internal control. Luc: Luciferase, siRNA control

**Figure 5. Delivery to stellate cells in different injury models**

- **A** shows LNP mediated delivery to stellate cells in acute injury and acute injury model with bile-duct ligation for portal fibrosis.

- **B** illustrates relative Col1a1 mRNA levels after treating the animals with siRNA against Col1a1, SMAD2/3 or Luc.

**Figure 6. LNP delivery to stellate cells in chronic injury model**

- **A** shows LNP delivery to stellate cells in chronic injury model with two doses of CCl4 administered 7 days apart, and animals sacrificed 24 or 48 hours after treatment.

- **B** demonstrates relative Col1a1 mRNA levels after treating the animals with siRNA against Col1a1, SMAD2/3 or Luc.

**Figure 7. Col1a1 knockdown is mediated by RNAi.**

- **A** shows gel showing DNA bands for nested PCR. 80% of the clones from LNP-Col1a1 group showed expected cleavage product.

- **B** illustrates relative SMAD2 mRNA levels after treating the animals with siRNA against Col1a1, SMAD2/3 or Luc.

- **C** shows relative FVII mRNA levels after treating the animals with LNP-FVII or LNP-Luc.

**Figure 9. Cartoon depiction of TGF-β SMAD signaling pathway in liver fibrosis.** Different signaling pathways lead to production of extracellular matrix components including Collagen1.