Published in final edited form as:

J Control Release. 2011 October 30; 155(2): 326–330. doi:10.1016/j.jconrel.2011.06.037.

Targeted TFO Delivery to Hepatic Stellate Cells

Ningning Yang, Saurabh Singh, and Ram I Mahato*

Department of Pharmaceutical Sciences, University of Tennessee Health Science Center,
Memphis, TN 38103

Abstract

Triplex-forming oligonucleotides (TFOs) represent an antigene approach for gene regulation through direct interaction with genomic DNA. While this strategy holds great promise owing to the fact that only two alleles need silencing to impact gene regulation, delivering TFOs to target cells in vivo is still a challenge. Our recent efforts have focused on conjugating TFOs to carrier molecules like cholesterol to enhance their cellular uptake and mannose-6-phosphate-bovine serum albumin (M6P-BSA) to target TFO delivery to hepatic stellate cells (HSCs) for treating liver fibrosis. These approaches however are rendered less effective owing to a lack of targeted delivery, as seen with lipid-conjugates, and the potential immune reactions due to repeated dosing with high molecular weight BSA conjugated TFO. In this review, we discuss our latest efforts to enhance the effectiveness of TFO for treating liver fibrosis. We have shown that conjugation of TFOs to M6P-HPMA can enhance TFO delivery to HSCs and has the potential to treat liver fibrosis by inhibiting collagen synthesis. This TFO conjugate shows negligible immunogenicity owing to the use of HPMA, one of the least immunogenic copolymers, thereby making it a suitable and more effective candidate for antifibrotic therapy.

Keywords

Bioconjugation; HPMA; TFO; liver fibrosis

1. Introduction

Hepatic fibrosis is the scarring response by the liver to chronic insults and damages, resulting in accumulation of extra extracellular matrix (ECM) [1]. Major causal factors for liver fibrosis include alcohol abuse, nonalcoholic steatohepatitis (NASH) and viral hepatitis [2]. During liver injury, the infiltrated leukocytes and resident macrophages, continually release reactive oxygen species (ROS), inflammatory cytokines and growth factors, leading to the transformation of quiescent, vitamin A storing hepatic stellate cells (HSCs) into proliferating α -smooth muscle actin (SMA) positive myofibroblast-like cells [3]. Activated HSCs, in addition to secreting excessive collagen in fibrotic liver, are also the source of inflammatory cytokines. This creates a positive feedback loop that ensures continual HSC activation, and leads to inflammation, and increased immune cell infiltration. Further influx of bone marrow-derived fibrocytes in the damaged liver tissue results in the transformation

© 2011 Elsevier B.V. All rights reserved.

*Corresponding author: Ram I. Mahato, Ph.D., Department of Pharmaceutical Sciences, University of Tennessee Health Science Center, Cancer Research Bldg, RM 224, 19 South Manassas, Memphis, TN 38103-3308, Tel: (901) 448-6929, Fax: (901) 448-2099, rmahato@uthsc.edu, <http://www.uthsc.edu/pharmacy/rmahato>.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

of other liver cell types like cholangiocytes into myofibroblasts via epithelial to mesenchymal transition (EMT), a process in which epithelial cells lose their phenotypic characteristics and acquire features of mesenchymal cells such as fibroblasts [4]. All these pathogenic mechanisms result in the deposition of excess ECM, including type I collagen, which is the hallmark of fibrosis.

Excessive collagen synthesis interferes with the maintenance of normal liver function and prevents effective liver regeneration [2]. Strategies to treat liver fibrosis are based chiefly on reducing collagen synthesis [5], inhibiting the activation of HSCs to myofibroblasts [6], or protecting the injured hepatocytes by controlling inflammation [7]. In this review, we focus on triplex forming oligonucleotide (TFO) which represents an antisenescence therapy and causes transcription inhibition [8]. Unlike antisense oligonucleotides (ODNs) and siRNA approaches, TFO can form triplex with the specific region of genomic DNA [9]. We have been able to enhance both the specific delivery to HSCs and the circulation time of this TFO by utilizing a bioconjugation strategy. Triplex formation with DNA prevents both the binding of transcription factors to the gene promoter and unwinding of the duplex during transcription, making this an attractive therapeutic strategy for managing and treating various diseases, including fibrosis of the liver and other organs.

2. Triplex formation for transcription inhibition to treat liver fibrosis

Although DNA normally exists in a duplex form, under some circumstances it can assume triple helical (triplex) structures, which are either intramolecular or intermolecular. The intermolecular triplexes, formed by the addition of a sequence-specific third strand to the major groove of the duplex DNA, have the potential to serve as selective gene regulators [10]. Several genes contain potential triplex forming homopurine/homopyrimidine sequences, which can be the targets for gene regulation by TFOs.

Sequence composition and organization are essential for both triplex formation and its therapeutic effectiveness. Previous research, both *in vitro* and *in vivo*, has shown that antiparallel TFO was more efficient than parallel TFOs in reducing collagen gene expression [11, 12]. TFOs occupy the major groove of duplex DNA forming purine or pyrimidine motif triplex structures with the native purine strand. The most stable triplexes are formed when the third strand binds in antiparallel orientation to the homologous native strand. It has been shown that formation of triplexes with various promoters could result in transcription inhibition [13]. TFOs, usually 13–20 nucleotides long, are composed of either polypurine or polypyrimidine and have binding specificity only towards the purine-rich strand of their target DNA duplex in the major groove. Further, TFOs containing C and T nucleotides bind in a parallel while those containing G and A or T nucleotides bind in an anti-parallel orientation to the target strand, respectively (Figure 1) [2]. Because only purines are able to further establish two hydrogen bonds in the major groove of DNA, successful TFO design must follow precise principles that require consecutive purines on the same strand for stable binding [14, 15].

Type I collagen consists of two $\alpha 1(I)$ and one $\alpha 2(I)$ polypeptide chains encoded by the $\alpha 1(I)$ and $\alpha 2(I)$ genes and synthesized in a 2:1 ratio. The polypyrimidine/polypurine sequence (C1) of $\alpha 1(I)$ collagen gene can form triplexes if a third strand is added. It is a major structural component of ECM and thus an ideal target for the treatment of liver fibrosis. It has been demonstrated that up-regulated expression of type I collagen by activated HSCs can be at both the transcriptional and post-transcriptional levels [16]. It was further shown that both the synthesis and stability of $\alpha 1(I)$ collagen mRNA was significantly increased during hepatic fibrosis. Therefore, it may be possible to prevent and potentially reverse fibrosis by inhibiting the transcription of type $\alpha 1(I)$ collagen gene.

Mammalian $\alpha 1(I)$ collagen gene promoter contains two contiguous 30-bp polypurine tract, C1 and C2, located at -141 to -170 and -171 to -200 upstream from the transcription initial site [17]. Studies have also demonstrated that 18-, 25-, and 30-mer antiparallel phosphorothioate (APS) TFOs specific for C1 tract, inhibit transcription in cultured fibroblasts by forming triplex with the genomic DNA [12]. Using this approach, we have demonstrated good correlation between the extent of triplex formation with the degree of transcription inhibition in naked genomic DNA, isolated nuclei of HSC-T6 cells and whole cells [18]. Further, in a rat model of dinitrosamine (DMN) induced liver fibrosis, the 25-mer and 18-mer TFOs, designed to be specific for the upstream nucleotide sequence from -141 to -165, were effective in preventing collagen accumulation and in improving liver function tests [10].

Systemic delivery of TFOs has been applied for treating both genetic and acquired diseases. The major advantage of TFOs over antisense ODNs and siRNAs is their ability to interact with genomic DNA and shutting down transcription rather than silencing mRNA translation, for which hundreds or thousands of copies per cell may be present. Furthermore, specific mRNAs are continuously transcribed from genomic DNA, even though those in the cytoplasm may have been silenced. Therefore, inhibition of gene transcription using TFOs might offer significant advantage over other gene therapy techniques, at least in some cases.

The TFO investigated in our laboratory has two advantages compared to other TFO. Firstly, this TFO is a polypurine lacking any CpG motifs. In a study by Woolridge et al. [19], DNA with CpG motifs were shown to trigger innate immune defense mechanisms. This stimulation of immunostimulation may actually worsen liver fibrosis instead of curing it. Secondly, the TFO utilized in our studies can form DNA triplex under physiological conditions, thus facilitating triplex formation at target sites. The resulting triplex is thus more stable and makes gene silencing more efficient. Taken together, the proposed TFO against $\alpha 1(I)$ collagen can be used as a potent anti-fibrotic drug. Our experimental data confirmed our hypothesis when it was demonstrated that administration of this TFO to DMN induced hepatic fibrosis in rat can abrogate collagen accumulation and alleviate fibrosis. Compared to DMN-treated controls rats which showed moderate to severe deposition of collagen, TFO treatment reduced collagen deposition significantly [20].

3. Bioconjugation for site-specific delivery of TFO

TFO was widely distributed throughout the body following systemic administration, with higher uptake in the liver and kidney [20]. This pharmacokinetic profile is in accordance with previous reports utilizing phosphothioate (PS) and G-rich ODNs [21, 22]. Overall, TFO uptake by tissues was observed as a dose dependent phenomenon, but at high doses, saturated kinetic mode was observed. The hepatic uptake of the TFO was shown to be linear at low doses (0.2–1 mg/kg), while nonlinear at doses above 1 mg/kg [20]. The hepatic uptake curve of free TFO fits the saturation kinetic equation. The wide distribution and uptake by different liver cell types strongly suggests the need for targeted TFO delivery systems.

Particle size and surface charge play an important role in drug delivery to fibrotic liver as hepatic cytoarchitecture is severely disrupted during the course of the disease which leads to a shrinking of liver and closure of sinusoidal gaps. This phenomenon will not allow particulate drug delivery systems such as nanoparticles and liposomes to reach HSCs after systemic administration [23]. Cationic polymers that are frequently utilized to deliver nucleic acid in various experimental systems may not be suitable to achieve TFO delivery to HSCs as they form large complex and possess net positive charge. A previous effort utilizing galactose conjugated poly(L-lysine) demonstrated enhanced delivery to the liver

but the ODN biodistribution within different liver cell types was non-specific [24]. To avoid the use of polycations, Rajur et al. conjugated ODNs to asialoglycoprotein via a disulfide bond [25]. However, this strategy is not suitable for us because direct conjugation of molecules to the TFOs may disrupt their triplex-forming ability, making them ineffective as transcription inhibitory molecules. We also conjugated TFO with cholesterol which enhanced its uptake by liver but the intrahepatic distribution to different cells was non-specific [26], suggesting the need for targeted TFO delivery.

4. M6P-BSA mediated TFO Delivery to HSCs

The expression of mannose 6-phosphate/insulin like growth factor II (M6P/IGF II) receptors on HSCs is up-regulated during the course of chronic liver injury. These receptors ensure a preferential uptake of M6P conjugated human serum albumin (HSA) after systemic administration into rats [27]. To maximize TFO delivery to the liver in general and to HSCs in particular, we synthesized M6P-BSA and conjugated it to TFO via a disulfide bond. Our data showed enhanced targeted delivery of TFO to HSCs [28]. After systemic administration of M6P-BSA-³³P-TFO in rats, the TFO concentration in the HSCs was 1093 ng/mg cell protein, compared to only 215 ng/mg cell protein for free ³³P-TFO injected rats. There was also increase in TFO concentration, from 112 ng/mg cell protein for free ³³P-TFO injection to 670 ng/mg cell protein for M6P-BSA-³³P-TFO injection, in Kupffer and endothelial cells. Both M6P-BSA-³³P-TFO and free ³³P-TFO administration showed very low concentration in hepatocytes, thereby establishing efficient targeting abilities of this conjugated TFO.

As the liver uptake by HSCs is dependent on the number of M6P per BSA, we administered three different conjugates carrying 14, 20 and 27 M6P per BSA. The percentage of the injected dose accumulated in the liver was shown to increase with increase in M6P density, from 56.3% after (M6P)₁₄-BSA-TFO administration to 67.4% after (M6P)₂₇-BSA-TFO injection. However, there was a saturation in the hepatic uptake of M6P-BSA-TFO because no significant difference between (M6P)₂₀-BSA-TFO and (M6P)₂₇-BSA-TFO hepatic uptake was seen.

Competition experiments were also performed to determine whether the hepatic accumulation of M6P-BSA-³³P-TFO is mediated by M6P/IGF II receptor-mediated endocytosis.[28] After excessive amount of (M6P)₂₀-BSA was intravenously injected in both normal and fibrotic rats, hepatic disposition of M6P-BSA-³³P-TFO decreased in both rat groups, but more so in fibrotic rats. This may be due to the fact that M6P/IGF II receptors are upregulated during fibrosis, which should increase uptake by HSCs via receptor-mediated endocytosis due to saturation by excess amounts of M6P-BSA and cancel out any adverse effect created by decrease in sinusoidal gap.

5. M6P-HPMA-TFO for targeted delivery to HSCs

BSA has been utilized for many years as a carrier because it is neither phagocytosed by macrophages in the liver nor excreted by the kidney and can thus help increase the circulation time of conjugated drugs. However, repeated injections of M6P-BSA-TFO at high dose to treat liver fibrosis may be immunogenic due to the high molecular weight of BSA (MW=64000) [28]. This led us to replacing BSA with N-(2-Hydroxypropyl) methacrylamide (HPMA) copolymer, which has shown great potential for delivery of small molecular drugs, principally due to its non-immunogenicity. Even though other groups have utilized HPMA for ODN delivery, no targeting ligands were used to achieve site-specific delivery in these studies [29]. Therefore, we conjugated M6P to HPMA and then to TFO via a GFLG linker, which is known to be degraded lysosomally (Figure 2) [30]. An enzymatic dissociation experiment was applied to M6P-GFLG-HPMA-GFLG-TFO to determine whether TFO could be released from the conjugate. Papain was used as model enzyme

because it is a cysteine protease hydrolase enzyme and belongs to the same family as cathepsin B, which is the most important enzyme in the lysosomes to cleave GFLG spacer. It was shown that free TFO concentration increased with incubation time with papain. To ensure that TFO released from the conjugate can indeed form a triplex with the targeted duplex DNA, we incubated M6P-GFLG-HPMA-GFLG-TFO with papain for 24 h and then used the released TFO to form a triplex with the target DNA. Our results demonstrated that that TFO released from the conjugate indeed formed a triplex with DNA [30].

6. Effect of Collagen Transcription Inhibition by TFO on Liver Fibrosis

Liver fibrosis results from the overproduction of ECM, especially type I collagen by liver fibrogenic cells, including HSCs. To inhibit collagen production and treat fibrosis, we used antiparallel phosphorothioate (APS) TFOs and demonstrated efficient triplex formation in vitro with transcription inhibition of type 1 collagen in cultured immortalized rat HSC-T6 cells [31]. We have also demonstrated that psoralen modified TFOs could form triplexes with the C1 region of the $\alpha 1(I)$ collagen gene promoter and inhibit transcription in short duplex DNA, plasmid, naked genome, isolated nuclei, and intact HSC-T6 cells [18].

We then assessed the severity of DMN-induced liver fibrosis in rats by staining the liver sections with hematoxylin and eosin (H & E) and Sirius Red to assess tissue architecture and extent of fibrosis, respectively. Moderate to severe periportal or lobular fibrosis was observed with the DMN-treated rats. In contrast, in the DMN+TFO treated group, liver fibrosis was higher than the saline controls, but less than those given DMN alone [20]. Antifibrotic effects of the TFOs in DMN-induced liver fibrosis may result from a direct effect on the liver fibrogenic cells and/or from its secondary anti-inflammatory effects. Therefore, we used a common bile-duct ligated (CBDL) rat model to determine the therapeutic effect of free TFO formulations, since this model has low degree of cell damage and inflammation. Systemic administration of TFO could decrease liver injury, inflammation and fibrosis [32].

7. Therapeutic effects of M6P-HPMA-TFO

To confirm that TFO conjugation to M6P-HPMA had no adverse effect in its ability to form triplex with type I collagen promoter, an in vitro study using cultured HSC-T6 cells was carried out. The inhibitory effect of M6P-GFLG-HPMA-GFLG-TFO on type I collagen transcription was similar to that of free TFO. Our results also demonstrated efficient escape of TFO from the conjugate in sub-cellular compartments, thereby ensuring its availability for efficient gene silencing.

The effect of M6P-GFLG-HPMA-GFLG-TFO on treating liver fibrosis in rats was determined using CBDL rats. Following the systemic administration of M6P-GFLG-HPMA-GFLG-TFO to CBDL rats three times a week for two weeks, samples were analyzed to determine the extent of liver fibrosis. M6P-GFLG-HPMA-GFLG-TFO helps not only the injured liver to reduce collagen synthesis but also the damaged hepatocytes to recover, compared to unconjugated TFO. Taken together, our data demonstrates significant reduction in collagen deposition and fibrosis, and this outcome closely matched the down-regulation of the gene transcripts for $\alpha 1$ collagen gene, thereby establishing a causal link between TFO-mediated gene silencing and abrogation of fibrosis in vivo.

8. Role of TGF- $\beta 1$ in liver fibrosis

In addition to inhibition of collagen biosynthesis by TFO, we have been working on complementary strategies to treat liver fibrosis. A number of inflammatory cytokines and growth factors are involved in the initiation and maintenance of hepatic fibrogenesis, of

which TGF- β 1 appears to be the most important [33–35]. TGF- β 1 in the liver is secreted by hepatocytes, Kupffer cells, stellate cells, endothelial cells and infiltrating mononuclear cells [35]. TGF- β 1 in the liver is secreted by hepatocytes, Kupffer cells, stellate cells, endothelial cells and infiltrating mononuclear cells [35]. Silencing of TGF- β 1 may not only inhibit matrix production but also accelerate its degradation [23].

Although TGF- β 1 is upstream of type 1 collagen, we found that transcriptional inhibition of type 1 collagen can also cause silencing of TGF- β 1 gene expression (Fig. 3A). Since TFO is a polypurine and forms G quartet and requires a nuclear translocation for triplex formation with genomic DNA, we also applied siRNA strategy to target and silence TGF- β 1. This not only affected TGF- β 1 gene silencing, but also restored collagen gene inhibition (Fig. 3B) and thus has the potential to treat liver fibrosis [36]. To achieve prolonged and HSC-specific TGF- β 1 gene silencing, we constructed plasmids based anti-TGF- β 1 shRNAs (Fig. 3C) driven by glial fibrillary acidic protein (GFAP)-promoter, a known biomarker for HSCs of fibrotic livers [37].

9. Concluding Remarks

Till date, there are limited pharmaceutical interventions available for treating fibrotic disease, in part because these drugs either do not accumulate in the target cells or have unacceptable toxicity and side effects. We have proposed changing the pharmacokinetic profiles of potential antifibrotic drugs by utilizing a novel bioconjugation approach to generate M6P-HPMA-TFO with GFLG-linkers which can be degraded in the lysosomes, thereby releasing free TFO inside the target cells. We have shown that this approach will lead towards antifibrotic therapeutics that are less immunogenic and thus are more effective.

Although M6P-HPMA-TFO showed higher silencing efficiency than free TFO, the subcellular fate of conjugated TFO is still unclear. It is yet to be determined if they escape from lysosome in major part or still remain in the capsules? From our recent unpublished data, the difference between conjugated TFO and free TFO is even higher on the HSC cellular uptake efficiency than on the silencing effects which are overall higher for conjugated TFO, albeit non-significantly. Some of these effects are definitely due to the complicated biological environment present during pathological liver fibrosis, but part of it may still result from poor lysosomal escape.

Significant work has been done towards establishing novel TFOs as potential therapeutic agents. For various reasons however, the early promises offered by this strategy have not been entirely successful, and targeted delivery to specific cell types remains the major obstacle in its wider application. Our M6P-HPMA-TFO delivery system is novel and represents a new generation of antigene therapeutics utilizing TFOs. We continue to work and improve on this antigene approach to ensure better therapeutic effects for clinical settings.

Acknowledgments

This work was supported by R01 EB003922 and DK69968 from the National Institute of Health (NIH).

References

1. Friedman SL. Pathogenesis of Liver Fibrosis. Annual Review of Pathology: Mechanisms of Disease. 2011; 6
2. Ye Z, Houssein HSH, Mahato RI. Bioconjugation of oligonucleotides for treating liver fibrosis. Oligonucleotides. 2007; 17:349–404. [PubMed: 18154454]

3. Friedman SL. Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver. *Physiological reviews*. 2008; 88:125. [PubMed: 18195085]
4. Zeisberg M, Yang C, Martino M, Duncan MB, Rieder F, Tanjore H, Kalluri R. Fibroblasts derive from hepatocytes in liver fibrosis via epithelial to mesenchymal transition. *J Biol Chem*. 2007; 282:23337–23347. [PubMed: 17562716]
5. Cheng K, Mahato RI. Gene modulation for treating liver fibrosis. *Crit Rev Ther Drug Carrier Syst*. 2007; 24:93–146. [PubMed: 17725523]
6. Fu R, Wu J, Ding J, Sheng J, Hong L, Sun Q, Fang H, Xiang D. Targeting transforming growth factor {beta} RII expression inhibits the activation of hepatic stellate cells and reduces collagen synthesis. *Experimental Biology and Medicine*. 2011; 236:291. [PubMed: 21378033]
7. Iredale JP. Models of liver fibrosis: exploring the dynamic nature of inflammation and repair in a solid organ. *Journal of clinical investigation*. 2007; 117:539. [PubMed: 17332881]
8. Ye Z, Houssein HS, Mahato RI. Bioconjugation of oligonucleotides for treating liver fibrosis. *Oligonucleotides*. 2007; 17:349–404. [PubMed: 18154454]
9. Son G, Hines IN, Lindquist J, Schrum LW, Rippe RA. Inhibition of phosphatidylinositol 3-kinase signaling in hepatic stellate cells blocks the progression of hepatic fibrosis. *Hepatology*. 2009; 50:1512–1523. [PubMed: 19790269]
10. Koilan S, Hamilton D, Baburyan N, Padala MK, Weber KT, Guntaka RV. Prevention of liver fibrosis by triple helix-forming oligodeoxyribonucleotides targeted to the promoter region of type I collagen gene. *Oligonucleotides*. 2010; 20:231–237. [PubMed: 20818932]
11. Joseph J, Kandala JC, Veerapanane D, Weber KT, Guntaka RV. Antiparallel polypurine phosphorothioate oligonucleotides form stable triplexes with the rat alpha1(I) collagen gene promoter and inhibit transcription in cultured rat fibroblasts. *Nucleic Acids Res*. 1997; 25:2182–2188. [PubMed: 9153319]
12. Nakanishi M, Weber KT, Guntaka RV. Triple helix formation with the promoter of human alpha1(I) procollagen gene by an antiparallel triplex-forming oligodeoxyribonucleotide. *Nucleic Acids Res*. 1998; 26:5218–5222. [PubMed: 9801322]
13. Vasquez KM, Narayanan L, Glazer PM. Specific mutations induced by triplex-forming oligonucleotides in mice. *Science*. 2000; 290:530–533. [PubMed: 11039937]
14. Radhakrishnan I, Patel DJ. DNA triplexes: solution structures, hydration sites, energetics, interactions, and function. *Biochemistry*. 1994; 33:11405–11416. [PubMed: 7522550]
15. Praseuth D, Guieysse A, Hélène C. Triple helix formation and the antigene strategy for sequence-specific control of gene expression. *Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression*. 1999; 1489:181–206.
16. Lindquist J, Marzluff W, Stefanovic B III. Posttranscriptional regulation of type I collagen. *American Journal of Physiology-Gastrointestinal and Liver Physiology*. 2000; 279:G471. [PubMed: 10960344]
17. Joseph J, Kandala J, Veerapanane D, Weber K, Guntaka R. Antiparallel polypurine phosphorothioate oligonucleotides form stable triplexes with the rat alpha 1 (1) collagen gene promoter and inhibit transcription in cultured rat fibroblasts. *Nucleic Acids Res*. 1997; 25:182–188.
18. Ye Z, Guntaka RV, Mahato RI. Sequence-specific triple helix formation with genomic DNA. *Biochemistry*. 2007; 46:11240–11252. [PubMed: 17845009]
19. Wooldridge JE, Ballas Z, Krieg AM, Weiner GJ. Immunostimulatory oligodeoxynucleotides containing CpG motifs enhance the efficacy of monoclonal antibody therapy of lymphoma. *Blood*. 1997; 89:2994. [PubMed: 9108420]
20. Cheng K, Ye Z, Guntaka RV, Mahato RI. Biodistribution and hepatic uptake of triplex-forming oligonucleotides against type alpha1(I) collagen gene promoter in normal and fibrotic rats. *Mol Pharm*. 2005; 2:206–217. [PubMed: 15934781]
21. Yu RZ, Geary RS, Monteith DK, Matson J, Truong L, Fitchett J, Levin AA. Tissue disposition of 2 O (2 methoxy) ethyl modified antisense oligonucleotides in monkeys. *Journal of pharmaceutical sciences*. 2004; 93:48–59. [PubMed: 14648635]
22. AGRAWAL S, TEMSAMANI J, Galbraith W, TANG J. Pharmacokinetics of antisense oligonucleotides. *Clinical pharmacokinetics*. 1995; 28:7–16. [PubMed: 7712663]

23. Friedman SL. Liver fibrosis—from bench to bedside. *Journal of hepatology*. 2003; 38:38.
24. Mahato RI, Takemura S, Akamatsu K, Nishikawa M, Takakura Y, Hashida M. Physicochemical and disposition characteristics of antisense oligonucleotides complexed with glycosylated poly (-lysine). *Biochemical pharmacology*. 1997; 53:887–895. [PubMed: 9113108]
25. Rajur S, Roth C, Morgan J, Yarmush M. Covalent protein-oligonucleotide conjugates for efficient delivery of antisense molecules. *Bioconjugate chemistry*. 1997; 8:935–940. [PubMed: 9404669]
26. Cheng K, Ye Z, Guntaka RV, Mahato RI. Enhanced hepatic uptake and bioactivity of type alpha1(I) collagen gene promoter-specific triplex-forming oligonucleotides after conjugation with cholesterol. *J Pharmacol Exp Ther*. 2006; 317:797–805. [PubMed: 16452392]
27. Beljaars L, Molema G, Weert B, Bonnema H, Olinga P, Groothuis GM, Meijer DK, Poelstra K. Albumin modified with mannose 6-phosphate: A potential carrier for selective delivery of antifibrotic drugs to rat and human hepatic stellate cells. *Hepatology*. 1999; 29:1486–1493. [PubMed: 10216133]
28. Ye Z, Cheng K, Guntaka RV, Mahato RI. Targeted delivery of a triplex-forming oligonucleotide to hepatic stellate cells. *Biochemistry*. 2005; 44:4466–4476. [PubMed: 15766277]
29. Wang L, Kristensen J, Ruffner DE. Delivery of antisense oligonucleotides using HPMa polymer: synthesis of A thiol polymer and its conjugation to water-soluble molecules. *Bioconjugate chemistry*. 1998; 9:749–757. [PubMed: 9815169]
30. Yang N, Ye Z, Li F, Mahato RI. HPMa polymer-based site-specific delivery of oligonucleotides to hepatic stellate cells. *Bioconjug Chem*. 2009; 20:213–221. [PubMed: 19133717]
31. Ye Z, Cheng K, Guntaka RV, Mahato RI. Receptor-mediated hepatic uptake of M6P-BSA-conjugated triplex-forming oligonucleotides in rats. *Bioconjug Chem*. 2006; 17:823–830. [PubMed: 16704223]
32. Panakanti R, Pratap A, Yang N, Jackson JS, Mahato RI. Triplex forming oligonucleotides against type alpha1(I) collagen attenuates liver fibrosis induced by bile duct ligation. *Biochem Pharmacol*. 2010; 80:1718–1726. [PubMed: 20816672]
33. Bataller R, Brenner DA. Liver fibrosis. *J Clin Invest*. 2005; 115:209–218. [PubMed: 15690074]
34. Gressner AM, Weiskirchen R, Breitkopf K, Dooley S. Roles of TGF-beta in hepatic fibrosis. *Front Biosci*. 2002; 7:D793. [PubMed: 11897555]
35. Bauer M, Schuppan D. TGFbeta1 in liver fibrosis: time to change paradigms? *FEBS letters*. 2001; 502:1. [PubMed: 11478937]
36. Cheng K, Yang N, Mahato RI. TGF- 1 gene silencing for treating liver fibrosis. *Molecular pharmaceutics*. 2009; 6:772–779. [PubMed: 19388665]
37. Yang N, Mahato RI. GFAP Promoter-Driven RNA Interference on TGF-beta1 to Treat Liver Fibrosis. *Pharm Res*. 2011; 28:752–761. [PubMed: 21347569]

Figure 1. Triplex forming oligonucleotides

A. The blue and green bands refer to the double strands of DNA molecule. The red one is triplex forming oligonucleotides (TFOs). B. Principles of triplex formation. A third polynucleotide sequence can bind to double-stranded DNA at the major groove to form triplex structure via formation of Hoogsteen/reverse Hoogsteen hydrogen bonds. TFOs can only bind to polypurine strand of target DNA by either (G, A)-motif or (C, T)-motif.

Figure 2. Synthesis and biodistribution of M6P-HPMA-TFO. (A) Synthesis scheme and, (B) Intrahepatic distribution. Reproduced from Yang et al. (2009) *Bioconjugate Chem* 20:213–21.

Figure 3. TGF- $\beta 1$ gene silencing for treating liver fibrosis

(A) Effect of TFO. (B) Effect of siRNA sequence and (C) Effect of shRNA construct.

Reproduced from Cheng et al. (2009) Mol Pharm 6: 772–9 and Yang et al. (2011) Pharm Res 28: 752–61.