MicroRNA’s in chronic hepatitis B and C virus infection
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SUMMARY

The main focus of this thesis was to evaluate therapeutic miR-122 targeting in chronic hepatitis C patients and to improve our understanding of the role of miRNAs and viral factors in chronic hepatitis B infection.

I: Targeting microRNA-122 in chronic hepatitis C patients

miR-122 is an important host factor for HCV replication. In 2013, a phase 2 study was performed including 36 treatment naïve, HCV genotype 1 infected chronic hepatitis C patients. These patients were treated with 5 weekly doses of miravirsen, an antisense oligonucleotide targeting miR-122, or placebo over a 29-day period. Miravirsen dosing resulted in a substantial but transient decline in HCV RNA levels.

In chapter 2 we assessed whether plasma miRNA levels changed in patients dosed with miravirsen. Plasma levels of 179 miRNAs were determined by qPCR before, during and after treatment and were compared between patients dosed with miravirsen or placebo. At week 1, 4, 6 and 10/12, patients dosed with miravirsen had respectively a median 72-fold, 174-fold, 1109-fold and 552-fold lower expression of miR-122 than at baseline (p=0.001, as compared to patients receiving placebo). Plasma levels of other miRNAs were not significantly affected by antagonising miR-122.

Chapter 3 describes a retrospective follow-up study of the 36 chronic hepatitis C patients who participated in the previously described phase 2 study with miravirsen. Up to 35 months following therapy, no long-term safety problems were observed among the 27 chronic hepatitis C patients that were treated with miravirsen. None of the miravirsen treated patients developed HCC or cirrhosis related morbidity such as ascites or variceal bleeding. In addition, antiviral therapy with peg-IFN and ribavirin (standard of care at time of this study) following miravirsen resulted in SVR in 58% of patients.

Chapter 4 describes the safety, pharmacokinetics, and antiviral effect of a single dose of RG-101 in chronic hepatitis C patients. RG-101 is an oligonucleotide inhibitor of miR-122 that is linked to a N-acetylgalactosamine carbohydrate structure designed to enhance uptake of the oligonucleotide by hepatocytes. In this randomised, placebo-controlled, phase 1b study we included 32 chronic hepatitis C patients with HCV genotype 1, 3 or 4 infection. The first cohort received a single subcutaneous injection of 2 mg/kg RG-101 (n=14) or placebo (n=2); the second cohort received a single subcutaneous injection of 4 mg/kg (n=14) or placebo (n=2). A single dose of RG-101 was well tolerated and no serious adverse events were observed. Median viral load reductions at week 4 were 4.42 and 5.07 log 10 IU/ml for 2 and 4 mg/kg treatment respectively, and HCV RNA levels were undetectable in three patients at week 76 of follow-up after a single dose of RG-101. Viral rebound at or prior to week 12 was associated with the appearance of mutation(s) in miR-122 binding regions in 5’ UTR of the HCV genome.
In chapter 5 we analysed the effects of RG-101 on antiviral immunity in chronic hepatitis C patients. We showed that IP-10 levels, which are elevated in chronic hepatitis C patients as a consequence of continuous immune activation, decreased following RG-101 dosing. Furthermore, the frequency of natural killer (NK) cells increased, the proportion of NK cells expressing activating receptors normalized, and NK cell interferon-γ production decreased after RG-101 dosing. No restoration of HCV-specific T cell function was observed after viral load decline, nor after long term HCV RNA negativity.

Chapter 6 describes an investigator-initiated study wherein 29 chronic hepatitis C patients with HCV genotype 1, 3 or 4 were treated with sofosbuvir and daclatasvir with or without ribavirin for 12 to 24 weeks. Of these 29 patients, 18 patients had participated in the previously described phase 1b study and had received a single dose of RG-101. All patients, with or without previous RG-101 dosing, were successfully treated and achieved a sustained virological response. In chronic hepatitis C patients without previous RG-101 dosing miR-122 levels were elevated at baseline and normalized when sustained virological response was achieved. After successful treatment with direct-acting antivirals, we showed that broad immune activation reduced in chronic hepatitis C patients and that the magnitude and functionality of ex vivo HCV-specific T cell responses did not increase.

II: Role of microRNA's and viral factors in chronic hepatitis B virus infection
For the second part of this thesis we have used data of a study cohort of 92 chronic hepatitis B patients (44 HBeAg-positive and 48 HBeAg-negative) with HBV DNA levels >100,000 copies/mL (>20,000 IU/mL) who were previously treated with a combination of peg-IFN and adefovir for 48 weeks. Two years after treatment ended, HBsAg loss was observed in both HBeAg-positive (11%) and HBeAg-negative (17%) patients.

Chapter 7 describes the identification of plasma miRNAs associated with HBeAg status and response to antiviral therapy in this cohort of chronic hepatitis B patients. We showed that HBeAg-positive patients had higher plasma levels of miR-122, miR-125b, miR-192, miR-193b, and miR-194 as compared to HBeAg-negative patients, and that levels of these miRNAs were associated with HBV DNA and HBsAg levels. Higher pre-treatment levels of miR-301a and miR-145 were associated with a favourable response (combined response and/or HBsAg loss) to antiviral treatment. Some of our identified HBV associated miRNAs were present in exosomes and HBsAg particles secreted by hepatoma cell lines.

In chapter 8 we used 454 deep sequencing to study amino acid differences in HBV core protein. The HBV core protein self-assembles to form viral capsids, predominantly consisting of 120 homodimers, which packages the HBV genome. Sequence Harmony identified a number of amino acid changes associated with HBeAg status and response to antiviral treatment. Residues with amino acid differences between HBeAg positive and negative patients were located at the inner surface or intra-dimer interface of HBV core
protein, whereas residues with amino acid differences at the outer surface of HBV core protein were associated with treatment response.

Finally, in chapter 9 we describe the long-term outcome of the previously described 92 chronic hepatitis B patients treated with peg-IFN and adefovir. At year 5 of follow-up, 19% of HBeAg-positive and 16% of HBeAg-negative patients had HBsAg loss, and no HBsAg sero-reversion was observed. The majority of patients without HBsAg loss were retreated with NUCs at 5 years of follow-up.

GENERAL DISCUSSION
The place of targeting miR-122 in antiviral treatment of chronic hepatitis C virus infection
Antiviral treatment for chronic hepatitis C virus infection has drastically changed in the last 25 years. From subcutaneous administration of interferon-α multiple times per week for 6 to 12 months resulting in SVR rates of less than 10%, to current all-oral IFN-free DAA combination treatment for 8 to 24 weeks resulting in SVR in more than 90% of patients. Despite the emergence of these highly potent DAA regimens, HCV will remain a public health concern for many years. The identification of infected patients, global access to therapy, and delivering treatment regimens at lower costs are still major challenges.1,2 Moreover, some limitations of DAA therapy remain, including DAA failure and viral resistance.2 Most DAAAs rapidly select for resistant viruses, and therefore treatment regimens combining several DAAs are necessary. Because miR-122 inhibitors target conserved host proteins, not variable viral proteins, they have a pan-genotypic potential and high barrier to resistance.3–5 Although a single dose of RG-101 resulted in sustained viral load reduction for 76 weeks (1.5 years) in three chronic hepatitis C patients (chapter 4), the majority of patients had a virological rebound. Therefore, it is not expected that monotherapy will lead to viral cure in large numbers of patients.6,7 According to HCV guidelines, these three patients could be considered HCV cured (undetectable HCV RNA more than 12-24 weeks after cessation of therapy). However, these results should be interpreted with caution considering the long half-life of anti-miR-122 oligonucleotides and the observation of very late viral rebounds (up to 52 weeks) after RG-101 dosing (chapter 4). Future studies are needed to establish the optimal duration of follow-up before an SVR is reached in anti-miR-122 treated chronic hepatitis C patients. In the future, targeting miR-122 may be used complementary to DAAs and may be able to shorten treatment duration from 12 weeks to 4-6 weeks, which might be of interest in view of the high costs of these drugs. The addition of miR-122 antagonism to DAA treatment was shown to have additive or synergistic antiviral effects in preclinical studies, and helped to prevent the emergence of DAA-resistant mutants.8 However, anti-miR-122 therapy also has drawbacks such as subcutaneous administration in contrast to oral dosing of DAAs. The potential benefits
of targeting miR-122 should be weighed in the context of available treatment options. It is likely that miR-122 antisense oligonucleotides will not play a key role in future antiviral treatment of HCV.

**Mechanism of antiviral effect by targeting miR-122**

The exact role of miR-122 in HCV replication is still not fully understood. The most recent data support two mechanisms: protection against degradation by cellular exoribonucleases, and promoting viral RNA synthesis. Sequestration of miR-122 by an antisense oligonucleotide in chronic hepatitis C patients therefore may result in increased viral RNA degradation and/or less viral RNA synthesis. Hitherto, two proof-of-principle studies wherein chronic hepatitis C patients were treated with a miR-122 antisense oligonucleotide (miravirsen and RG-101) demonstrated a substantial and long-lasting antiviral effect. The crucial difference between miravirsen and RG-101 is an altered chemistry of RG-101 by conjugation to an N-acetylgalactosamine structure enhancing its hepatocyte uptake and potency. This altered chemistry might have rendered RG-101 more efficient: whereas miravirsen dosing resulted in viral load reductions of 2-3 log 10 IU/mL and in longest undetectable HCV RNA levels for 7 months in one patient (chapter 3), a single dose of RG-101 led to viral load reductions of 4-5 log 10 IU/mL and in undetectable HCV RNA levels in three patients up to 1.5 years after dosing (chapter 4). Preclinical studies demonstrated that both miravirsen and RG-101 have a tissue half-life of 2 to 4 weeks. Although the tissue half-life of these miR-122 antisense oligonucleotides was not assessed in humans, it might be substantial given the prolonged pharmacodynamic effects that were observed, including increased alkaline phosphatase levels and decreased cholesterol levels (chapter 4). Suppression of the biogenesis of miR-122 at the primary and precursor miRNA level by antagonising miR-122 may also contribute to the long-lasting effects of treatment. However, it is unknown if inhibiting miR-122 by an antisense oligonucleotide in chronic hepatitis C patients is capable to sequester all hepatic miR-122. In cell culture studies, it was observed that the HCV RNA was reduced by approximately 80% after dosing with a miR-122 antisense oligonucleotide. In chapter 4, we observed that some patients had long-lasting suppression of HCV RNA levels, but that HCV RNA levels did not become undetectable in these patients, a so-called “low-replication state”. We assumed that other factors such as immune responses may contribute to the antiviral effect of anti-miR-122 treatment. Although our data in chapter 5 strongly suggests that restoration of the HCV specific CD8+ T cell compartment does not occur after RG-101 dosing, restoration of the NK cell compartment does occur, a phenomenon that has been documented in several studies investigating the effects of IFN-free DAA therapies on innate and adaptive immunity. Our results do not identify whether the changes in the NK cell compartment are a direct result of miR-122 antisense oligonucleotide therapy or whether they comprise a bystander effect on account of viral load reduction. Therefore the involvement of NK cells in viral control remains uncertain.
Relevance of mutations in 5’UTR HCV RNA miR-122 binding sites

Viral resistance to DAA therapy may be of major importance in a small subgroup of chronic hepatitis C patients. Since the underlying antiviral mechanism of miR-122 inhibition is completely different than that of DAAs, patients with DAA viral resistance may potentially benefit from this treatment. \(^2^1\) Although no mutations were found in the S1 and S2 miR-122 binding sites, a change of cytosine to uridine at position 3 (C3U) with or without a change of cytosine to guanine at position 2 (C2G) of the HCV RNA was present in chronic hepatitis C patients with viral rebound following anti-miR-122 dosing (chapter 4). \(^2^1\) The viral fitness of the C3U mutation has been tested before in vitro. \(^2^0\) It was shown that an HCV variant with C3U mutation replicated less well than a virus without a mutation (wild-type) in the presence of miR-122, and that it was fully susceptible to all DAAs tested. \(^2^1\) In chapter 6 we have shown that the C3U and C2G+C3U mutations spontaneously disappear within several weeks after their identification in chronic hepatitis C patients, suggesting that the mutated viruses were competed out by the fitter wild-type variant. Furthermore, we have shown that DAA therapy is highly effective in chronic hepatitis C patients who received previous anti-miR-122 treatment (chapter 6). Therefore, we expect that the relevance of the mutations in 5’UTR miR-122 binding sites will be limited in future clinical practice. More interesting is the question why these mutations appear and if they have a replication benefit compared to wild-type virus in an environment lacking miR-122. Cell culture studies showed low level HCV replication in cells with undetectable miR-122 levels, \(^2^2\) and have identified several mutations that result in reduced reliance of HCV on miR-122. \(^2^3,^2^4\) The in vivo identified mutations in the 5’ UTR miR-122 binding sites of the HCV genome in chapter 4 may enable successful HCV replication in absence of miR-122. One could speculate that mutations in the first nucleotides of the HCV RNA could protect the viral RNA from degradation, alter recruitment of host cell RNA-binding proteins, or affect RNA folding resulting in a more stable structure. However, functional studies are needed to elucidate how these mutations confer miR-122 independence.

Long-term safety of miR-122 inhibition

miR-122 modulates the expression of a large number of proteins within the hepatocyte, some of which have been implicated in hepatocarcinogenesis. Several animal studies demonstrated that short-term inhibition of miR-122 using antisense oligonucleotides was well tolerated without the evidence for liver histopathological changes. \(^2^5–^2^7\) However, knockout and liver-specific knockout of mouse miR-122 resulted in hepatic steatosis, hepatitis, and the development of tumours resembling hepatocellular carcinoma (HCC). \(^2^8,^2^9\) Therefore, the safety of transient miR-122 antagonism in chronic hepatitis C patients should be well studied. The follow-up data up to 35 months following inhibition of miR-122 by an antisense oligonucleotide described in chapter 3 showed no safety issues, however this data was retrospectively collected and therefore may have missed important safety problems. \(^1^6\) A prospective long-term follow-up study including adverse
event reporting, laboratory testing, and abdominal ultrasounds should be performed to determine long-term safety of antagonising miR-122 in chronic hepatitis C patients. To better valuate the risk of transient miR-122 inhibition, we assessed the duration of the decrease in plasma miR-122 levels after anti-miR-122 dosing in chronic hepatitis C patients. The increasing trend in plasma miR-122 levels three months following miravirsen dosing (chapter 2) suggest that miR-122 levels return to normal values within several months after anti-miR-122 dosing. This was confirmed by normalised plasma miR-122 levels six months after dosing with RG-101, to levels comparable to levels of healthy controls and successfully treated chronic hepatitis C patients (chapter 6). From a safety perspective, it is favourable that antisense inhibition of miR-122 did not affect plasma levels of other miRNAs (chapter 2), suggesting a specific inhibitory effect for miR-122. Recently, it was shown that not only the “guide strand” of miR-122 (miR-122-5p), but also the “passenger-strand” namely miR-122* (miR-122-3p) is expressed in liver cells and may act as a tumour suppressor. It is unknown whether miR-122 antagonism results in miR-122* accumulation or suppression. Also, it was postulated that HCV recruits large fractions of miR-122 in HCV-infected hepatocytes (Figure 1). This results in reduced availability of miR-122 to bind and repress its cellular mRNA targets, the so-called “sponge effect”. Thereby, HCV itself alters the host’s gene expression in a way that may promote liver fibrosis and carcinogenesis. The question remains whether this de-repression of miR-122 targets by HCV in vitro is also relevant in vivo.

Will miRNAs solve the mystery of HBV infection?

Attempts to improve treatment response by combining peg-IFN with NUCs have not resulted in high functional cure rates (defined as HBsAg loss with or without the formation of anti-HBs antibodies) in chronic hepatitis B patients. A functional cure was achieved in less than 20% of chronic hepatitis B patients five years after treatment with peg-IFN and NUC combination, and the majority of patients had to be retreated with NUC therapy (chapter 9). Therefore, there is a need to develop novel antiviral agents that directly interfere with HBV replication and/or modulate the immune system. To achieve true cure of HBV, it would be needed to eliminate cccDNA, or prevent the transcriptional activity of cccDNA, and to provoke an adequate host immune response. Various compounds with different antiviral mechanisms are under development such as entry inhibitors, siRNAs targeting viral transcripts, capsid assembly inhibitors, and TLR-7 agonists (Figure 2). One of the most promising antiviral targets is the HBV core protein because of its important role in capsid assembly and involvement in almost all other steps of the HBV replication cycle. Mutations in HBV core protein may affect the secretion of virions, reverse transcription, or change important immunogenic regions of the protein. The naturally occurring HBV core protein mutations associated with HBeAg status and treatment response identified in chapter 8 may help to gain better insight in the properties of HBV core protein and
thereby contribute to the development of potent capsid assembly inhibitors. Could there be a role for miRNAs as novel antiviral targets in HBV treatment? Although numerous cellular miRNAs that (directly or indirectly) interact with HBV replication have been identified, it is likely that miRNAs do not play an equally crucial role in HBV replication as they do in HCV replication. In chapter 7 we have identified several miRNAs, which are actively packaged into exosomes and HBsAg particles, and are associated with HBeAg status, HBV DNA levels and HBsAg levels. This suggests that these miRNAs could play a role in viral replication. To determine the precise role of miRNAs in HBV replication and to assess their potential as a therapeutic target, the effect of miRNA overexpression as well as downregulation on different steps in the HBV replication cycle should be elucidated. Future studies should also focus on the identification of miRNAs, and other host or viral...
factors, involved in the epigenetic regulation of cccDNA which could lead to the aspired eradication of the cccDNA pool in hepatocytes.

Future therapeutic applications of anti-miRNAs

Although it is expected that antisense oligonucleotides will not play a major role in the future of antiviral treatment of HCV, anti-miRNAs have the potential to play a role in the treatment of other viral infections. The outcome of the virus-miRNA interplay can have either a positive (proviral) or negative (antiviral) effect on the virus. Therefore, two approaches could be used to modulate miRNA activity: (i) inhibiting the function of a miRNA, and (ii) restoring function of a miRNA.\textsuperscript{35–37} In addition to viral infections, miRNAs are involved in other human diseases such as cancer, cardiac disease, and metabolic disease. One of the key factors and challenges in successful miRNA-based treatment is the delivery of miRNA modulators to the cell type or tissue of interest.\textsuperscript{35–37} This can be achieved by association with nanoparticles or liposomes, or chemical modification of an oligonucleotide, such as the N-acetylgalactosamine carbohydrate conjugated oligonucleotide for hepatocyte-targeted delivery as shown in chapter 4.\textsuperscript{37} Although challenges in miRNA targeted therapy remain, such as the limitation of off-target effects and the long-term safety of miRNA modulation in humans, miRNA-based therapeutics have the potential to become an important new drug class in future medicine.
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