REVIEW

The Need for New Anti-Hepatitis C Virus Therapeutic Strategies
Targeting the cellular micro-ribonucleic acids?

Elias A Said

الحاجة لاستراتيجيات علاج جديدة لمرض التهاب الكبد – ج
استهداف الحمض النووي الريبي الخلوي الدقيق

إلياس سعيد

الملخص: تعتبر الإصابة بفيروس التهاب الكبد – ج مشكلة عالمية. يتزايد عدد المصابين بالشكل المزمن لهذا الوباء والذين لا يستجيبون للعلاج المعياري. هناك حاجة ملحة لعلاج جديد أكثر فاعلية و تحملاً. هذه المراجعة تناقش النتائج الجديدة التي تظهر أن استهداف الحمض النووي الريبي الخلوي الدقيق، الذي يزيد تكاثر فيروس التهاب الكبد – ج، هو علاجٌ جديدٌ يشكل حاجزاً أمام قدرة الفيروس على المقاومة.

مفتاح الكلمات: فيروس التهاب الكبد ج، الحمض الريبي النووي الدقيق، إنترفيرون، الكبد، العدوى، العلاج.

ABSTRACT: Infection with the hepatitis C virus (HCV) is a worldwide problem. Patients with chronic HCV infection who are non-responders to standard therapy represent a growing population within the HCV epidemic. Novel, more efficient and tolerable therapies are urgently needed. This review discusses the recent results showing that targeting miR-122, a micro-ribonucleic acid (MicroRNA) that enhances HCV replication, is a new anti-HCV therapy with a high barrier to resistance.

Keywords: Hepatitis C virus; MicroRNAs; Interferon; Liver; Infection; Therapy.

T**he hepatitis C virus (HCV) is a** worldwide public health problem affecting an estimated 200 million individuals. There are approximately 3–4 million new cases of HCV infection each year. HCV is a serious infectious problem in the Middle East with Egypt having the highest prevalence of HCV infection and Oman being in the intermediate category.[1,2]

HCV is a flavivirus comprising 6 genotypes with numerous subtypes. Genotype 1 is the most prevalent in Europe and North America and the most difficult to treat. Genotypes 2 and 3 appear to be more prevalent in the Far East. Of the other genotypes, genotype 4 is common in Africa and the Middle East, whereas genotypes 5 and 6 are predominant in South Africa and South-East Asia, respectively.[3-5]

Only 10% - 40% of acute HCV-infected individuals can be cured by spontaneous viral clearance and 60% - 90% of infected individuals develop chronic infection.[6] There is a very slight chance of clearing the virus spontaneously in chronic HCV carriers (0.5% - 0.74%); however, the majority of patients with chronic hepatitis C infection will not clear the infection without treatment. Progression of liver diseases usually takes decades and up to 20% of those infected may develop complications including cirrhosis, liver failure, or hepatocellular carcinoma.[7] End-stage liver disease, due to infection with HCV, currently represents the major indication for liver transplantation, and the virus universally recurs after transplantation.

Acute HCV infection is characterised by a delay of 6–8 weeks in the induction of adaptive immunity. This is despite active viral replication; this therefore suggests the failure of innate immunity to contain viral replication and the presence of a blockade in the cross-talk between innate and adaptive

Department of Microbiology & Immunology, Faculty of Medicine, Montreal University; Montreal, Quebec, Canada; and Montreal University Hospital Research Center (CR-CHUM), St-Luc Hospital, Montreal, Quebec, Canada.

Email: elias_a_said@yahoo.fr

immunity. CD8+ (cluster of differentiation 8) and CD4+ (cluster of differentiation 4) HCV-specific T-cell responses are essential to prevent viral persistence[8,9] and it has been demonstrated that infections that progress to chronicity are characterised by a limited immune response either in frequency, magnitude or the number of effector functions that can be detected.[10] Unfortunately, the mechanisms underlying the inefficient priming of HCV-specific T-cells in infections with chronic evolution are poorly understood.

In this review we will discuss how targeting MicroRNA-122 (miR-122), which enhances HCV replication with specific oligonucleotidic sequences, can be a new anti-HCV therapy with a high barrier to resistance.

The Efficacy of Current Anti-HCV Treatments

There is currently no effective anti-HCV vaccine available. The use of interferon-α (IFN-α) for treatment was based on the fact that this cytokine is an important element of the innate antiviral immune response; however, success with the IFN-based therapy was modest. Treatment with IFN-α induced a quick decrease of HCV ribonucleic acid (RNA) levels in the serum, and the long-term responses were characterised by a sustained absence of HCV RNA from the serum and liver, and resolution of the chronic infection. The use of ribavirin and pegylated (peg-) IFN-α, where IFN-α was linked to polyethylene glycol (peg) in order to increase the half-life of IFN, significantly increased the proportion of patients achieving sustained antiviral response (SVR).[6,7] Long-term studies have shown that SVR indicates clearance of the virus and cure of the disease. Unfortunately, although anti-HCV therapy combining peg-IFN-α with ribavirin has significantly improved, it leads to sustained clearance in only about 50% of patients and the treatment is frequently associated with severe side effects.[6,7] Multiple factors can modulate the response to therapy, i.e. viral genotype and patient characteristics. Rates of SVR range from 42% - 46% in patients with genotype 1. In contrast, SVR rates for patients with genotypes 2 and 3 are 76 - 80% and the clearance can be reached with a shorter course of therapy and lower doses of ribavirin. The host determinants of therapy are still not well known.

Certain patient populations hardly respond to treatment. A transient response with relapse occurs in 10 - 25% of patients. Non-response to treatment occurs in about one-third of chronic HCV-infected patients. These patients never reach clearance, although viral loads might fall at some stage in treatment.[6,7] However, treatment in the acute phase of the infection is accompanied by a high rate of response where IFN-α therapy reduces the chronicity rate to 10% or lower. Nonetheless, non-responders among the HCV-infected represent a growing population within the HCV epidemic. The majority of these patients is frequently labelled as "difficult to treat". They are infected with genotype 1 and have a high viral load and advanced fibrosis or cirrhosis. The best therapy available has been unsuccessful in inducing a virologic response in these patients. Clarification of the reasons of non-response to IFN-α therapy is a main challenge to research on HCV.

The mechanism of how IFN-α inhibits HCV replication remains poorly understood. It has been reported that IFN-α has potent antiviral activity, but does not act directly on the virus or the replication complex. Instead, it acts by inducing IFN-stimulated genes (ISGs), which establish a non-virus-specific antiviral state within the cell.[7] Recently, microarray analyses were performed on liver biopsies taken before therapy from a cohort of HCV-infected patients treated with peg-IFN-α and ribavirin. The results showed that patients who were subsequently identified as non-responders had higher baseline expression of ISGs than responders to therapy (and those who relapsed); the latter resembled healthy controls more closely. Interestingly, peg-IFN-α did not induce the expression of ISGs to levels higher than those observed at the pre-treatment stage in the non-responders.[7] These findings suggest that non-responders have an up-regulated and largely ineffective IFN response. Coincidentally, chimpanzees with chronic HCV infection have high expression levels of ISGs; however, neither type-1 nor type-2 IFNs are induced during the infection.[11,12] Although IFN-α could induce ISGs expression in healthy chimpanzees, those chronically infected do not respond to IFN treatment and thus appear to be an extreme representative of non-responders in human HCV-infected patients. This makes chimpanzees an ideal model for the study of non-responsiveness to IFN-α therapy and

for the development of novel antiviral agents.

Overall, although IFN therapy has considerably improved, 50% of the infected individuals with chronic disease do not achieve sustained clearance of HCV. Recent studies suggest that the duration of the treatment with peg-IFN plus ribavirin therapy must be determined in each patient according to the on-treatment virological response, i.e. the viral load at 4 weeks and the genotype.[13] Currently, there are no approved treatment options available for patients who have failed to respond to previous treatments. Novel, more efficient and tolerable therapies are urgently needed. A greater understanding of the viral life cycle has led to an increase in the number of possible targets for antiviral intervention. It has resulted in the development of several agents that target specific stages of the life cycle. Potential processes for viral inhibition include virus entry into the host cell, proteolytic processing, RNA replication and the assembly and release of the new viral particles. Those agents include protease inhibitors, i.e., BILN 2061 (the trials were stopped due to severe side effects in the animal model); proceprevi SCH 503034 and telaprevir (both tested in phase II); polymerase inhibitors (i.e., valopicitabine, BILB 1941– the trials were stopped due to gastrointestinal-related side effects); immune modulators (i.e., ANA 975 for TLR7) and host factor inhibitors (i.e., NIM-811 for cyclophilin B). A very recent study showed the potential clinical anti-HCV effect of BMS-790052, an inhibitor of NS5A, which is an HCV protein with no known enzymatic function.[14] A list of other trials, including two other forms of IFN (albuferon [albumin IFN]) and locteron (a newly developed controlled-release formulation of lemna-derived free (unpegylated) recombinant interferon-α2b), can be found on the website of the HIV and Hepatitis Treatment Advocates, Inc.[15] However, the generation and selection of resistant variants can allow the virus to escape the antiviral pressure exerted by treatment. Indeed, mutations in both the polymerase and protease enzymes have already been identified.[16] The HIV infection model suggests that the combination of different anti-viral therapies might decrease HCV chances to develop escape mutations; however recent studies on HIV-infected patients demonstrated the emergence of drug resistance during receipt of combined therapy and described a relatively common virological failure by 8 years of combination antiretroviral therapy (cART).[17-19] Therapies that target essential host functions for HCV may provide a high barrier to resistance and, thus, could present an effective approach for the development of new HCV antiviral drugs.

Sequestrating Cellular MicroRNAs is a Promising Strategy

New hope can be given by targeting cellular microRNAs that are implicated in the HCV life cycle. MicroRNAs are a class of small non-coding RNA molecules that function through post-transcriptional regulation of gene expression. Those small RNAs are important in development and disease.[20] Therefore, they represent a potential new class of targets for therapeutic intervention. MicroRNA 122 (MiR-122) is specifically expressed in the liver, where it constitutes 70% of the total microRNA population.[21] Three miR-122 binding sites in the HCV genome have been reported: one is located in 3' non-coding region (NCR) of the HCV genome and its function remains unknown; the other two binding sites are closely located in the 5' NCR and separated by a highly conserved 14-nucleotides sequence.[21] Those sequences are highly conserved among the six HCV genotypes. MiR-122 binds to sequences in the 5' NCR of HCV RNA, i.e. S1 and S2, resulting in the up-regulation of viral RNA levels [Figure 1].[22] Interaction of miR-122 with the HCV genome is essential for the accumulation of viral RNA in the cultured liver cells and all target sites are required for modulation of HCV RNA abundance. Although treatment with IFN has been shown to decrease miR-122 levels, no correlation between miR-122 levels in the liver and the viral load was observed upon treatment with IFN.[23,24] However, lower levels of miR-122 were observed in non-responders.[24] Of note, the CD56+ T-cells were shown to inhibit HCV replication by decreasing miR-122 expression. Whether this effect is due to the up-regulation of type-I IFN expression by hepatocytes remains to be clarified.[25]

RNA oligonucleotides with exact complementarity to miR-122 have been shown to sequester miR-122 leading to significant decrease in HCV RNA accumulation [Figure 1]. This observation underscored miR-122 as a potential therapeutic target for the treatment of
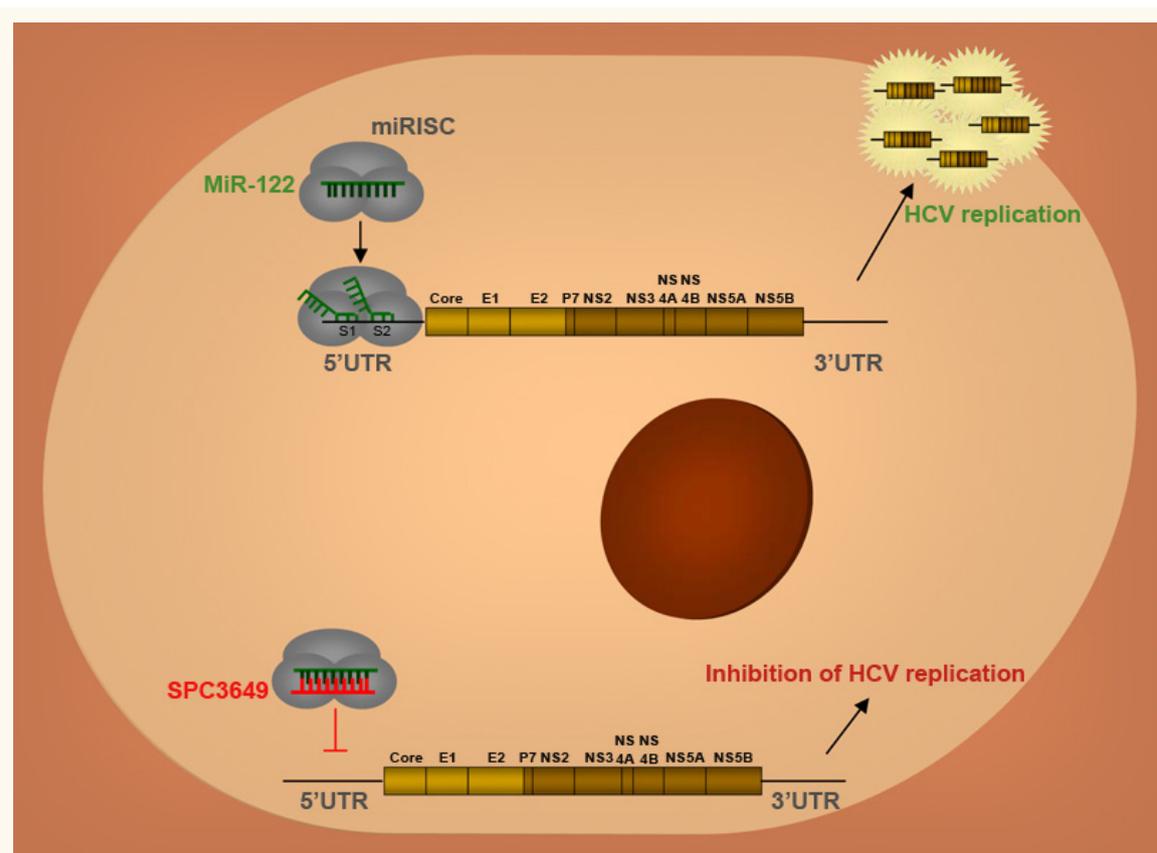
Figure 1: The binding of MiR-122 to hepatitis C virus (HCV) genome and its inhibition by SPC3649. The attachment of miR-122 to target sites, S1 and S2, in the 5'UTR of HCV ribonucleic acid (RNA) induces an increase in the amount of viral RNA and thus viral replication. The locked nucleic acid (LNA)-anti-miR-122, SPC3649, binds to MiR-122 and prevents its interaction with HCV RNA, which inhibits the replication of HCV.

HCV infection. Locked nucleic acid (LNA) technology[20] has been introduced into the development of an efficient approach for miR-122 targeting *in vivo* and high-affinity LNA-modified DNA oligonucleotides (LNA anti-miRs) complementary to miR-122 were developed.[26] LNA-modified nucleosides are a class of nucleic acid analogues in which the ribose ring is "locked" by a methylene bridge connecting the 2'-O atom and the 4'-C atom. By "locking" the molecule with the methylene bridge, the LNA-modified nucleosides are constrained in the ideal conformation for Watson-Crick binding.[20] When incorporated into a DNA oligonucleotide, LNA therefore enhances the speed of the pairing with a complementary nucleotide strand and increases the stability of the resulting duplex. Phosphorothioate modifications have been shown to provide good pharmacokinetic and tissue uptake properties in antisense oligonucleotides along with protection against nucleases. Moreover, it has been reported that specific miR-122 silencing *in vivo* using a

LNA-modified phosphorothioate oligonucleotide (SPC3649) complementary to the 5'-end of miR-122 leads to long-lasting decrease of serum cholesterol in mice and African green monkeys, which make it possible to inhibit the miR-122 function *in vivo*. Thus, it was tempting to investigate the potential of miR-122 antagonism as a new anti-HCV therapy.

The potential role of SPC3649 as a new anti-HCV treatment was tested in chronically HCV-infected chimpanzees.[26] Treatment with high doses of SPC3649 induced a decrease of 2.3 $\log^{10}$ in HCV RNA levels in the liver of the animals. These results showed that MiR-122 is essential for accumulation of HCV RNA *in vivo*. Moreover, the absence of viral resistance to therapy during treatment with SPC3649 has been demonstrated. This lack of viral resistance to SPC3649 is in striking contrast to what has been observed with direct acting antiviral drugs in HCV-infected chimpanzees. Within two days of treatment with a non-nucleoside polymerase inhibitor, 67% of the HCV clones already possessed known resistance mutations, with 10% of the clones

having two resistance mutations, which triggered a rapid rebound in viraemia.[26] The reduction in viraemia upon treatment with SPC3649 was clearly associated with a down-regulation of most IFN-regulated genes (IRGs) in chimpanzees. This correlated with serum levels of the chemokine IP-10 (CXCL10), a highly induced IRG during HCV infections.[26] These data show that the endogenous IFN pathway in the liver is rapidly normalised in response to inhibition of HCV RNA accumulation even when therapy does not completely eradicate detectable viral RNA. Thus, the treatment with SPC3649 results in the normalisation of IRG levels suggesting that this therapy could be used to convert the IFN non-responders, described above, to responders by reducing the viral load. To assess the safety of the treatment with SPC3649, complete blood counts, blood chemistries, coagulation markers, urinalysis, and complement activation were determined throughout the study, as were lymphocyte subsets, circulating cytokine-chemokine profiles and additional safety parameters. No SPC3649-related abnormalities were observed for any of the measurements.[26] Alanine aminotransferase (ALT) was reduced to normal levels, likely due to reduction in the viral load, and was again elevated at the end of the follow-up period when viraemia returned to baseline. Histological examinations of the baseline liver biopsies revealed HCV-specific changes including mild hepatocellular swelling with disruption of hepatocellular sinuses and cords. Improved liver histology was observed after treatment at week 19, indicating a response to prolonged suppression of viraemia and normalisation of the IFN pathway.[26]

Indeed, none of the treated chimpanzees achieved SVR, thus a longer course, higher dose and optimisation of the delivery methods should be considered. However, these results show the feasibility and safety of the prolonged administration of an LNA oligonucleotide drug that antagonises the function of a specific microRNA in a highly relevant disease model and pave the way for new anti-HCV treatments that are greatly needed. Although targeting miR-122 had no major side effects, modulating other microRNAs might have non-desirable impacts as they might inhibit or enhance one or more cell functions rendering the therapeutic molecule toxic. Other molecules targeting miR-122 are currently being explored and could decrease HCV replication.[27] Of note, two other microRNAs, miR199a and miR-196, were identified as possible targets for anti-HCV therapy as, in contrast to miR-122, the overexpression of these two molecules leads to the inhibition of HCV replication in the replicon system *in vitro*.[28,29] However, the effect of these molecules should be validated on real HCV infection *in vitro* prior to testing them in the animal model and then in clinical trials.

Conclusion

Many therapeutic strategies against HCV infection are available, such as the combination of peg-IFN and ribavirin. However, the failure of these therapies, because of viral escape or the non-response of many patients to these therapies, makes the use of LNA-modified phosphorothioate oligonucleotide (SPC3649), which blocks miR-122, a very potent anti-HCV treatment with no side effects or escape mutations.

References

1. Hoofnagle JH. Course and outcome of hepatitis C. Hepatology 2002; 36:S21–9.

2. Hepatitis C. Geneva: World Health Organization, 2002. pp. 1–69 (WHO/CDS/CSR/LYO/2003) From: http://www.who.int/csr/disease/hepatitis/Hepc.pdf. Accessed: March 2010.

3. Gish RG, Lau JYN. Hepatitis C virus: eight years old. Viral Hep Rev 1997; 3:17–37.

4. Maertens G, Stuyver L. Genotypes and genetic variation of Hepatitis C virus. In: Harrison TJ, Zuckerman AJ, Eds. The Molecular Medicine of Viral Hepatitis. Hoboken, NJ: Wiley, 1997. pp. 183–233.

5. McOmish F, Yap PL, Dow BC, Follett EA, Seed C, Keller AJ, et al. Geographical distribution of hepatitis C virus genotypes in blood donors: an international collaborative survey. J Clin Microbiol 1994; 32:884–92.

6. Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, et al. Peg-interferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. Lancet 2001; 358:958–65.

7. Sarasin-Filipowicz M, Oakeley EJ, Duong FH, Christen V, Terracciano L, Filipowicz W, et al. Interferon signaling and treatment outcome in chronic hepatitis C. Proc Natl Acad Sci USA 2008; 105:7034–9.

8. Dustin LB, Rice CM. Flying under the radar: the

immunobiology of hepatitis C. Annu Rev Immunol 2007; 25:71–99.

9. Shoukry NH, Cawthon AG, Walker CM. Cell-mediated immunity and the outcome of hepatitis C virus infection. Annu Rev Microbiol 2004; 58:391–424 .

10. Gruener NH, Lechner F, Jung MC, Diepolder H, Gerlach T, Lauer G, et al. Sustained dysfunction of antiviral CD8+ T lymphocytes after infection with hepatitis C virus. J Virol 2001; 75:5550–8.

11. Bigger CB, Guerra B, Brasky KM, Hubbard G, Beard MR, Luxon BA, et al. Intrahepatic gene expression during chronic hepatitis C virus infection in chimpanzees. J Virol 2004; 78:13779–92.

12. Lanford RE, Guerra B, Bigger CB, Lee H, Chavez D, Brasky KM. Lack of response to exogenous interferon-alpha in the liver of chimpanzees chronically infected with hepatitis C virus. Hepatology 2007; 46:999–1008.

13. Zeuzem S, Poordad F. Pegylated-interferon plus ribavirin therapy in the treatment of CHC: individualization of treatment duration according to on-treatment virologic response. Curr Med Res Opin 2010; 26:1733–43.

14. Gao M, Nettles RE, Belema M, Snyder LB, Nguyen VN, Fridell RA, et al. Chemical genetics strategy identifies an HCV NS5A inhibitor with a potent clinical effect. Nature 2010; 465:96–100.

15. HCV Experimental Treatments HIV and Hepatitis Treatment Advocates, Inc. From: http://www.hivandhepatitis.com/hep_c/hepc_news_alter.html. Accessed: Sept 2010.

16. Kieffer TL, Kwong AD, Picchio GR. Viral resistance to specifically targeted antiviral therapies for hepatitis C (STAT-Cs). J Antimicrob Chemother 2010; 65:202–12.

17. UK Collaborative Group on HIV Drug Resistance. Long-term probability of detecting drug-resistant HIV in treatment-naive patients initiating combination antiretroviral therapy. Clin Infect Dis 2010; 50:1286–7.

18. Gupta R, Hill A, Sawyer AW, Pillay D. Emergence of drug resistance in HIV type 1-infected patients after receipt of first-line highly active antiretroviral therapy: a systematic review of clinical trials. Clin Infect Dis 2008; 47:712–22.

19. Lihana RW, Khamadi SA, Lubano K, Lwembe R, Kiptoo MK, Lagat N, et al. HIV type 1 subtype diversity and drug resistance among HIV type 1-infected Kenyan patients initiating antiretroviral therapy. AIDS Res Hum Retroviruses 2009; 25:1211–7.

20. Fluiter K, Mook OR, Baas F. The therapeutic potential of LNA-modified siRNAs: reduction of off-target effects by chemical modification of the siRNA sequence. Methods Mol Biol 2009; 487:189–03.

21. Niepmann M. Activation of hepatitis C virus translation by a liver-specific microRNA. Cell Cycle 2009; 8:1473–7.

22. Jangra RK, Yi M, Lemon SM. Regulation of hepatitis C virus translation and infectious virus production by the microRNA miR-122. J Virol 2010; 84:6615–25.

23. Pedersen IM, Cheng G, Wieland S, Volinia S, Croce CM, Chisari FV, et al. Interferon modulation of cellular microRNAs as an antiviral mechanism. Nature 2007; 449; 919–22.

24. Sarasin-Filipowicz M, Krol J, Markiewicz I, Heim MH, Filipowicz W. Decreased levels of microRNA miR-122 in individuals with hepatitis C responding poorly to interferon therapy. Nat Med 2009; 15:31–3.

25. Ye L, Wang X, Wang S, Wang Y, Song L, Hou W, et al. CD56+ T cells inhibit hepatitis C virus replication in human hepatocytes. Hepatology 2009; 49:753–62.

26. Lanford RE, Hildebrandt-Eriksen ES, Petri A, Persson R, Lindow M, Munk ME, et al. Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. Science 2010; 327:198–201.

27. Young DD, Connelly CM, Grohmann C, Deiters A. Small molecule modifiers of microRNA miR-122 function for the treatment of hepatitis C virus infection and hepatocellular carcinoma. J Am Chem Soc 2010; 132:7976–81.

28. Hou W, Tian Q, Zheng J, Bonkovsky HL. MicroRNA-196 represses Bach1 protein and hepatitis C virus gene expression in human hepatoma cells expressing hepatitis C viral proteins. Hepatology 2010; 51:1494–504.

29. Murakami Y, Aly HH, Tajima A, Inoue I, Shimotohno K. Regulation of the hepatitis C virus genome replication by miR-199a. J Hepatol 2009; 50:453–60.