

# Design, delivery and efficacy testing of therapeutic nucleic acids used to inhibit hepatitis C virus gene expression *in vitro* and *in vivo*

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Despite major achievements in the treatment of chronic hepatitis C with the combination of interferons and the nucleoside analog ribavirin<sup>[1]</sup> the majority of patients with chronic hepatitis C virus (HCV) infection cannot be treated effectively. To improve this response rate we used antisense technologies to inhibit HCV translation as possible additional option for experimental treatment. Antisense oligodeoxynucleotides (ODN) are short nucleic acid oligomers, which bind complementary to the single-stranded HCV-RNA with plus (+) strand polarity and can induce hybrid arrest at the ribosome as well as cellular RNase H activity, which cleaves the RNA template in DNA-RNA hybrids. The 5'-non-coding region (NCR) is highly conserved among the 6 main genotypes and exerts the function of an internal ribosomal entry site (IRES), which is important for translation<sup>[2]</sup> and possibly also for viral replication (Figure 1).

Various antisense oligodeoxynucleotides varying from 8 to 23-mers and covering stem-loop structures within the 5'-NCR and the adjacent core gene were synthesized<sup>[3]</sup>. ODN<sub>4</sub> comprises HCV 5'-NCR nucleotides (nts.) 326-348 including the start AUG of the polyprotein coding sequence. Since easy-to-handle and generally a available infection or replication systems for HCV are lacking. We used model systems (Figure 2), in which the HCV 5'-NCR and 66 nts. Of the core gene were fused to the firefly luciferase gene for *in vitro* transcription/

translation from the bacteriophage T7 promoter. A similar construct with the cytomegalovirus(CMV) immediate early promoter was constructed for transfection experiments of hepatoma and non-liver cell lines<sup>[4]</sup>.

As compared to mismatch or sense controls completely phosphorothioate-modified ODN 4 showed the best inhibitory effect of luciferase activity in the rabbit reticulocyte lysate, which ranged in the order 96% ± 1% at about 4μM concentration. The effectiveness of ODN 4 is possibly due to its interference with a pseudonot structure (Figure 1) in HCV RNA which is disrupted, when ODN4 binds to the target RNA. In HepG2 tissue cultures comparable specific effects were obtained. However, fluorescence labeling of this ODN demonstrated that only a small percentage of transfected cells did take up the therapeutic ODN.

In order to improve the capability of membrane permeation by variation of their lipophilicity chemically modified ODN such as methylphosphonate and benzylphosphonate ODN 4 were compared with phosphorothioate ODN 4, which had been used in completely modified form in the previously described experiments. For the following experimental setting partially modified ODN4, which carried 6 modifications over a length of 23 nts. either in terminal localization or scattered along the molecule (Table 1). Toxicological testing revealed acceptable IC<sub>50</sub> concentrations between 0.2 and 5.8μM and the best therapeutic indices of 3.8 for terminally modified benzylphosphonate (tB-) ODN 4 and terminally modified phosphorothioates (tS-) ODN4. While both scattered (s) and terminal (t) modifications inhibited HCV gene expression in case of phosphorothioate (S)-ODN 4, only terminally modified methylphosphonate (tM)-ODN 4 and benzylphosphonate (tB)-ODN 4 showed specific and effective inhibition. This is possibly correlated to an induction of RNaseH activity and the steric hindrance of this enzyme by differently modified molecules<sup>[5,6]</sup>. In cell culture, tB-ODN 4 showed the best inhibition of about 96%, leaving, however, 4% of reporter gene expression unaffected.

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**Table 1** Characterization of partially modified antisense oligodeoxynucleotides

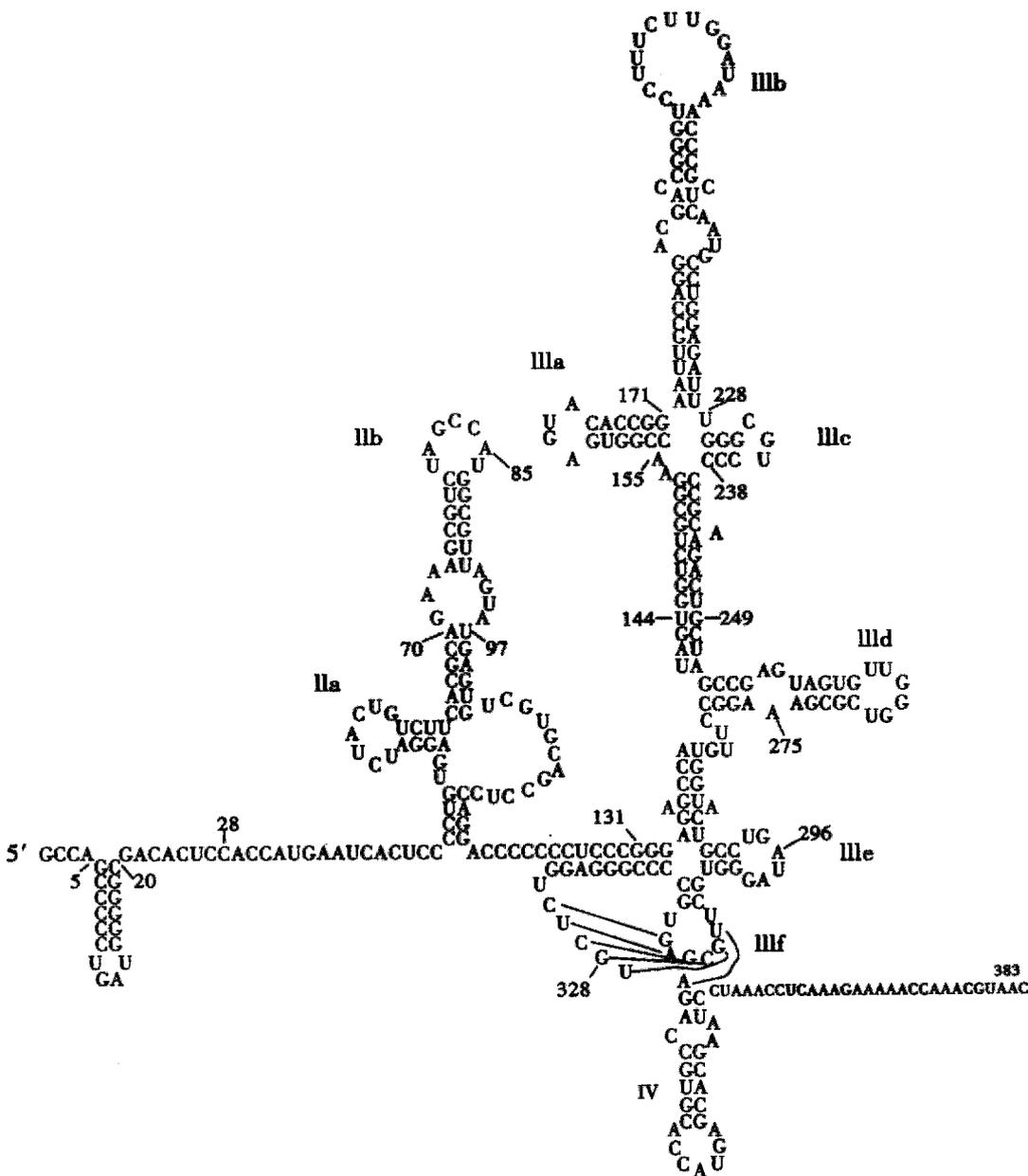
5'-TGC TCA TGG TGC ACG GTC TACA-3'	4
5'-TGC TGA TGC TGC ACG CTC TAG GA-3'	K
5'-TCG TAG ACC GTG CAC AT GAG CA-3'	S
5'-TGC TCA TGG TGC ACG GTC TAC GA-3'	4
5'-TGC TGA TGC TGC ACG CTC TAG GA-3'	K
5'-TCG TAG ACC GTG CAC CAT GAG CA-3'	S

Site of modification in bold font.

4: ODN 4, K: mismatch control, S: sense control ODN.

Therefore, katalytically active antisense RNAs, so-called hammerhead ribozymes (RZ), which cleave at NUH recognition sites (N: any nt., U: uridine, H: any nt. except guanosine) of a RNA template, were synthesized. Taking these recognition motifs into account, one of the RZ was directed towards similar stem-loop structures as the previously tested ODN. After purification of the RZ by denaturing polyacrylamide gel electro-

phoresis and reverse phase chromatography their katalytic activity was tested with short 14-mer substrates under defined cleaving conditions. Best cleavage was obtained for RZ A328-R which cleaves between HCV nts. positions 330 and 331. Its Michaelis Menton constant  $K_m$  was  $69.1 \pm 19.3$  nM. The katalytic constant  $K_{cat}$  was  $4,3 \text{ min}^{-1}$  indicating multiple turnover. The katalytic effectiveness  $K_{cat}/K_m$  was  $6.4 \times 10^7 \text{ M}^{-1} \times \text{min}^{-1}$ . The cleavage with longer (452 nts.) HCV-luciferase fusion RNA substrates was best effective, if a high molar excess of RZ were used. In direct comparison with antisense ODN or not katalytically active antisense RNAs targeting exactly identical HCV-RNA sequences RZ A328-R displayed the best about 90% inhibition of luciferase activity *in vitro*.



**Figure 1** HCV 5'-non-coding region. The pseudonot region and stem loop IV including the start AUG are indicated in bold font as optimum target sequences for ODN binding.



To further extend the amount of possible target regions on the HCV genome a A $\geq$ I exchange at nt. position 15.1 was introduced (Figure 3), which reverts the polarity of an important H-bond in the hammerhead structure and allows efficient cleavage at a non-natural NCH recognition site (N: any nt., C: cytidine, H: any nt. except guanosine). For stability reasons various 2'O-allyl or amino-ribonucleotide modifications had been introduced in these RZ<sup>[7]</sup>, which cleave between HCV nts. 346 and 347 (Figure 3). An inactive cleavage antisense RZ and an unspecific RNA completely unrelated to HCV were used as controls. Regardless of prehybridisation of the RNA molecules with their target sequence, the best inhibitions of luciferase activity were obtained with the NCH recognizing RZ. The inhibition was less pronounced, when inactive cleavage antisense RZ were used. This data could be reproduced in HCV-5'-NCR/core-luciferase-recombinant CCI13 cell lines, which stably express the HCV core-luciferase fusion protein above from the CMV immediate early promoter. *In vivo* testing using CMV-HCV 5'-NCR/core-luciferase-transgenic mice are presently going on.

Coupling of effective antisense molecules to biomolecules such as bile acids which are specifically transported into the hepatocyte by bile acid transporters are in progress (Figure 4). The size of the antisense ODN coupled to cholic acid or taurocholic acid has been optimized with respect to efficient and specific inhibition of HCV-luciferase translation *in vitro*. Experiments with cell lines overexpressing the organic anion transporting polypeptide (OATP) and the sodium taurocholate cotransporting polypeptide (NTCP) are in progress.

## SUMMARY

Antisense oligodeoxynucleotides, RNAs and ribozymes can effectively inhibit HCV translation in model systems *in vitro* and *in vivo*. Chemical modifications such as phosphorothioate or benzylphosphonate modifications of oligonucleotides as well as O-allyl or amino-ribonucleotide modifications of ribozymes guarantee sufficient stability towards nuclease degradation. The specific exchange of single ribonucleotides in synthetic ribozymes permits to extend the ribozyme cleavage specificity to non-natural NCH motifs. If problems of hepatocyte-directed delivery and sufficient intracellular effector concentration can be solved, therapeutic nucleic acids may turn out to be a safe and powerful treatment option in the multimodal therapy concept of chronic hepatitis C.

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