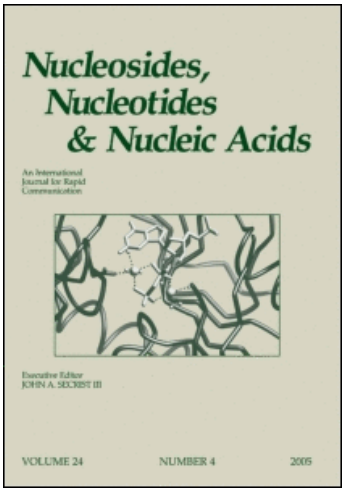

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DESIGN AND PROPERTIES OF HEPATITIS C VIRUS ANTISENSE OLIGONUCLEOTIDES FOR LIVER SPECIFIC DRUG TARGETING

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ABSTRACT

Different backbone modified antisense oligonucleotides (AS-ODNs) directed against the hepatitis C virus genome were 5′-conjugated to cholesterol, cholic acid or taurocholic acid to enhance liver specific drug targeting and hepatocellular uptake. The lipophilic character of modified AS-ODNs was determined from RP-HPLC retention times and duplex stability was correlated with T_m-values from UV melting curves.

Recently we synthesized terminally backbone modified phosphorothioates, methyl- and benzylphosphonates covering stem-loop structures within the 5′-NCR of the hepatitis C virus (HCV) genome and the adjacent core region (nucleotides 326–348). These 23 mer AS-ODNs contain three modifications at each end of the sequence and inhibited viral gene expression in a dose dependant manner combined with experimental evidence for RNase H activity (1,2,3). Starting from these well investigated 23 mers we truncated this sequence in steps of three nucleotides to find the minimal inhibitory sequence. Comparable inhibition of HCV-luciferase translation *in vitro* could also be obtained with 20 mers and 17 mers, whereas further truncated sequences led to lower inhibition levels.

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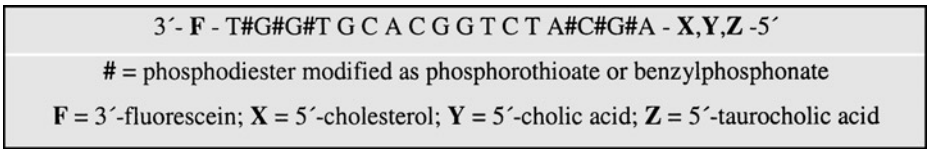

Figure 1. Design of AS-ODNs directed against the HCV genome.

To improve liver specific drug targeting and hepatocellular uptake we coupled effective 17 mer AS-ODNs to biomolecules such as cholesterol or bile acids, which are specifically targeted to the liver and specifically transported into hepatocytes. Coupling of cholesterol, cholic acid or taurocholic acid to the 5′-end of AS-ODNs was effected *via* the 3-hydroxy group of the steroid scaffold. The 3-hydroxy group is present in all natural bile acids, but their loss or exchange does not influence the interaction to the hepatic Na^+/bile acid cotransporter (4,5).

For effective coupling of bile acids to base labile methyl- or benzylphosphonates a new synthesis with mild deprotection of the esterified carboxyl function was developed. The synthesis of these bile acid phosphoramidites and automated oligonucleotide synthesis will be published elsewhere (6). Figure 1 shows the modifications, which were introduced to a 17 mer AS-ODN directed against nucleotides 326–342 of strain 1b of the non coding region of the HCV genome (7). For future determination of cell uptake the AS-ODNs were partly labeled with fluorescein at their 3′-position. At each end of the sequence three phosphodiesters were modified as phosphorothioates or benzylphosphonates to obtain sufficient nucleolytic stability. The 5′-end was coupled to cholesterol or bile acids:

The lipophilic character of different modified AS-ODNs directed against nucleotides 326–342 of the hepatitis C virus genome was investigated by RP-HPLC. An acetonitrile gradient from 0% to 60% in 0,1 M TEAA from 8 to 28 min was used to determine the elution times of cholesterol-, cholic acid-, taurocholic acid- and fluorescein-modified AS-ODNs. Besides the influence on lipophilicity of terminally modified benzylphosphonates compared to phosphorothioates was examined.

The modification of the phosphodiester backbone with three benzylphosphonates at each end of the sequence enhances the lipophilicity equally like the conjugation of a bile acid to a terminally modified phosphorothioate. Coupling of a bile acid to a benzylphosphonate yields additional lipophilicity as represented by the delayed elution times shown in Table 1. As expected coupling of cholesterol leads to the largest enhancement of lipophilicity. In this case a lipophilic benzyl-backbone shows no influence on elution times. The effect of the fluorescein dye on lipophilicity is negligible.

UV melting curves of different modified AS-ODNs hybridized to DNA and RNA target sequences of different length were measured. The concentration of each strand was 2 μM in phosphate buffer (140 mM sodium chloride, 10 mM phosphate,

Table 1. HPLC Elution Times: ° = Phosphorothioate; * = Benzylphosphonate; **F** = Fluorescein; **X** = Cholesterol; **Y** = Cholic Acid; **Z** = Taurocholic Acid

Phosphorothioates°	Benzylphosphonates*	Elution time [min]	Acetonitrile %
5′-T°G°G°TGCACGGTCTA°C°G°A	−3′	15,0	21
5′-T*G*G*TGCACGGTCTA*C*G*A	−3′	18,3	31
5′-X-T°G°G°TGCACGGTCTA°C°G°A	−3′	25,7	53
5′-X-T*G*G*TGCACGGTCTA*C*G*A	−3′	25,7	53
5′-Y-T°G°G°TGCACGGTCTA°C°G°A	−3′	18,0	30
5′-Y-T*G*G*TGCACGGTCTA*C*G*A	−3′	20,7	38
5′-Z-T°G°G°TGCACGGTCTA°C°G°A	−3′	18,0	30
5′-Z-T*G*G*TGCACGGTCTA*C*G*A	−3′	20,8	38
5′-T°G°G°TGCACGGTCTA°C°G°A-F	−3′	16,0	24
5′-X-T°G°G°TGCACGGTCTA°C°G°A-F	−3′	25,7	53
5′-Y-T°G°G°TGCACGGTCTA°C°G°A-F	−3′	18,2	30
5′-Z-T°G°G°TGCACGGTCTA°C°G°A-F	−3′	18,4	31

pH = 7,0). The target strands were of the same length compared to the AS-ODNs or had an overlap of three nucleotides at each end:

sense DNA 17:	5′-TCGTAGACCGTCCACCA	−3′
sense DNA 23:	5′-GTCTCGTAGACCGTGCACCATGA	−3′
sense RNA 17:	5′-UCGUAGACCGUGCACCA	−3′
sense RNA 23:	5′-GUCUCGUAGACCGUGCACCAUGA	−3′

In DNA/DNA duplices the T_m-values of benzylphosphonates are up to 2,4°C lower than those of their phosphorothioate analogs. This can be due to steric hindrance of the uncharged benzyl residues in the duplex. The influence of 3′-fluorescein and/or 5′-steroid modification is negligible in both cases - hybridization to the 17 mers and the overlapping 23 mers. Melting points of DNA/RNA heteroduplices are in general somewhat lower.

Table 2. T_m-values of AS-ODNs with DNA and RNA Targets: °; *; F; X; Y; Z = See Table 1; underlined Nucleotides = Mismatches in Control ODNs; — = No Melting Point Found; n.d. = Not Determined

Phosphorothioates°	Benzylphosphonates*	DNA (RNA) 17 mer [°C]	DNA (RNA) 23 mer [°C]
5′-T°G°G°TGCACGGTCTA°C°G°A	−3′	65,1 (65,1)	64,6 (63,7)
5′-T°G°C°TGCACGCTCTA°G°G°A	−3′	28,2 (n.d.)	—
5′-T*G*G*TGCACGGTCTA*C*G*A	−3′	62,7 (n.d.)	63,1 (58,2)
5′-T*G*C*TGCACGCTCTA*G*G*A	−3′	28,5 (n.d.)	—
5′-T°G°G°TGCACGGTCTA°C°G°A-F	−3′	65,2 (n.d.)	64,1 (64,3)
5′-X-T°G°G°TGCACGGTCTA°C°G°A	−3′	65,3 (62,7)	66,4 (61,9)
5′-Y-T°G°G°TGCACGGTCTA°C°G°A	−3′	65,7 (n.d.)	65,2 (62,6)
5′-Z-T°G°G°TGCACGGTCTA°C°G°A	−3′	66,6 (64,9)	66,2 (61,6)

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In summary, the bile acid or cholesterol modified AS-ODNs showed an enhanced lipophilicity as investigated by RP-HPLC and no significant loss of duplex stability due to nearly identical UV-melting points as compared to unmodified phosphorothioates. Additionally enhanced nucleolytic stability and activation of RNase H could be found (3). These properties makes them useful as potential liver specific antisense therapeutics.

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