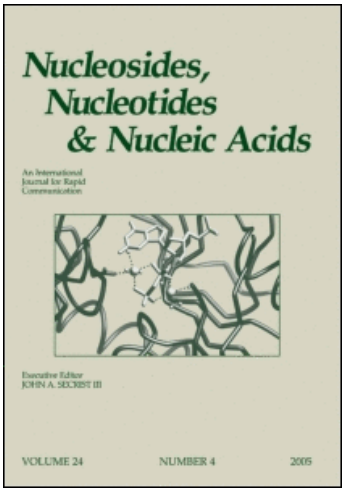

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Backbone Modified Antisense Oligodeoxynucleotides Directed Against the Hepatitis-C-Virus (HCV)-RNA

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BACKBONE MODIFIED ANTISENSE OLIGODEOXY-NUCLEOTIDES DIRECTED AGAINST THE HEPATITIS-C-VIRUS (HCV)-RNA

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ABSTRACT We synthesized (23 mer) ODN with different modifications, directed against nt 326-348 of HCV-RNA. The ODN contains 6 modified nucleotides. The types of modification we tested are nonionic (methylphosphonates, benzylphosphonates) and ionic phosphothioates.

Previously we have shown, that a fully thioate modified antisense oligodeoxynucleotide directed against a nucleotide stretch (nt 326-348) of **NCR** (**n**on **c**oding **r**egion)[1,2] in the 5'-end of the viral RNA efficiently inhibits HCV gene expression *in vitro* and in cell culture[3]. Phosphorothioate ODN are known to exert some undesired side effects like unspecific protein binding *in vivo* as a result of its polyanionic character and the presence of the thioate moiety. The synthetic Antisense oligodeoxynucleotides which we tested contain 3 modifications at the 5'- and the 3'-end of the sequences, or 6 modifications scatterted along the ODN-sequence.

In TABLE 1 are listed the ODN-sequences and there abbreviations. We could show that ODN with nonionic modifications inhibited the viral gene expression in an *in vitro* translations system specifically and dose-dependently only when the modifications are located at the ends of the ODN. The maximal inhibition for the methylphosphonate ODN and the benzylphosphonate ODN at a concentration of 10 μM was 92,3 ± 1,9% and 88 ± 3,7%, respectively: Under the same conditions the inhibition by sense and mismatch control ODN was only 36,5 ± 16,3%. In a cellular translation system the terminally

TABLE 1. **s** = scattered; **t** = terminal; **X** = **M**ethyl, **B**enzyl[4], [**S** (thioate)]; **K** = mismatch control; **S** = Sense-Strand; ° = position of modification

SEQUENCE	ODN	LENGTH
5'UCG UAG ACC GUG CAC CAU GAG CA 3'	**ODN 4-sense RNA**	23 nt
5'TCG TAG ACC GTG CAC CAT GAG CA 3'	**ODN 4-sense**	23 nt
5'T**G**°CT**C**°AT**G**°GTGCACG**G**°TC**T**°AC**G**°A 3'	**sX-ODN 4**	23 nt
5'T**G**°CT**G**°AT**G**°CTGCACG**C**°TC**T**°AG**G**°A 3'	**sX-ODN K**	23 nt
5'T**C**°GT**A**°GA**C**°CGTGCAC**C**°AT**G**°AG**C**°A 3'	**sX-ODN S**	23 nt
5'**T°G°C**°TCATGGTGCACGGTCT**A°C°G**°A 3'	**tX-ODN 4**	23 nt
5'**T°G°C**°TGATGCTGCACGCTCT**A°G°G**°A 3'	**tX-ODN K**	23 nt
5'**T°C°G**°TAGACCGTGCACCATG**A°G°C**°A 3'	**tX-ODN S**	23 nt

modified benzylphosphonate ODN were the most efficient and specific inhibitors of HCV gene expression. At a concentration of 5μM the inhibition of translation was 96,3 ± 0,7%. Under the same conditions the control ODN show no effect FIG. 1.

These data support that HCV gene expression can be inhibited effectively with modified antisense oligodeoxynucleotides. Furthermore we have experimental evidence that the mechanism is correlated with RNase H activity. Melting temperature experiments were performed at 260 nm using a Varian Cary 1/3 UV-Visible Spectrophotometer with a thermoblock. For this kind of experiments we dissolve 0,5 OD_{260} of the modified Antisense ODN and 0,5 OD_{260} of the Sense-Strand in a 1 ml cuvette. The solvens for the ODN is a buffer-system. It contained 140 mM NaCl and 10 mM HEPES, pH = 7,5. The Absorbance of ODN were measured every 0,5 °C from 10 °C to 80 °C. Melting temperatures were obtained from the maximum value of the first derivative plots of absorbance versus temperature. We analysed the duplex-stability in two groups of experiments one with an unmodified DNA-Sense-Strand and the other with an unmodified RNA-Sense-strand as target sequence for the modified Antisense ODN. This Datas are shown in TABLE 2 and they agree well with our previous results[5].

The complexes between unmodified ODN and the target RNA are a substrate for cellular RNase H that degrades the RNA portion in the DNA-RNA duplex. For this experiment a radiolabled 35-mer target RNA was incubated with the modified ODN in the reticulocyte lysate. The terminally modified methyl- and benzylphosphonate ODN

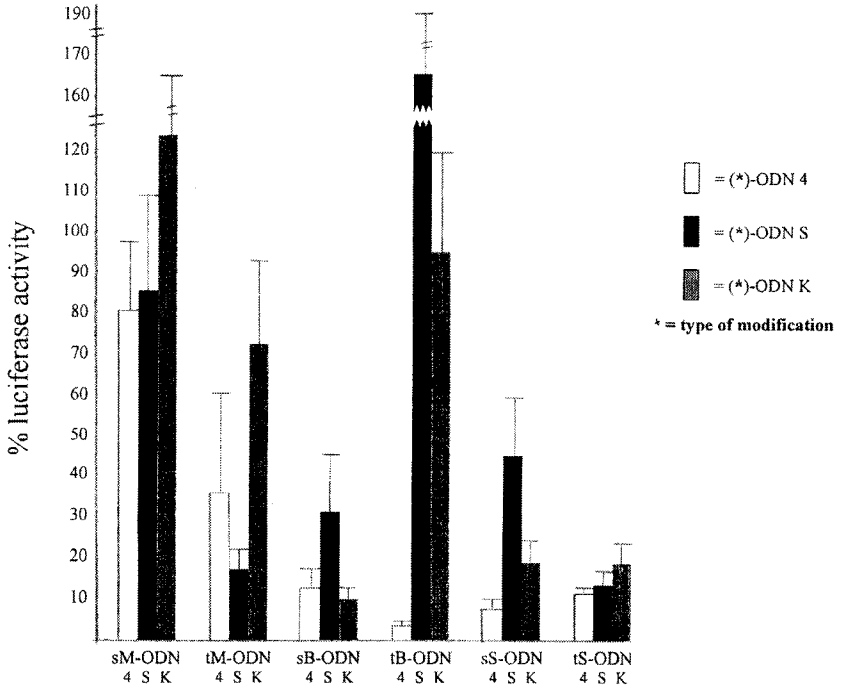

FIG. 1 Inhibition of HCV gene expression by modified ODN in cell culture. The luciferase activity was determined and compared to controls to which no ODN had been added (100% luc activity).

TABLE 2. T_m-values for DNA/DNA-duplex and RNA/DNA-duplex; * the relative tolerance is about ± 0,5°C

target-sequence	antisense-ODN	modification	T_m-value* [°C]
DNA/DNA-duplex			
ODN 4-sense	ODN 4	unmodified	73.7
ODN 4-sense	sM-ODN 4	methyl	68.3
ODN 4-sense	tM-ODN 4	methyl	70.5
ODN 4-sense	sB-ODN 4	benzyl	69.4
ODN 4-sense	tB-ODN 4	benzyl	69.0
ODN 4-sense	sS-ODN 4	thioat	76.7
ODN 4-sense	tS-ODN 4	thioat	76.1
RNA/DNA-duplex			
ODN 4-sense RNA	ODN 4	unmodified	70.7
ODN 4-sense RNA	sM-ODN 4	methyl	58.0
ODN 4-sense RNA	tM-ODN 4	methyl	63.5
ODN 4-sense RNA	sB-ODN 4	benzyl	57.5
ODN 4-sense RNA	tB-ODN 4	benzyl	57.6
ODN 4-sense RNA	sS-ODN 4	thioat	65.4
ODN 4-sense RNA	tS-ODN 4	thioat	65.6
ODN 4-sense RNA	ODN K	unmodified	32.7

tM-ODN and tB-ODN and both types of phosphorothioates (sS-ODN and tS-ODN) that specifically inhibited the viral translation in the *in vivo* translation system, mediated an efficient cleavage of the target RNA. No cleavage was observed in controls in which either no RNase H or no ODN was added to the *in vitro* translation reaction. Therefore these data suggest that ODN which mediate an RNase H-associated destruction of the target RNA are specific and efficient inhibitors of viral translation in the rabbit reticulocyte in vitro translation system.The incorporation of six modified nucleotides in the Antisense ODN shows an effect in thermal duplex-stability. The sterically hindered benzyl group shows the highest temperature-depression (TABLE 2). In case of the methyl modified ODN the temperature depression is about one degree lower. The highest melting temperature of the duplex shows the posphorothioate ODN. We can demonstrate that the duplex between the benzyl modified ODN and the RNA-target-strand has the lowest melting temperature. This kind of modification seems to be the best substrate for the RNase H enzymatic degradation. One can also see that the position of the modification in the Antisense ODN influences the Rnase H activity indicating a preferred cleavage of weaker duplices.

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