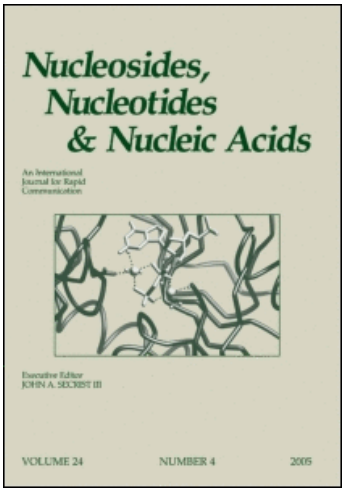


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Online publication date: 09 August 2003

**To cite this Article** Amberg, S. , Tamke, A. , Caselmann, W. H. and Engels, J. W.(2003) 'Specific Inhibition of Hepatitis C Viral Gene Expression by Non-polar (Phenylalkyl)phosphonates', Nucleosides, Nucleotides and Nucleic Acids, 22: 5, 1631 — 1634
**To link to this Article: DOI:** 10.1081/NCN-120023086
**URL:** http://dx.doi.org/10.1081/NCN-120023086

NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDS
Vol. 22, Nos. 5–8, pp. 1631–1634, 2003

# Specific Inhibition of Hepatitis C Viral Gene Expression by Non-polar (Phenylalkyl)phosphonates

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## ABSTRACT

Different phenylalkyl backbone modified antisense oligonucleotides complementary to the Hepatitis C virus (HCV) RNA nucleotides 326–342 were synthesized. The lipohilic character of modified oligonucleotides was determined from RP-HPLC retention times. The inhibitory effect of these antisense oligonucleotides on HCV gene expression was analyzed in an in vitro test system.

## INTRODUCTION

Hepatitis C Virus (HCV) is a positive-stranded RNA virus that is a frequent cause of chronic viral hepatitis in humans. The initiation of translation on the positive sense RNA genome of HCV is directed by an internal ribosomal entry site (IRES). The highly conserved 5′-noncoding region (NCR) forms a characteristic secondary structure which represents a promising target to inhibit viral gene expression by an antisense approach.[1] Previously synthesized terminally modified

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DOI: 10.1081/NCN-120023086
1525-7770 (Print); 1532-2335 (Online)




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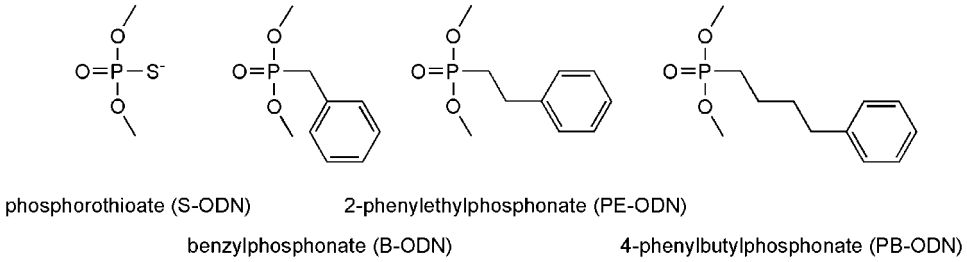


***Figure 1.*** Types of backbone modifications.

phosphorothioate antisense oligonucleotides (ODNs) directed against nucleotides 326–342 of the 5′-NCR can specifically inhibit HCV translation in vitro and in cell culture.[2] Unfortunately, cellular uptake is low (the use of an uptake enhancer like lipofectin is necessary) and not specific for hepatocytes. Recently synthesized non polar benzyl- modified oligonucleotides have shown better inhibitory activity against HCV gene expression than the polar phosphorothioates ODN's.[3] Therefore, it might be possible that modifications which are more lipophilic show the same or even better inhibitory activity and could be taken up by the cells more effectively. We investigated the lipophilicity and the inhibitory capacity of HCV specific 17-mer ODN's complementary to the HCV RNA nucleotides 326–342, which contained only six modified nucleotides either three at each end. Modified nucleotides were polar phosphorothioates (S-ODN) and non polar benzyl- (B-ODN), 2-phenylethyl- (PE-ODN) and 4-phenylbutylphosphonates (PB-ODN) (Fig. 1).

## RESULTS AND DISCUSSION

The antisense oligonucleotides were synthesized via the phosphoramidite method. For the synthesis of non polar oligonucleotides, benzyl-, 2-phenylethyl- and 4-phenylbutyl- modified building blocks were used.[4] After deprotection the oligonucleotides were purified by RP-HPLC. The pure oligonucleotides were desalted (Sephadex-G25) and characterized by MALDI-TOF mass spectra.

The lipophilic character of different non polar (phenylalkyl) phosphonates [5′-d(T∗G∗G∗TGCACGGTCTA∗C∗G∗A), ∗ = place of modification] directed against nucleotides 326–342 of the hepatitis C virus genome compared with the phosphorothioate and natural analogue were investigated by RP-HPLC (Fig. 2). An acetonitrile gradient from 0% to 60% in 0.1 M TEAA (pH 7.0) buffer within 30 min was used to determine the elution times. The broad peaks of (phenylalkyl) phosphonates result from their diastereomerical mixtures (Rp and Sp). As expected, the lipophilicity increased from a retention time of 15.15 min for benzyl- (B-ODN) to 19.51 min for 4-phenylbutyl- (PB-ODN) modified oligonucleotides compared to the parent 17mer (10.17 min) and the phosphorothioate- containing one (S-ODN; 10.92 min). Summarising, lipophilicity can be increased significantly and predictably by phenylalkyl modifications so they were tested in vitro as antisense oligonucleotides against HCV gene expression.





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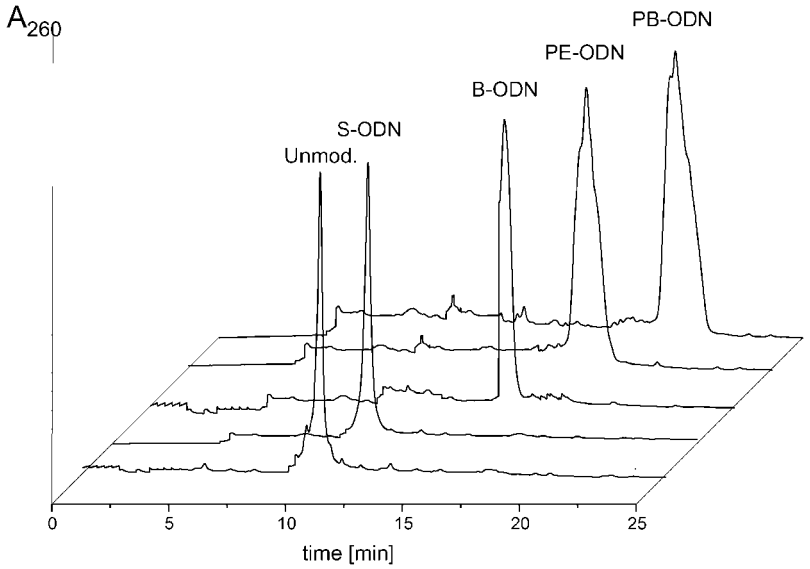


***Figure 2.*** RP-HPLC profile of antisense 17mer oligonucleotides.

The in vitro test system used in this study has been described previously.[2] Briefly, we used a T7-polymerase driven DNA construct consisting of HCV 5′-NCR, 66 nucleotides core fused to the firefly luciferase coding sequence. 50 ng of in vitro transcribed RNA was incubated with increasing concentrations of various antisense oligonucleotides in 12 μL rabbit reticulocyte lysates for one hour at

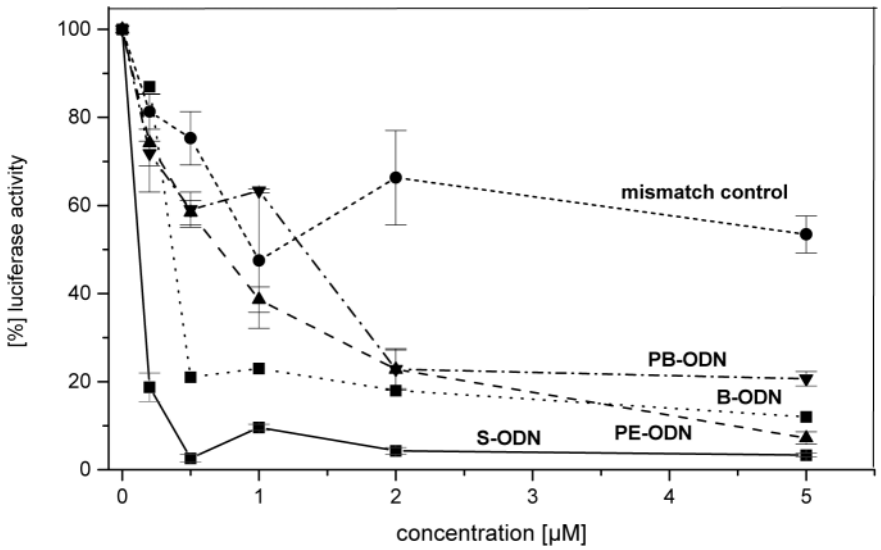


***Figure 3.*** Inhibitory activity (in vitro) of modified oligonucleotides 17mers.





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37°C. One third of the reaction was added to 100 μL luciferase assay reagent and the relative light units were determined for 30 sec in a luminometer. For all differently modified oligonucleotides a dose-dependent inhibition of viral translation was observed (Fig. 3). The maximal inhibition of 96% ± 2% was observed with phosphorothioate modified oligonucleotides at 5 μmol/L concentration. A good inhibition was obtained with the non polar 2-phenylethyl- (PE-ODN; 92% ± 3%) and benzyl- (B-ODN; 83% ± 4%) modified oligonucleotides. The 4-phenylbutyl- modified oligonucleotide (PB-ODN) showed a lower inhibitory effect (80% ± 5%). These data demonstrate that non polar phenylalkyl modified oligonucleotides are potent inhibitors for HCV gene expression in vitro. Further investigations like cell culture inhibition measurements and determination of cellular uptake of these non polar antisense oligonucleotides are under way.

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