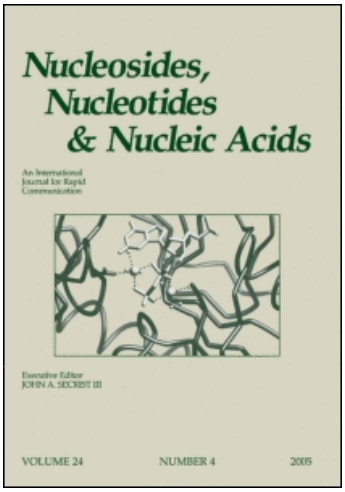

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ANTIVIRAL ACTIVITY OF ANTISENSE OLIGONUCLEOTIDES AGAINST VARIOUS TARGETS OF HERPES SIMPLEX VIRUS 1 (HSV1) AND COXSACKIEVIRUS B3 (CVB3) GENOME

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ABSTRACT: The influence of chemical modification on the antiviral activity of oligonucleotides was studied on Green monkey kidney cells (GMK) using a known antisense oligodeoxynucleotide (AS-ODN) directed against the IE-110 gene of HSV1. The highest antiviral activity was observed with ODNs carrying exclusively phosphothioate internucleotide linkages. CVB3-specific ODNs of this type were synthesized and successfully tested for antiviral activity on HeLa cells.

INTRODUCTION: It is well established that sense and antisense ODNs can bind in a sequence specific manner to complementary regions of viral mRNA in infected cells and thus selectively inhibit virus genome expression. The success of antiviral treatment mainly depends on cellular uptake, binding efficiency, and intracellular stability of the oligonucleotide applicated. Several chemical modifications were used to increase the stability of ODNs against cellular nucleases[1,2]. Phosphorothioate analogues of oligodeoxyribonucleotides (PS-ODN) have shown to be more resistant to nuclease degradation than natural DNA and maintain binding to complementary nucleic acid sequences[3,4,5,6,7]. Furthermore, PS-ODNs have successfully been used to block gene expression[8] and replication of viruses in vitro e.g. of human immunodeffiency virus 1[9,10,11,12], HSV1[13,14,15], influenzavirus A[16]. As a first step in our antiviral investigations the influence of chemical modifications on antiviral activity of oligonucleotides was studied with a known AS-ODN directed against the IE110 gene of HSV1[13].

So far, there have not been published any reports on the effects of oligonucleotides on coxsackievirus B infection in vitro. CVB3 belongs to the Picornaviridae family. The virus genome consists of an approximately 7500 nucleotide long positive RNA. Nucleotide sequence[17] and most parts of the gene functions[18,19] are known and provided the possibility to define CVB3 RNA regions that could act as target sites for inhibition of virus replication by S-ODN and/or AS-ODN. In our study 20mer oligonucleotides directed against different target genes of CVB3 were investigated for antiviral activity.

RESULTS: The automated synthesis of phosphorothioates was performed by the phosphoramidite approach[20,21]. Phosphorothioate bonds were introduced into oligodeoxyribonucleotides

using a 0.5 M solution tetraethylthiuram disulfide in anhydrous acetonitrile[22] (Sulfurization time: 15 min). The reagent solution is commercially available from Applied Biosystems Inc. (Foster City, USA).

Oligonucleotides with one, two, or three phosphothioates at both the 5´ and 3´ ends or with a complete phosphorothioate backbone were synthesized on Applied Biosystems DNA/RNA synthesizers (model 380B and model 394, Applied Biosystems Inc.) using standard phosphoramidite chemistry in 0.2 or 1,0 µmole scale. To introduce phosphorothioate bonds sulfurization using tetraethylthiuram disulfide was performed directly after coupling but prior to capping. Cleavage from support (CPG 50 nm lcaa) and final deprotection were carried out with 28 - 30 % aqueous ammonia at 55°C for 5 to 12 h. All oligonucleotides were purified by ethanol precipitation in presence of sodium acetate. Purity of all oligonucleotides was greater than 90 - 95 % by HPLC (FIG. 1). Isolation of small aliquots of PS-ODNs was performed by affinity purification of their 5´-DMT derivatives on OPC purification cartriges (Applied Biosystems Inc.) according to the instructions of the manufacturer for DNA purification with the modification of an increased (20 to 35 %) of acetonitrile in the elution buffer of the final step.

Repetitive and overall coupling yields were estimated from absorption measurements of the dimethoxytrityl protecting group at 495 nm in deblocking solutions acidified with p-toluene-sulfonic acid.

Phosphorothioates are significantly more hydrophobic than their phosphodiester counterparts. The sulfurizing reaction is not stereospecific at the chiral phosphorous center, yielding a large number of chemically distinct, diastereomeric products. In Rp18 HPLC the diastereomers manifest in a slightly increased elution time as well as a slight broadening of the peaks. Gel capillary electrophoresis is the most useful technique for verification of the purity of phosphorothioate oligonucleotides (crude or purified state). The final purification of large ODN amounts (more than 25 OD_{260} units) were performed by preparative HPLC with a Bio-Rad Model 2700 (Bio-Rad CA,USA) device fitted with Software Series 800 HRLC-System,Version 2.30.1a. The apparatus was equipped with the Automatic Sampling System Model AS-100T HRLC. Detection was performed by UV-1806 (Bio-Rad) at 260 nm. Resource RPC (3 ml) purchased from Pharmacia LKB Biotechnology (Uppsala, Sweden) and Eurospher 100 C_{18} from Knaur (Germany) were used as columns with similar performance using the following conditions:

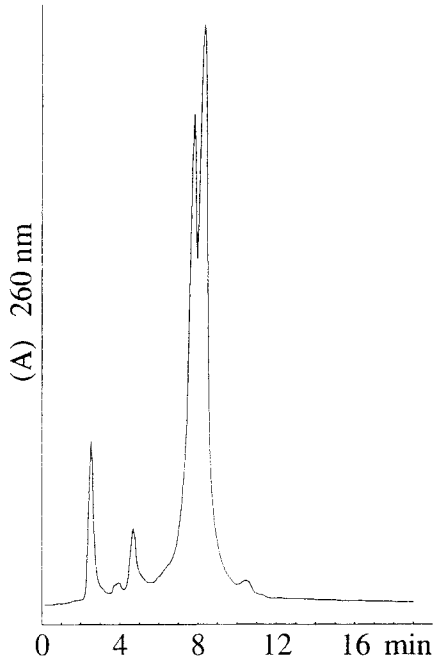

FIG. 1: HPLC on RPC (3 ml) columns elution profile of the ODN XII (CVB3) 20mer phosphorothioate with 5´ FITC

Flow-rate: 1 - 1.5 ml/min

Eluent: Solvent A = 0.1 M triethylammonium acetate in water (pH 7)

Solvent B = acetonitrile

TABLE 1: Description of oligonucleotides used in this study

Entry	Virus	Sequence (5′ ——> 3′)	Target gene (function)
I.	HSV1	GCG GGG CTC CAT GGG GGT CG	IE - 110 ^{AS}
II.	HSV1	19-PS-GCG GGG CTC CAT GGG GGT CG	IE - 110 ^{AS}
III.	HSV1	G_{PS}CG GGG CTC CAT GGG GGT C_{PS}G	IE - 110 ^{AS}
IV.	HSV1	$G_{PS}C_{PS}$G GGG CTC CAT GGG $GG_{PS}T$ C_{PS}G	IE - 110 ^{AS}
V.	HSV1	$G_{PS}C_{PS}G_{PS}$ GGG CTC CAT GGG $GG_{PS}T_{PS}$ C_{PS}G	IE - 110 ^{AS}
VI.	HSV1	FITC-19-PS-GCG GGG CTC CAT GGG GGT CG	IE - 110 ^{AS}
VII.	CVB3	19-PS-ACC ACA CAG AAC CAA GAT GC	VP1 ^{S}
VIII.	CVB3	19-PS-GCA TCT TGG TTC TGT GTG GT	VP1 ^{AS}
IX.	CVB3	19-PS-CCC CCT CCC CCA ACT GTA AC	5′ noncoding region ^{S}
X.	CVB3	19-PS-CCA GCT CTC AAC TCC CCC AC	VP4 ^{S}
XI.	CVB3	19-PS-GTG GGG GAG TTG AGA GCT GG	VP4 ^{AS}
XII.	CVB3	FITC-19-PS-CCC CCT CCC CCA ACT GTA AC	5′ noncoding region ^{S}
			S = Sense AS = Antisense

Gradient for RPC 3 ml columns

Linear gradient by Trityl - OFF synthesis: 0 % A to 50 % B in 30 min

Gradient for Eurospher 100 C_{18}

Linear gradient by Trityl - ON synthesis: 15 % B to 35 % B in 30 min

Linear gradient by Trityl - OFF synthesis: 10 % B to 30 % B in 30 min

In order to determine the most effective chemical modification for further antiviral investigations the study was started with a known HSV1 specific AS-ODN with varying numbers of phosphothioate internucleotide linkages (TABLE 1).

As a general parameter the cytotoxic effect of oligonucleotides on cells was determined in a dye uptake assay after 72 h treatment (at 37°C). No growth inhibition of GMK cells could be detected for chemically modified HSV1 A-ODN at concentrations up to 80 µM (data not shown).

The antiviral activity of the PS modified AS-ODN against HSV1 was studied in an assay of inhibition of cytopathogenic effect (CPE) on GMK cells. Briefly, GMK cells were seeded into 96 well culture plates (1,7 x 10^4 cells/well) in 100 µl of Eagle's minimal essential medium (MEM) supplemented with 10 % foetal calf serum (FCS, PAA Laboratories, Linz, Austria). The culture medium was removed from the confluent cell monolayers after 48 h of incubation at 37°C in a 5 % CO_2 atmosphere. 50 µl of various concentrations of oligonucleotides (0,3 - 80 µM) as well as 50 µl of virus suspension resulting in a multiplicity of infection of 0,1 (MOI) were applicated. After 48 h of further incubation at 37°C and 5 % CO_2 a complete cell death was observed microscopically in the untreated virus control. At this time all cells were fixed and stained with a 0,25 % crystal violet/formalin solution. Inhibition of viral CPE was quantitated spectrophotometrically with a plate reader (Dynatech, Guernsey, GB) at 550/630 nm.

All tested modifications of the oligonucleotide I inhibited the HSV1 induced CPE in a dose dependent manner (FIG. 2). The introduction of 1, 2, or 3 PS-linkages at the end of the HSV1 AS-ODN moderately improved the antiviral activity of the oligonucleotide I. A clear enhancement (up to 80 x) of antiviral activity was obtained after introduction of PS-linkages at any nucleotide of the AS-ODN I.

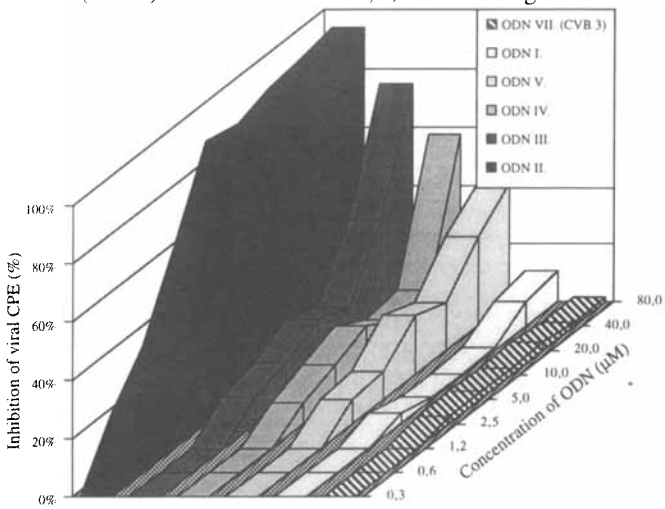

FIG. 2: Influence of chemical modifications on the antiviral activity of HSV1 specific AS-ODN studied by inhibition of virus-induced CPE on GMK cells

These results correspond well with data obtained from plaque reduction assays (PRA) in 24-well culture plates on GMK cells, in which the ODN concentrations were given in a MEM overlay containing 0,5 % agarose after 1 h of virus adsorption at 4°C (FIG. 3). In contrast, no plaque reduction was found if the AS-ODN with PS-linkages at any nucleotide was present only during virus adsorption. Furthermore, none of the CVB3-specific PS-ODNs exhibited any antiviral activity against HSV1 neither in the CPE inhibition test nor in the PRA (for example PS-ODN VII). Based upon these data we suggested, that the inhibition of HSV1 replication by the tested anti-HSV AS-ODN with PS-linkages at any nucleotide is very specific.

Therefore ODNs directed against different target genes of the CVB3 genome were synthesized with

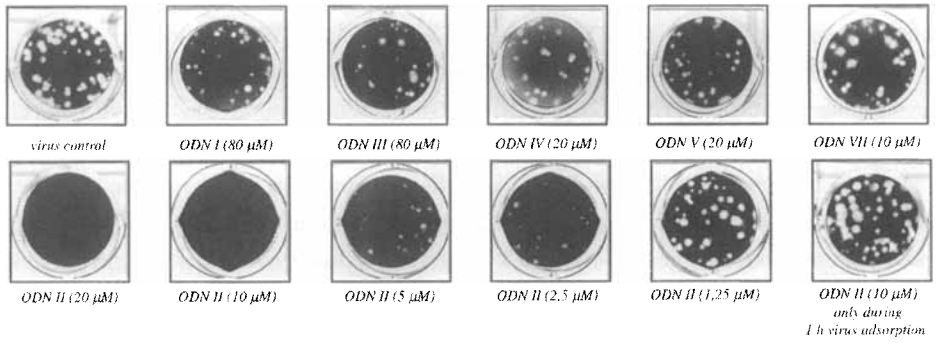

FIG. 3: Results obtained by plaque reduction assays with HSV1 on GMK cells

PS-linkages between all nucleotides (TABLE 1). To make the results comperable only PS-ODN of 20 nucleotides lenght were used. The 50 % cytotoxic dose for HeLa cells was ≥ 80 µM. The antiviral activity of the oligonucleotides studied was determined by CPE inhibition (FIG. 4) as well as plaque reduction. Essentially, both antiviral assays were performed as described for HSV1. Briefly, HeLa cells (strain Ohio) were cultivated in Eagel's MEM containing 10 % newborn calf serum (NCS, PAA Laboratories, Linz, Austria). CVB3-specific PS-ODN were applicated 48 h before virus infection (MOI 1) until the staining of the tests (24 h in CPE inhibition assay and 48 h in PRA).

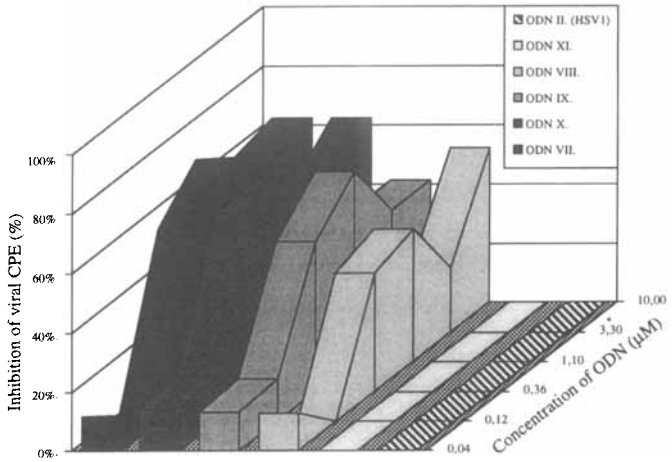

FIG. 4: *Antiviral activity of CVB 3 specific ODN studied by inhibition of virus-induced CPE on HeLa cells*

0,3 µM of sense PS-ODN directed against the VP1 coding region and 0,3 µM of sense PS-ODN directed against the VP4 coding region of the CVB3 genome inhibited the virus-induced CPE by 50 %. Surprisingly, the application of PS-ODN with sense orientation directed against the 5´ noncoding region of CVB3 also resulted in a strong suppression of CPE production (ID_{50} = 0,36 µM). No CPE inhibition was measured after treatment with PS-ODN XI and the HSV-specific PS-ODN II underlaying a specific mode of action for the oligonucleotides tested. As for HSV1 this conclusion was supported by the results from PRA (data not shown).

Finally, we studied the penetration and intracellular distribution of PS-ODN on GMK cells (FIG. 5). The immunofluorescence photographics demonstrated a clear dose and time depended penetration of FITC-labeled PS-ODN V. 24 h after treatment with 20 µM of anti HSV1 PS-ODN in nearly all cells granulated cytoplasmatic fluorescence and single nucleoli with bright fluorescence were detected.

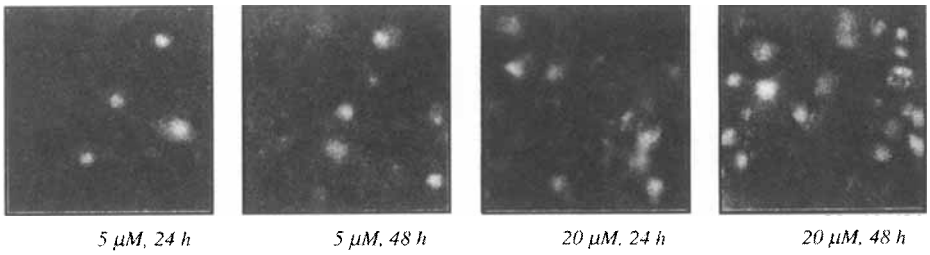

FIG. 5: *Time dependent penetration and intracellular distribution of FITC-conjugated PS-ODN VI observed with an immunofluorescence microscope at 24 h and 48 h after ODN application*

After 48 h the intensity of cytoplasmatic fluorescence and the number of bright lighting nucleoli was significantly enhanced. The application of 5 µM PS-ODN led to delayed penetration into GMK cells.

CONCLUSION: The results obtained indicate that the introduction of PS-linkages at any nucleotide allowed an effective selection of sequence-specific antiviral active ODNs, using low amounts of these substances. This chemical modification was successful applied in antiviral investigations against CVB3.

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