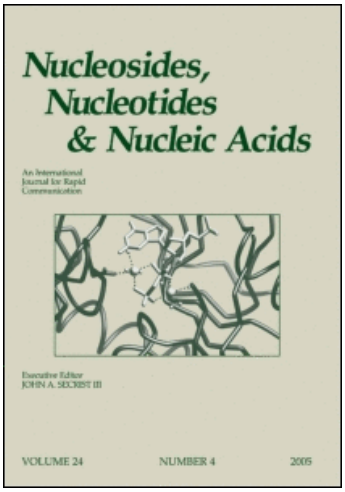

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PENETRATION AND ANTIVIRAL ACTIVITY OF COXSACKIEVIRUS B3 (CVB3)-SPECIFIC PHOSPHOROTHIOATE OLIGODEOXYNUCLEOTIDES (PS-ODN)

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ABSTRACT: The antiviral activity of PS-ODNs, complementary to different regions of the CVB3 genome, was investigated under *in vitro* conditions. Inhibition of CVB3 replication was detected only after prolonged pretreatment of HeLa cells with antiviral active PS-ODNs, but not when virus and PS-ODN were applicated simultaneously. Results from flow cytometric analysis indicate that a low cellular uptake of anti-CVB3 oligonucleotides into HeLa cells might be a reason for their moderate antiviral activity.

INTRODUCTION

One possibility to inhibit selectively the virus genome expression is the application of short oligodeoxynucleotides containing a complementary base sequence to virus RNA.[1] Viruses are intracellular pathogens and replicate only in certain cell lines or organs. Therefore, cellular uptake, binding efficiency, and nuclease stability determine the success of antiviral treatment with oligonucleotides.[2, 3, 4]

Previously, we could show that the introduction of phosphorothioate-linkages at any nucleotide allowed an effective selection of ODNs with specific antiviral activity using small amounts of these substances.[5] Furthermore, we detected a lower extent of inhibition of CVB3 replication by the investigated virus-specific PS-ODNs in comparison to the inhibition of herpes simplex virus type 1 (HSV1) multiplication by a published anti-IE110 PS-ODN.[6] In order to find better targets within the CVB3-genome, we synthesized further GC-rich oligonucleotides directed against different regions of the CVB3 genome. The definition of specific target sites was based on the published nucleotide sequence[7]

and known gene functions[8, 9] of the picornavirus CVB3. Cytotoxicity, antiviral activity, and cellular uptake of these PS-ODNs were determined under *in vitro* conditions and compared with results using an anti-HSV1 PS-ODN published by Peymann et al. 1995.[6]

RESULTS AND DISCUSSION

Oligonucleotide synthesis and purification. To make the results comparable, only 20mer ODNs with identical chemical modifications were used in this study. ODNs directed against different target genes of CVB3 were synthesized with a complete phosphorothioate backbone to avoid their rapid degradation by cellular or serum nucleases.

Oligodeoxyribonucleotides with different amounts (up to 100 %) of phosphorothioate bonds in the backbone were synthesized by phosphoramidite chemistry with an Expedite model 8909 syntheziser (Perseptive Biosystems, Inc. Wiesbaden-Nordenstadt, Germany) in 1 µmole scale. The support consisted of polystyrene grafted onto polytetrafluoroethylen with a graft degree of 5 % and a nucleoside load of 48 µmole/g.[10] Phosphorothioate bonds were introduced in each cycle using a 0.05 M solution of the sulfurization agent 3H-1,2-benzodithiol-3-one-1,1-dioxide (Glen Research, Eurogenetec, Seraing, Belgium) in acetonitrile using a silanized bottle. Fluorescence labels were attached to the 5'-terminus during the synthesis by means of 5'-fluorescein phosphoramidite with coupling times prolonged to 3 minutes.

The purification of large amounts of phosphorothioate oligonucleotides was performed by preparative HPLC on a Shimadzu LC10 apparatus (Shimadzu, Europe GmbH, Duisburg, Germany). In a first step, the material resulting from the deprotection reaction was prepurified by reverse-phase HPLC on ResourceRPC (Pharmacia, Uppsala, Sweden) or, with similar results, on Eurospher 100 C18 (Knauer, Berlin, Germany). During this purifications, fractionated elution was accomplished by application of a linear acetonitrile gradient (0 - 50 % within 40 minutes) against aqueous 0.1 M triethylammoniun acetate (pH 7.0) at a flow rate of 1 ml/min. This procedure removed any contaminating phosphorodiester oligonucleotide contamination and yielded the fluorescein-labelled oligonucleotides in 100 % and the desired nonlabelled oligonucleotides in 85 - 90 % purity. In a second step, truncated sequences were removed by anion-exchange chromatography on MonoQ HR 5/5 (Pharmacia, Uppsala, Sweden) using a flow rate of 1.5 ml/min (Eluent

TABLE 1: Description of PS-ODNs complementary to HSV1 and CVB3 RNA

Entry	Virus	Sequence	Target gene/function
1*	HSV1	19-PS-GCGGGGCTCCATGGGGGTCG	IE - 110 (antisense = As)
2*	CVB3	19-PS-CCCCCTCCCCCAACTGTAAC	5′noncoding region (sense = S)
3*	CVB3	19-PS-GTTACAGAAGGGGGAGGGGG	5′noncoding region (As)
4	CVB3	19-PS-AAAATGGGAGCTCAAGTATC	start codon (S)
5	CVB3	19-PS-GGAGCTCAAGTATCAACGCA	start codon (S)
6	CVB3	19-PS-TGCGTTGATACTTGAGCTCC	start codon (As)
7	CVB3	19-PS-ACCACACAGAACCAAGATGC	VP1 (S)
8	CVB3	19-PS-GCATCTTGGTTCTGTGTGGT	VP1 (As)
9	CVB3	19-PS-CCAGCTCTCAACTCCCCCAC	VP4 (S)
10	CVB3	19-PS-GTGGGGGAGTTGAGAGCTGG	VP4 (As)
11	CVB3	19-PS-GGCTTAACCCTACTGTGCTA	3D pol (S)
12	CVB3	19-PS-TAGCACAGTAGGGTTAAGCC	3D pol (As)
13	CVB3	19-PS-GACCATGGGGGGTGAAGGCG	2A prot (S)
14	CVB3	19-PS-CGCCTTCACCCCCCATGGTC	2A prot (As)
15	CVB3	19-PS-CCAGGGCCCAGTGGAAGACG	VP3/1 (S)
16	CVB3	19-PS-CGTCTTCCACTGGGCCCTCC	VP3/1 (As)
17	CVB3	19-PS-GGGACGTGGGGCTAC	VP3 (S)
18	CVB3	19-PS-GTAGCCCCACGTCCCC	VP3 (As)

* ODNs, which were labelled with fluorescein (FITC) at the 5′-terminus

A: 10 mM NaOH, 0.1 M NaCl; Eluent B: 10 mM NaOH, 1 - 2 M NaCl). Within 40 minutes linear gradients from 0 to 100 % of Eluent B were applied to fractionally elute the bound components. The phosphorothioate backbone bonds require 2 M NaCl concentration to be eluted. After desalting on NAP-25 columns (Pharmacia, Uppsala, Sweden), the purity of the final products was 95 to 97 % without contamination by phoshorodiester containing oligonucleotides.

Cytotoxicity of PS-ODNs on confluent cell monolayers and inhibition of cell growth. Before starting the antiviral investigations, the 50 % cytotoxic concentration (CC_{50}) and maximal tolerated dose (MTD) of oligonucleotides were determined on green monkey kidney (GMK) or HeLa cells. For that purpose, serial dilutions of PS-ODN concentrations (2.5 to 80 μM, dilution factor 2) were added to confluent cell monolayers. 72 h after PS-ODN application, cell viability was evaluated as the percentage of mean value of optical density resulting from 6 mock-treated cell controls which was set 100 %. For this purpose, a semiautomatic crystal violet uptake assay was used. This test is based on

the determination of optical density at 540/630 nm with a microplate reader (Dynatech, Guernsey, GB) after dye elution. No cytotoxic effects were detected using this method for PS-ODN1 on confluent GMK cells at concentrations up to 80 μM. The MTD for ODN2-18 on confluent HeLa cell monolayers ranged from 25 to 50 μM. The CC_{50} determined for these CVB3-specific PS-ODNs was ≥ 80 μM.

Additionally, the antiproliferative effect of PS-ODN1 and 2 was investigated. For this experiment, logarithmically growing GMK or HeLa cells were seeded in 96-well plates at a density of 1 x 10^4 cells per well. After 4 h incubation at 37°C, medium containing PS-ODNs was added. Cells were incubated for additional 3 days. Cell number as well as viability were determined with a cell counter and analyzer system (CASY, Schärfe System GmbH, Reutlingen, Germany). FIG. 1 shows that no significant effect on HeLa cell growth was observed using PS-ODN2 at concentrations of 10 μM or less. Higher degrees of inhibition of cell growth were determined with this method for PS-ODN1 on GMK cells. The 50 % inhibitory dose amounted to 2,5 μM.

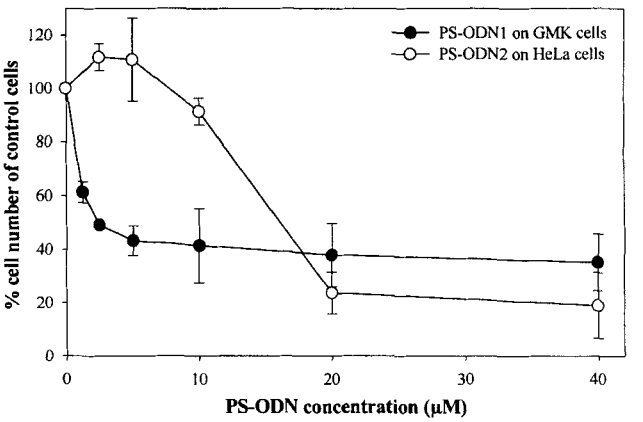

FIG. 1: Effect of PS-ODN treatment on cell proliferation. The average cell number for duplicate samples per oligonucleotide concentration was determined. Values are expressed as the percentage of mean cell number for 3 untreated control cells. Curves represent the data ± SD from one of two independent performed experiments.

Antiviral activity of CVB3- and HSV1-specific PS-ODNs. Noncytotoxic concentrations of oligonucleotides were used to investigate the antiviral activity against CVB3 in HeLa cells with a cytopathic effect (CPE) inhibition assay. The inhibition of HSV1-replication by PS-ODN1 was determined on GMK cells using the same assay conditions. The method based on the protection of cells from cytolysis by antiviral active PS-ODN

and was described previously.[5] In these studies, IC_{100} and IC_{50} concentrations of the herpes virus antiviral phosphonoformic acid (PFA) and of the CVB3 antiviral guanidino-hydrochlorid (Gua-HCl) were included as controls.

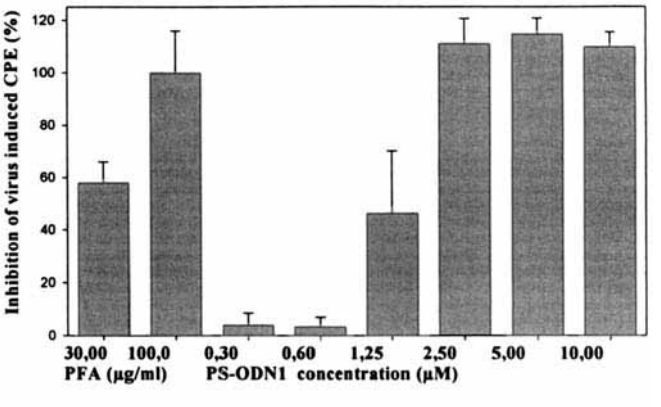

FIG. 2: Inhibition of HSV2-induced CPE by PS-ODN. Values are expressed as the percentage of mean optical density from 3 untreated cell controls after crystal violet staining. Results represent the average ± SD from 3 independent experiments performed in the same manner.

At first, virus and PS-ODNs were added simultaneously to confluent cell monolayers. The results shown in FIG. 2 demonstrate that the PS-ODN1 inhibited the HSV1-induced CPE in a dose-dependent manner without additional pretreatment of GMK cells before virus application. In contrast, none of the tested CVB-specific PS-ODNs exhibited any antiviral activity against HSV1 at concentrations of 10 µM and lower. In contrast, no reduction of CVB3 production was observed if PS-ODN2-18 and virus were added simultaneously to HeLa cell monolayers (data not shown).

The published studies antiviral PS-ODNs often involved a 2 to 3 h pretreatment of cells with sense or antisense oligonucleotides.[6, 11, 12] to increase the inhibition of virus multiplication. Therefore, we determined the influence of preincubation of HeLa cells with oligonucleotides on the extent of inhibition of virus-induced CPE. Cells were incubated with oligonucleotides at various concentrations for 24 or 48 h at 37°C. Then, the growth medium was removed and replaced by fresh maintain medium with serial dilution's of PS-ODN at the same concentrations as those used during preincubation. Prolonged pretreatment of HeLa cells with antiviral active PS-ODNs led to enhanced inhibition of CVB3-multiplication in these cells. Maximal antiviral effects were observed when antiviral oligonucleotides were added 48 h before virus inoculation. For example, the influence of preincubation time on the inhibition of CVB3-induced CPE by PS-ODN2, 3

and 4 is shown in FIG. 3. Depending on the target site and the orientation of PS-ODNs, 30 to 60 % inhibition of CPE was determined when PS-ODNs were added to HeLa cell monolayers 48 h prior virus inoculation (FIG. 4). The specificity of the anti-CVB3 PS-ODN was confirmed by the lack of inhibition of CVB3 multiplication by PS-ODN1 at 10 μM.

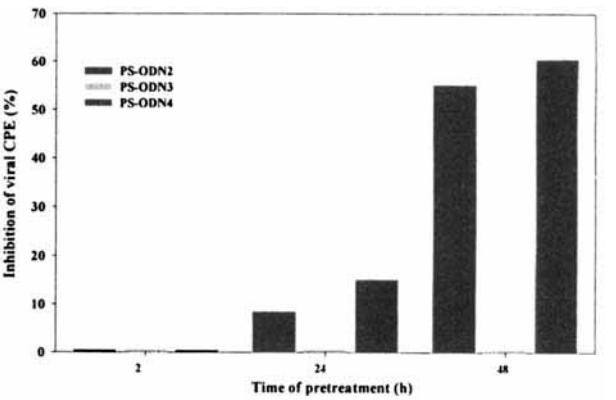

FIG. 3: Influence of pretreatment of cells with PS-ODNs on the extent of CPE-inhibition. Medium containing 10 μM of PS-ODN 2, 3 or 4 was added to HeLa cells. After the indicated time periods medium was removed. The percentage of CPE inhibition was determined in duplicate samples as described in the text.

The published values of inhibition of viral multiplication by virus-specific oligonucleotides ranged from 25 - 100 %. Besides the chemical modification and the lenght of oligonucleotides, the virus type as well as the experimental conditions (assay, cell line, mode of oligonucleotide application) seems to influence the degree of reduction of virus multiplication. Until now, the most promising results were achieved for HIV[13] and members of the herpesviridae.[6, 11, 12] These viruses replicate relatively slow in comparison to such RNA viruses such as measles-, influenza A-, foot-and-mouth disease virus (FMDV), and CVB3 which produce high numbers of virus copies in a short multiplication time. Both, the inefficient cellular penetration of oligonucleotides and the fast viral multiplication resulting in low viral RNA/ODN ratios may be responsible for the determined moderate antiviral activity of oligonucleotides in these examples.[14, 15, 16, 17] For instance, even microinjection of PS-ODN complementary to the AUG codon of FMDV resulted only in transient inhibition of viral VP1 expression in

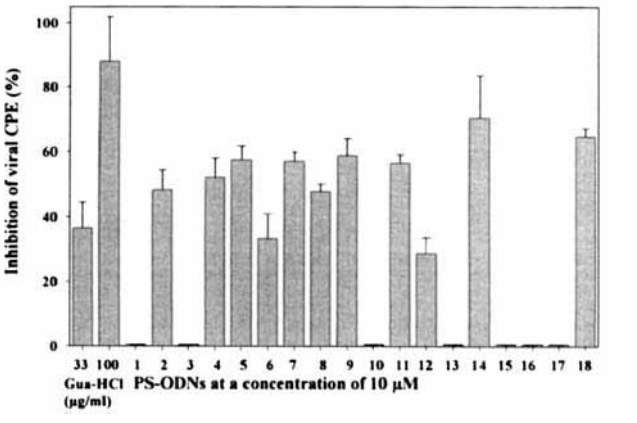

FIG. 4: Inhibition of CVB3-induced CPE in dependence on target site of PS-ODN. The degree of inhibition of virus-induced CPE was investigated on HeLa cells which were incubated with the indicated oligonucleotides 48 h before CVB3 application.

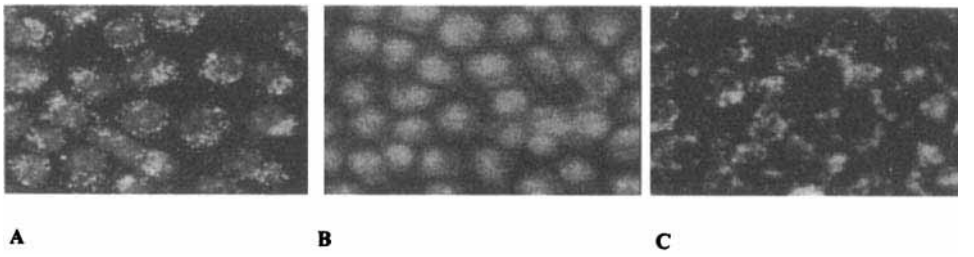

FIG. 5: Fluorescence pattern after internalization of 10 μM FITC-labeled PS-ODN1* (A), 2* (B), and 3* (C) into GMK (A) and HeLa cells (B+C) detected by fluorescence microscopy 24 h after application.

BHK1 cells.[16] Significant inhibitions in infectious FMDV yield, ranging from 35 - 52 %, were obtained using oligonucleotide concentrations of 125 - 250 μM at 5 h post infection. Moreover, it was proved that inhibition of FMDV replication was dose-dependent and was achieved at molar ratios of RNA to transcripts that were higher than 1 : 20.[16] The maximal capacity (30 - 60 % inhibition) of viral transcript to inhibit virus plaque formation was attained with an RNA/transcript ratio of 1 : 2000. An increased inhibition was found when viral RNA's and transcripts were allowed to reanneal before transfection.

Time- and concentration-dependent cellular uptake of PS-ODN1, 2 and 3. The internalization of phosphorothioate oligonucleotides was investigated by treating GMK- and HeLa cells with 2 and 10 μM of fluorescein-conjugated oligonucleotides. In a first approach, the fluorescence label within the cells was visualized by fluorescence microscopy (Wild Leitz GmbH, Wetzlar, Germany). After 24 h and 48 h of incubation of cell monolayers with either 2 or 10 μM oligonucleotide, significant signals of internalized fluorescence were visualized as shown in FIG. 5.

For PS-ODN1* and 3* the fluorescent signals appeared as a granular pattern surrounding the nuclei of treated cells. In contrast, only diffuse FITC-signals were detected after pre-incubation of cells with PS-ODN2*. The fluorescence intensity was stronger in cells treated with 10 μM than in those treated with 2 μM of oligonucleotides and increased with prolonged incubation time (data not shown).

To determine the time and concentration-dependent uptake of PS-ODN1*, 2* and 3* in more detail, GMK- and HeLa-cell monolayers grown in 24-well culture plates were

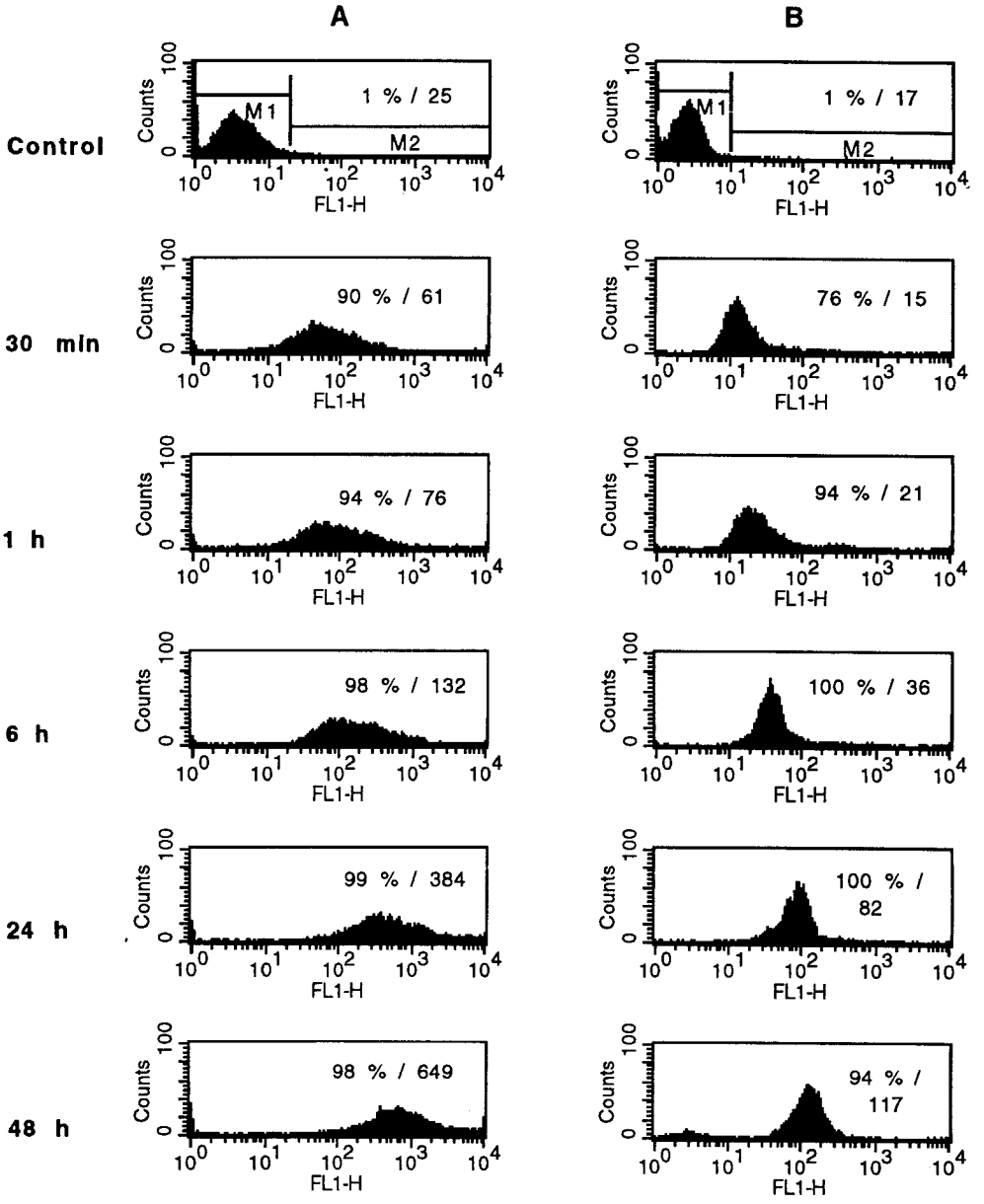

FIG. 6: Cellular uptake of FITC-labeled PS-ODN1* by GMK cells (column A) and of PS-ODN2* by HeLa cells (column B) at concentrations of 10 μM. FITC fluorescence (FL1) is depicted on the x-axis. The values in the histograms represent the percentage of ODN internalizing cells and the median of FL1 intensity.

incubated for indicated time intervals at 37°C in culture medium containing the FITC-conjugated anti-HSV1 or anti-CVB3 oligonucleotides at 2 or 10 μM concentrations. Cells incubated without ODNs served as controls. Then, the cells were washed 4 times with PBS, trypsinized and fixed with 0,4 % paraformaldehyde. Finally, GMK as well as HeLa cell suspensions were diluted with culture medium and flow cytometrical analysis was performed with a FACS-Calibur flow cytometer and CellQuest software (Becton Dickinson, Heidelberg, Germany). Each sample was run in a setup mode until 10000 events of the selected gate were acquired. Histogram statistics software was used to quantify the transformation rate and the median value which reflect the percentage of ODN internalizing cells and their relative median green channel fluorescence intensity (FL1). Histogram markers were set in such a way that ODN-free controls reached transformation rates of 1 % or less. The results from our study show that binding and/or penetration of PS-ODN1*, 2*, and 3* was significantly enhanced after treatment of cells at 10 μM concentrations in comparison to 2 μM. The percentage of oligonucleotide internalizing cells reached a plateau at approximately 6 h after application of 2 μM PS-ODN (data not shown). In contrast, the use of FITC-conjugated phosphorothioate oligonucleotides at a concentrations of 10 μM led to 90 - 99 % fluorescent cells within 30 to 60 min. (FIG. 6). The relative median fluorescence intensity was also increased by adding 10 μM oligonucleotides in comparison to 2 μM. Surprisingly, the median fluorescence intensity reached much higher values after treatment of GMK cells with PS-ODN1* than of HeLa cells with PS-ODN2* and 3*indicating the different capacities of these cell lines to take up PS-ODNs. No differences were determined with regard to the median value between PS-ODN2* and 3*. Therefore, only results obtained with PS-ODN2* were shown.

CONCLUSION: A moderate inhibition of CVB3 replication was observed using exogenous PS-ODNs directed against different regions of the virus genome. Obviously, the intracellular oligonucleotide concentrations are the limiting factor for the antiviral activity of the investigated PS-ODNs.

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