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Inhibition of herpes simplex virus in culture by oligonucleotides composed entirely of deoxyguanosine and thymidine

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Abstract

Oligodeoxynucleotides (ODNs) composed entirely of deoxyguanosine and thymidine, but not specifically designed to act as antisense agents, were able to significantly inhibit herpes simplex virus growth in acute infection assay systems. The guanosine/thymidine (GT) ODNs which demonstrated this antiviral activity contained either natural phosphodiester (PO) or phosphorothioate (PS) modified internucleoside linkages. In all experiments, the antiviral activity of the ODNs was enhanced when the backbone was modified to contain the PS linkages. When present during the time of virus addition, the ODNs were able to block the adsorption of virus to Vero cells. In this assay the PS-containing ODNs had ID_{50} values of approximately 0.020 μM for HSV-2 and of 0.3 μM for HSV-1. When these same PS-containing ODNs were used against HSV-2 in single-cycle viral yield assays, designed to minimize the effects due to external blockage of virus, the ID_{50} values rose to 0.2 μM. Analysis of viral DNA obtained 14 h post-HSV-2 infections in the single-cycle assay, revealed a decrease in replicated viral DNA in cells treated with PS-ODNs. Analysis of viral mRNA obtained 4 h post-HSV-2 infection revealed, in cells treated with the PS-ODNs, a decrease in measurable HSV-2 α- and β-mRNAs. Although the mechanism of action of the antiviral activity (beyond adsorption blocking) is not fully understood, the toxicity of these compounds was low, giving high therapeutic indices for the GT-rich PS-ODNs. The good therapeutic index of GT-ODNs make this a class of compounds which warrant investigation as therapeutic agents to be used against herpes viruses.

Keywords: Herpes simplex virus; Oligonucleotide; Deoxyguanosine; Thymidine; Phosphodiester; Phosphorothioate

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1. Introduction

Oligodeoxynucleotides (ODNs) are being investigated as possible therapeutic agents directed against viral and cellular gene targets, as well as research tools for the inactivation of selected genes. The use of ODNs containing a natural phosphodiester (PO) backbone, however, has been limited by the molecule's sensitivity to nucleases (Milligan et al., 1993; Stein and Cheng, 1993). To overcome the ODN nuclease sensitivity problem, many investigators have attempted to chemically modify the natural PO backbone, the 3′-terminal hydroxyl group or the bases themselves (Birg et al., 1990; Orson et al., 1991; Milligan et al., 1993). Therefore, many studies using ODNs to inhibit gene expression in tissue culture assays have used ODNs in which the natural PO backbone has been chemically modified by replacing one of the non-bridging oxygen atoms with a sulfur atom. The resulting phosphorothioate (PS) ODNs are more stable to nucleases than standard PO-ODNs, yet they retain uptake properties similar to those of unmodified ODNs, and in the case of antisense ODNs, the ability to form RNase H-sensitive duplexes (Milligan et al., 1993). Antisense PS-containing ODNs have been used to inhibit the expression of a variety of viral and cellular genes, including human immunodeficiency virus type 1 (HIV-1) *rev* protein (Matsukura et al., 1987), *bcl*2 (Reed et al., 1990), c-*myc* (Watson et al., 1991), and *cdc*2 (Lapidot-Lifson et al., 1992). However, problems are associated with the use of rationally designed PS-ODNs including reduced thermal stability of PS-ODN containing duplexes (Stein and Cohen, 1988) and increased non-specific interactions with RNA (Cazenave et al., 1989) and proteins (Ghosh et al., 1993). In addition, when the PO backbone of an antisense ODN is modified to a PS backbone in antiviral assays, the inhibitory activity increases for the antisense molecule as well as for ODNs used as negative controls (Milligan et al., 1993).

In addition to the rationally designed mechanisms by which ODNs exert their effects, recent reports have indicated that a variety of other potential mechanisms exist by which ODNs inhibit viral infections. For example, a PS-containing homopyrimidine (oligodeoxycytidine, poly-SdC) has been found to inhibit herpes simplex virus type 2 (HSV-2) and HIV-1 in acute culture assay systems (Matsukura et al., 1987; Gao et al., 1990b; Marshall et al., 1992). When Gao et al. (1989, 1990a,b) used poly-SdC_{28} to inhibit HSV-2 in culture, a number of different antiviral mechanisms were determined for this molecule including adsorption blocking and inhibition of HSV DNA polymerase. Matsukura et al. (1987), also using poly-SdC, showed that the inhibition of HIV-1 in culture was dependent on the size (length) of the ODN. One potential mechanism for this anti-HIV-1 activity, competitive inhibition of HIV-1 reverse transcriptase, was postulated by Marshall et al. (1992) while more recently Stein et al. (1993) characterized the interaction of poly-SdC_{28} with the v3 loop of HIV-1 gp120. Often poly-SdC or other control ODNs, with little or no homology to a specific gene target, show significant levels of activity, including the ability to inhibit AMV reverse transcriptase (RT), Pol I (Klenow fragment) and human polymerase α (Gao et al., 1989; Marshall et al., 1992), β and γ (Gao et al., 1989). In addition, oligonucleotides have been reported to bind to the cellular CD4 receptor, the coat glycoprotein of HIV-1 (Ojwang et al., 1994b), to cause transcription arrest at unintended sites (Praseuth et al., 1993), and to effect cellular growth and morphology (Yaswen et al., 1993) all without prior rational design.

In the present study, we describe a class of ODNs composed entirely of deoxyguanosine (G) and thymidine (T) which are particularly effective anti-HSV agents. While the natural PO backbone versions of these compounds show a limited level of antiviral activity, in acute assay systems, the PS versions of these same ODNs are able to inhibit both HSV-1 and HSV-2 with ID_{50} values in the submicromolar range. Some of the antiviral activity of these compounds is directly attributable to adsorption blocking of the virus to the outside of the target cell, but a portion of the overall antiviral profile is contributed by other, as yet undetermined, mechanisms of action.

2. Materials and methods

2.1. Reagents, cells and viruses

Radiochemical ([α-^{32}P]ATP), was purchased from New England Nuclear. The Random-Primed DNA labeling kit was purchased from Boehringer Mannheim Biochemicals (BMB). Nytran filter membranes were purchased from Schleicher and Schuell and Nick columns were obtained from Pharmacia. Acyclovir was obtained from Burroughs Wellcome. Phosphonoacetic acid (PAA) was obtained from Sigma.

The recombinant plasmid, pGR18, containing a 4.7 kb fragment of the HSV-2 thymidine kinase gene was provided by Dr. G.R. Reyes (Ingenex, Inc.). Vero cells were obtained from the ATCC. HSV-2 strain HG52 and HSV-1 strain 17 were obtained from Dr. D.J. McGeoch, University of Glasgow.

2.2. Oligodeoxynucleotide synthesis and modification

All oligodeoxynucleotides were synthesized on an Applied Biosystem Inc. DNA synthesizer model 380B or 394, using standard phosphoramidite methods at 0.2 or 1.0 μmol scales. 5′-Protected nucleoside phosphoramidite monomers and other reagents were obtained from Milligen with the exception of acetonitrile, which was obtained from Baxter. All oligodeoxynucleotides were synthesized with a 3′-Amino Modifier (Glen Research), which results in the covalent attachment of a propanolamine group to the 3′-hydroxyl group (Nelson et al., 1989; Durland et al., 1991) or with a cholesterol moiety attached to the 3′-terminus via a triglycyl linker (Vu et al., 1993). Phosphorothioate containing oligodeoxynucleotides were prepared using the sulfurizing agent TETD (Vu and Hirschbein, 1991). The purity of the oligodeoxynucleotides was confirmed by analytical HPLC, electrophoresis of ^{32}P-labeled oligo on a polyacrylamide gel, or by capillary gel electrophoresis. The 3′-cholesteryl modified oligodeoxynucleotides were prepared and purified as described by Vu et al. (1993).

2.3. Cytopathic effect assay

To determine the effect of ODNs on herpes viruses in culture a variation of the CPE assay, described by Ehrlich et al. (1965), was used. Vero cells were seeded onto 96-well culture plates (4×10^4 cells/well) in 0.1 of minimal essential medium (MEM) with

Earle salts (GIBCO BRL) supplemented with 10% heat inactivated fetal bovine serum (FBS) (GIBCO BRL) and pennstrep (100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, GIBCO BRL) and incubated at 37°C in a 5% CO_2 atmosphere overnight. The medium was then removed and 50 μl of medium containing 30–50 plaque forming units (PFU) of virus diluted in test medium and various concentrations of ODNs were added to the wells. Test medium consisted of MEM supplemented with 2% FBS and pennstrep. The virus was allowed to adsorb to the cells, in the presence of ODNs, for 10 min at 37°C. The test medium was then removed and the cells were rinsed 3 times with fresh medium. A final 100 μl aliquot of test medium was added to the cells and the plates were returned to 37°C. Cytopathic effect was scored 40–48 h postinfection when control wells showed maximum cytopathic effect.

2.4. Viral yield assay

The virus yield assay was performed as modified for the use of 96-well plates with minor modifications (Prichard et al., 1990). Vero cells were plated at 4×10^4 cells/well. The media was replaced with test medium containing various concentrations of ODNs 5–8 h after the cells were seeded, and the cells were returned to 37°C, 5% CO_2 for 20–24 h. At the time of virus infection, the test medium was removed, the cells were rinsed 3 times with fresh medium, and 3×10^4 PFU of HSV-2 was added. Virus was allowed to adsorb for 10 min at 37°C. The virus was then removed and the cells were again rinsed 3 times with fresh medium before test medium containing various concentrations of the same ODNs was added. The infected cells were cultured for 14 h at 37°C at which time the culture medium was removed, the cells were rinsed twice with fresh medium and overlayed with 100 μl of MEM/2% FBS. The plate was then placed at −70°C and subjected to a single round of freeze–thaw lysis. The virus present in the cell lysate was titered in 96-well plates on Vero cells. Time of addition studies were performed as viral yield assays with the drug added to infected cells at the indicated time relative to infection with HSV-2.

2.5. Cytotoxicity

Growth inhibition of Vero cells by ODN was determined using the Promega CellTiter 96™ Assay. Cells were initially seeded at 500 cells/well and allowed to grow in the presence of ODN for 4 days, when the assay was performed according to manufacturer's instructions. The TD_{50} is defined as the amount of compound required to give a 50% reduction in the dye metabolism compared to control wells containing no test compound.

2.6. DNA preparation and analysis

Vero cells were plated at (1.5×10^6 cells/well) 24 h before infection with HSV-2 (3×10^5 PFU). Total intracellular DNA was extracted from cells, 1 or 14 h postinfection, by lysing the cells in 10 mM Tris-HCl (pH 8), 25 mM EDTA, 100 mM NaCl and 0.5% SDS. The lysed cells were digested with 100 μg/ml proteinase K at 55°C for 2 h and deproteinized by phenol : chloroform extraction. Nucleic acids were precipitated

with 2 vol. of ethanol, redissolved in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA and then treated with RNase A (100 μg/ml) at 37°C for 1 h. Concentrated ammonium acetate was added to the aqueous phase to yield a final concentration of 0.4 M. The DNA was again precipitated with ethanol and washed with 70% ethanol. Various concentrations of purified DNAs were denatured, immobilized on Nytran membranes using a BioRad dot slot manifold, and pretreated in a buffer containing 1 × SSC, 0.5% SDS for 30 min at 42°C. The filter membranes were then prehybridized in a buffer containing 6 × SSC, 5 × Denhardt's solution, 1% SDS, 50% formamide, 1% non-fat dry milk and 100 μg/ml of both denatured salmon sperm DNA and yeast tRNA. The filter was prehybridized at 42°C for 6 h. The 4.7-kb fragment of the HSV-2 thymidine kinase (TK) gene derived from plasmid pGR18 (Reyes et al., 1982) or total extracted cellular (Vero) DNA were radiolabeled using [α-^{32}P]ATP and the random priming kit. The radiolabeled probes were then purified away from unincorporated deoxynucleotide triphosphates using Nick columns and added to the prehybridization buffer (5.3×10^6 cpm/ml). The hybridization reactions were allowed to proceed for 16–18 h at 42°C at which time the filters were washed (twice with 2 × SSC, 0.1% SDS for 10 min at room temperature and then twice with 0.1 × SSC, 0.1% SDS for 15 min at 50°C), dried and either processed for autoradiography or quantitated using a Betascope blot analyzer (Betagen).

2.7. RNase protection assays

Vero cells (2×10^6 cells) were plated in T25 flasks in MEM/10% FBS and incubated at 37°C overnight. The medium was then removed, replaced with MEM/2% FBS (with or without B106-96 where indicated) and further incubated for 18 h. One hour before virus infection, PAA was added to the appropriate flasks. At the time of viral infection, medium was removed from the flasks, the cells rinsed three times and then infected with 1×10^5 PFU of HSV-2 in a volume of 1 ml/flask. The virus was allowed to adsorb for 10 min at 37°C at which time the medium was removed and the cells were rinsed once with acidic phosphate-buffered saline (PBS, pH 3.0) and twice with MEM/2% FBS. Fresh-medium containing the appropriate drug was then added to the cells in test medium. Each treatment and RNA isolation was performed in triplicate. RNA was then isolated from the cells at 4 or 10 h postinfection as described by Chomczynski and Sacchi (1987).

RNase protection assays were performed using the RPAII Ribonuclease Protection Assay Kit (Ambion), 2.5 μg of extracted RNA per reaction and the assay conditions specified by the manufacturer. Molecular probes were generated by polymerase chain reaction (PCR) amplification of specific regions of the HCMV IE63 (−58 to +188 relative to the mRNA start site) gene (Whitton et al., 1983) or TK (approximately −75 to +390 relative to the putative mRNA start site) gene (Kit et al., 1983) and cloning of these DNA fragments into the bacterial plasmid vector pCR II. Radioactive single-stranded probes generated using SP6 or T7 polymerase and [α-^{32}P]ATP were gel purified before use. For each gene, the probe spanned the putative transcription initiation site and the fragments protected were the predicted size. Reaction products were separated using a 5% denaturing polyacrylamide gel, dried and either processed for autoradiography or quantitated using a Betascope blot analyzer (Betagen).

3. Results

The initial ODNs used in this study were PO and PS versions of two similar sequences, each composed entirely of G and T (Table 1). These GT-rich ODNs were 36 nucleotides (nt) in length with a 2-hydroxy-propanolamine or triglycyl-linked cholesterol blocking group at the 3′-terminus to prevent degradation. Capping of the 3′-hydroxyl terminus of ODNs has been shown to greatly reduce degradation by both cellular and extracellular exonucleases (Birg et al., 1990; Orson et al., 1991). In addition GT-rich ODNs with capped 3′-termini have been reported to be stable both in vitro and in vivo (Durland et al., 1991; Orson et al., 1991; Zendegui et al., 1992; Ojwang et al., 1994a). Oligonucleotides B106-62, B106-85 and B106-96 synthesized for this study are identical in sequence but differ in either internucleoside backbone linkage or 3′-capping group (Table 1). In addition, they have the same base composition as ODNs B106-71 and B106-97, but with a slightly altered arrangement of the nucleotide sequence to minimize potential sequence-specific activities while maintaining the general structure of the ODN (Table 1).

3.1. Cytotoxicity

The GT-rich ODNs were tested for their ability to inhibit Vero cell growth using the dye metabolism assay described in Materials and methods. Vero cells were plated at a concentration that insured that during the period of the test, the cells would be in continuous growth. Under these conditions the ability of the culture to metabolize the dye is dependent on the ability of the cells to grow and divide in the presence of drug. In this assay system cells go through approximately 5 doublings in the presence of compound. In this assay the PS-ODN B106-96 was slightly more inhibitory than the

Table 1
Guanosine/thymidine oligonucleotide sequences synthesized for use in CPE and single-cycle viral yield assays

ODN	Backbone [a]	Sequence	Length (nt)
B106-62	PO	5′-gtggtggtggtgttggtggtggtttgggggggtgggg-3′	36
B106-85 [b]	PO	5′-gtggtggtggtgttggtggtggtttgggggggtgggg-3′	36
B106-96	PS	5′-gtggtggtggtgttggtggtggtttgggggggtgggg-3′	36
B106-140	PS	5′-gtggtggtggtgttggtggtggtttg-3′	26
B106-71	PO	5′-gtggttggtggtggtgtgtgggtttggggtgggggg-3′	36
B106-97	PS	5′-gtggttggtggtggtgtgtgggtttggggtgggggg-3′	36
I100-12	PS	5′-gttgggggttgttggtggggtggtgg-3′	26
I100-21	PS	5′-gtggtgggtgggtgggtggtgggtggtggttgtgggtgggtggtg-3′	45
G106-57	PS	5′-ggtgggtgggttgggggggtgggtgggg-3′	27
1162	PS	5′-ccgcgcggcatctcattagcgcccggc-3′	27
1207	PS	5′-gggggggggggggg-3′	15
1205	PS	5′-ccccccccccccccc-3′	15

[a] The internucleoside backbone linkages were either phosphodiester (PO) or phosphorothioate (PS).
[b] The 3′-terminus modification of B106-85 was a triglycyl-linked cholesterol moiety. All other molecules were protected at their 3′-terminus by the addition of a propanolamine group.

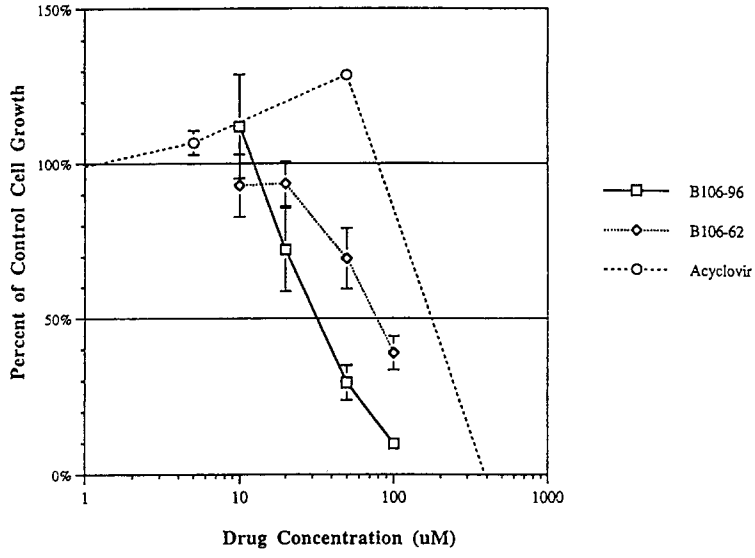


Fig. 1. Effect of PO- and PS-ODNs on the growth of Vero cells. Vero cells were cultured as described in Materials and methods. The data represent the number of viable cells, in the presence of drug, after 4 days in culture relative to untreated samples. The cells were plated at a concentration (500 cells/well) which ensured that cells would double 4–5 times, in untreated samples, during the course of the assay.

PO-ODN B106-62 with TD_{50} values of approximately 30 and 80 μM, respectively (Fig. 1, Tables 2 and 3).

3.2. Adsorption blocking / CPE inhibition

All of the ODNs listed in Table 1 (except B106-85) were tested in an abbreviated CPE assay designed to measure the ability of an ODN to block the interaction of HSV-1

Table 2
Blockage of HSV adsorption to Vero cells by PO- and PS-ODNs monitored using the cytopathic effect assay

ODN	Backbone [c]	ID_{50} (μM) [a]		TD_{50} [d]	Therapeutic index [b]	
		HSV-1	HSV-2		HSV-1	HSV-2
B106-62	PO	> 5.0	0.5	77.1 (9.0)	< 15	155
B106-96	PS	0.3	0.02	31.8 (6.2)	106	1590
B106-71	PO	> 5.0	0.5	n.d. [e]	n.d.	n.d.
B106-97	PS	0.3	0.02	n.d.	n.d.	n.d.

[a] The ID_{50} values were determined from multiple experiments scored for plaque reduction performed in duplicate or triplicate.
[b] The therapeutic index is the ratio of the TD_{50} to the ID_{50} (TD_{50}/ID_{50}).
[c] The internucleoside backbone linkages were either phosphodiester (PO) or phosphorothioate (PS).
[d] The TD_{50} values and standard deviations (given in parentheses) are in μM and represent the dose required to inhibit Vero cell growth by 50%.
[e] Value not determined (n.d.).

Table 3
Inhibition of HSV-2 in Vero cells by PO- and PS-ODNs monitored using the single-cycle viral yield assay

ODN	Backbone [b]	Anti-HSV-2 activity (μM) [a]		TD_{50} [c]	Therapeutic index [d]
		ID_{50}	ID_{90}		
B106-62	PO	17.3 (9.7)	> 20.0	77.1 (9.0)	4.4
B106-96	PS	0.16 (0.03)	0.55 (0.05)	31.8 (6.2)	198.7
B106-85	PO	3.4 (1.9)	> 20.0	> 100	> 29.0
B106-71	PO	> 5.0	> 5.0	n.d. [e]	n.d.
B106-97	PS	0.23 (0.12)	0.70 (0.18)	n.d.	n.d.
Acyclovir		0.84 (0.10)	15.65 (2.58)	144.2 (2.5)	171.4

[a] The dose required to inhibit virus growth by 50% (ID_{50}) or 90% (ID_{90}). The standard deviations (in μM) are given in parentheses.
[b] The internucleoside backbone linkages were either phosphodiester (PO) or phosphorothioate (PS).
[c] The dose required to inhibit growth of Vero cells by 50% (TD_{50}). The standard deviations (in μM) are given in parentheses.
[d] The therapeutic index is the ratio of the TD_{50} to the ID_{50} (TD_{50}/ID_{50}).
[e] Value not determined (n.d.).

or HSV-2 with Vero cells. In these assays, the ODN was present briefly at the time of virus adsorption only. It was then washed away, fresh medium was added to the cell culture, and the virus infection was allowed to progress in the absence of test compound. The PS-containing ODNs demonstrated a strong ability to block HSV-2 infection in this assay. The dose of PS-ODNs required to inhibit HSV-2 by 50% (ID_{50}) was approximately 20 nM, while the ID_{50} of the matched sequence PO-containing ODNs was approximately 10–20 fold higher at 500 nM (Table 2). In identical assays using HSV-1, there was less observable inhibition for all ODNs tested, though the PS-containing molecules still demonstrated significant activity while the PO-ODNs showed no inhibition of HSV-1 adsorption at the highest concentration tested (Table 2). In these experiments, the inhibition of virus by ODNs was not due to an irreversible effect on the virus particles during the short period of incubation. Short (1 h) preincubation of HSV-2 with ODN before infection did not severely affect the infectivity of the virus as long as the ODN was diluted at the time of infection (data not shown).

3.3. Single-cycle viral yield assay

To evaluate the antiviral activity of the oligonucleotides, independent of their ability to block adsorption of the virus, a single cycle virus yield assay was used. In this assay ODNs were added to the Vero cell culture medium for 20–24 h before infection with HSV-2, removed at the time of virus infection, reapplied to the medium for 14 h and then removed again for the subsequent assay round. These assay conditions eliminate the presence of ODN during the period of virus adsorption, both during the initial infection, and during the assay round of infection. At 14 h postinfection, the titer was determined for the cell-associated virus. Cell-associated virus constitutes > 95% of the total virus at this point in the virus replicative cycle (data not shown). All ODNs tested continued to show anti-HSV-2 activity though the ID_{50} values shift higher by approximately a factor

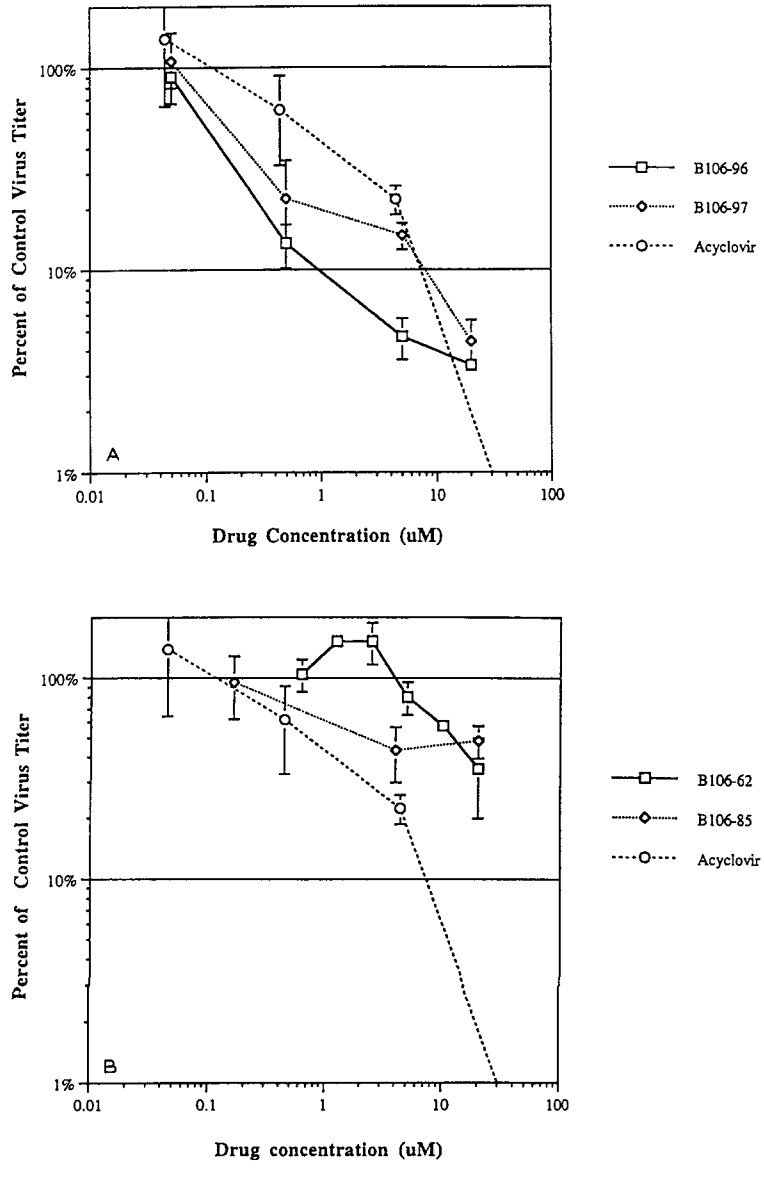


Fig. 2. Effect of PO- and PS-ODNs on HSV-2 growth. The data are presented as a fraction of the virus titer observed in control assays (no drug). The results obtained using PS-ODNs are depicted in A, while the results obtained using the PO molecules are presented in B. The cholesterol modified PO-ODN (B106-85) is shown in B.

of 10 for both the PO- and PS-ODNs (Fig. 2, Table 3). The ID_{50} values for the PS-ODNs B106-96 and B106-97 were approximately 0.2 μM in this assay (Table 3). It is interesting to note that the ID_{90} values for the PS-ODNs B106-96 and B106-97 were

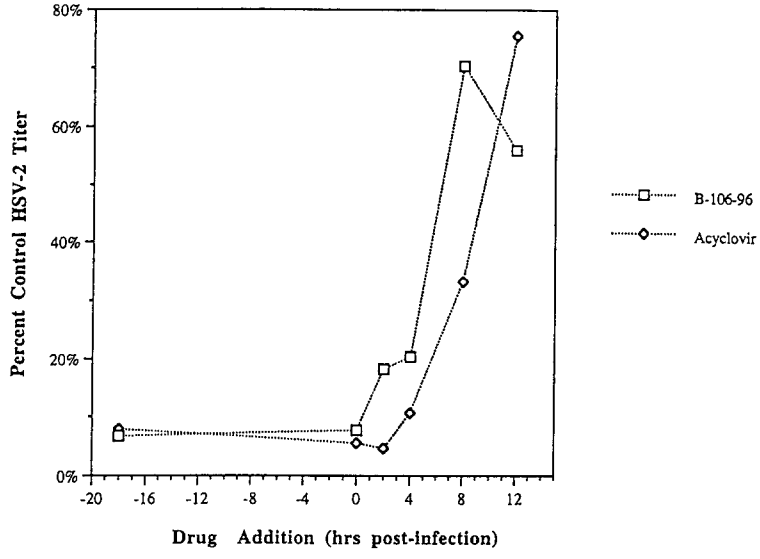


Fig. 3. Effect of timing of drug addition on HSV-2 viral yield. Drug (5 μM B106-96 or 4.4 μM ACV) was added at the indicated time, with $t = 0$ representing the end of the virus adsorption period. For the 18-h time point, drug was removed during the period of virus adsorption, then added again after extensively rinsing cells to remove residual virus. Data are presented as the viral yield as a fraction of the control (infected but not treated cells) well.

under 1 μM (Table 3). Results obtained from experiments in which the PS-ODNs were only added to the cells in culture after the viral infection, were similar to those presented in Table 3 (in which drug was added before and after, but not during, viral infection). In addition, B106-62 and B106-96 were able to inhibit HSV-2 infection of Hep-2 cells with the same efficacy as shown in the Vero cell assay system (data not shown).

In contrast to the reports of Letsinger et al. (1989) describing the enhanced efficacy of anti-HIV-1 ODNs containing a cholesterol modification, cholesterol addition to the 3′-terminus of a PO-ODN, B106-85, had only a slight enhancing effect on its activity against HSV-2. The discrepancy in results may be due to differences in the viruses studied or in the linkage schemes used for the attachment of the cholesterol moiety to the oligonucleotide.

The effect of the timing of addition of a PS-ODN was determined. B106-96 (5 μM) and ACV (4.4 μM) were added at various times before, during and after infection of Vero cells with HSV-2. Addition of ACV 4 h post-HSV-2 infection minimally affected the viral suppression; however, at the 8-h time point the antiviral profile of ACV was significantly reduced (Fig. 3). This result is consistent with the proposed mechanism of action for ACV (interference with viral DNA synthesis). In cells treated with B106-96, a small portion of the overall antiviral activity was lost when the drug was added up to 4 h postviral infection, and a major decrease in activity observed if the drug was added 8 h postinfection (Fig. 3). The early loss of activity may be due to residual adsorption blocking of the virus, while the later loss in activity may coincide with events leading up to or including viral DNA synthesis.

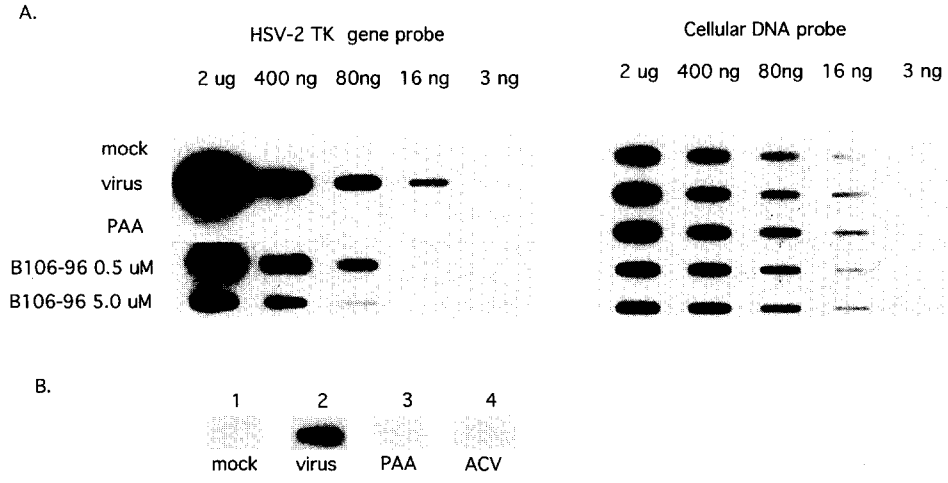


Fig. 4. Analysis of viral DNA in Vero cells treated with a PS ODN. (A) Total DNA extracted from HSV-2-infected Vero cells 14 h postinfection was applied (in various concentrations from 2 μg down to 3 ng) to a Nytran membrane and probed for the presence of HSV-2 DNA using the HSV-2 TK gene molecular probe or for the presence of cellular DNA using total Vero cell DNA as a probe. The samples in both panels, corresponding to uninfected Vero cells (virus$^-$), HSV-2-infected Vero cells (virus$^+$) or HSV-2-infected Vero cells treated with PAA (150 μg/ml) or B106-96 (0.5 and 5.0 μM), are labeled to the left of the HSV-2 TK gene probe panel. (B) In this panel 400 ng of DNA extracted from mock infected or HSV-2 Vero cells was applied to a Nytran membrane and probed for the presence of HSV-2 genomic DNA. PAA (150 μg/ml) or ACV at 44 μM (10 μg/ml) were used on the HSV-2-infected cells.

3.4. Effects of a PS-ODN on HSV-2 DNA and RNA in a single-cycle assay

To confirm that a component of the observed antiviral activity of the PS-ODNs was due to a mechanism other than blockage of viral adsorption, we isolated total DNA from B106-96 treated Vero cells 1 and 14 h postinfection with HSV-2. As a control for this experiment we used a drug (PAA) which is known to inhibit herpes virus DNA synthesis (Honess and Watson, 1977). In this experiment, Vero cells were infected with HSV-2 for 10 min, washed extensively and then incubated in complete medium containing 0.5 or 5.0 μM B106-96 or 150 μg/ml PAA. The results of this assay demonstrated that 1 h postinfection, the level of viral DNA present in untreated infected cells was equivalent to that obtained from infected cells treated with 106-96 at 5 μM (data not shown). In DNA samples extracted 14 h postinfection, less viral DNA was detectable in cells treated with B106-96 or PAA relative to virus-infected untreated Vero cells (Fig. 4A). In additional experiments, ACV, at 10 μg/ml (44 μM), was included as a control and, as expected, allowed little or no viral DNA synthesis (Fig. 4B). These results suggests that while all cells were infected equally with HSV-2 the cells treated with ODN were inhibitory to virus DNA replication. However, when compared to PAA or ACV, it was obvious that the level of reduction of viral DNA synthesis was not as dramatic.

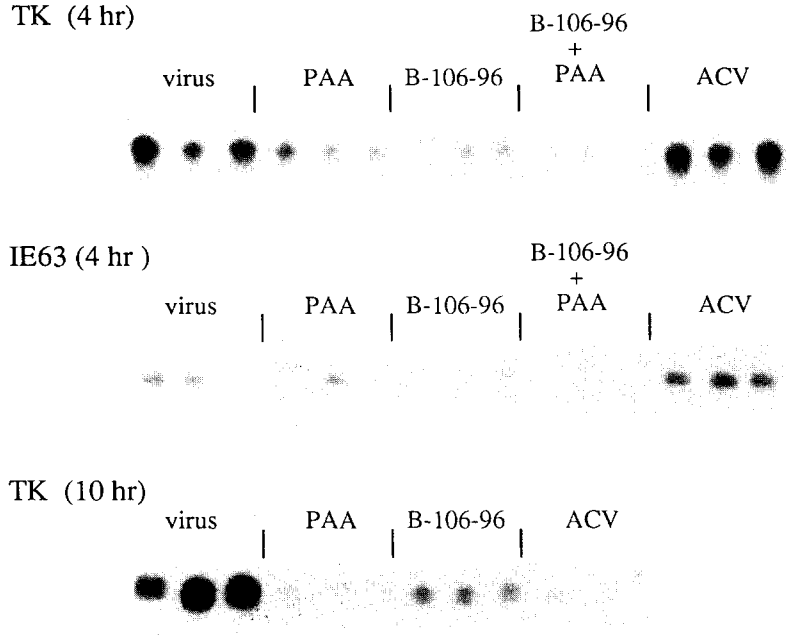
To determine the stage of the virus life cycle at which B106-96 was exerting its antiviral effect, mRNA levels were examined for representative HSV-2 genes of the α- and β-class. The α-gene studied was IE63 (immediate early gene) and the β-gene studied was the HSV-2 TK gene. The HSV-2 TK gene is transcribed before DNA synthesis, but slightly later than, and dependent upon, the transcription of the immediate early genes. In RNA samples harvested 10 h post-HSV-2 infection, a significant decrease in TK RNA levels was observed for all drugs tested (Fig. 5A). Like the DNA synthesis inhibitor PAA, the results obtained using B106-96 showed a decrease in both α- and β-mRNAs. Since this time point represents a period after the onset of DNA synthesis, this result is consistent with the data presented in Fig. 3 (timing of drug addition) in which inhibition of virus occurred when the drug was added up to the time of DNA synthesis. Inhibition of DNA synthesis would be expected to cause a decrease in the 10-h β-gene RNA levels. To examine events before the onset of DNA synthesis, RNA was harvested 4 h postinfection, and examined for the levels of IE63 and TK RNAs. Both acyclovir and PAA were used at concentrations which allowed little to no viral DNA synthesis to occur (Fig. 4B). As can be seen in Fig. 5B, though acyclovir, as expected, had no effect on this early time point RNA (pre-DNA synthesis), B106-96 gave reduced levels of both RNA classes studied. In combination with PAA, this reduction was further enhanced. Surprisingly, PAA alone was also able to depress the level of viral mRNA, albeit at a much reduced level compared with B106-96, though ACV gave no reduction in corresponding RNA levels. The ODN was therefore interfering in the virus cycle, to some extent, even before the onset of DNA synthesis. It is not possible to determine if the transcription inhibition seen early in the infection is sufficient to account for subsequent DNA synthesis inhibition, or if the interference with DNA synthesis results from a more direct interference with the DNA polymerase enzyme.

3.5. Anti-HSV-2 activity of size and sequence variants of B106-96

It was apparent from the single cycle and CPE inhibition assays that there was no difference in the antiviral profile for the PS-ODNs B106-96 and B106-97. However, because the ODNs tested were 36 nt in length, any differences in antiviral activity due to the sequence of these molecules may have been missed, because other mechanisms of action, dependent on the length of the ODNs, compensated for the difference. For this reason, GT-rich ODNs of various length and sequence, with PS backbones, were assayed for their ability to inhibit HSV-2 production in the single-cycle assay. The results of this experiment clearly show that several different PS-containing GT-rich ODNs (B106-140, I100-12 and G106-57), all 26 or 27 nt in length, were just as effective

Fig. 5. Effect of drug treatment on viral RNA levels. (A) Ribonuclease protection gels showing the levels of IE63 and TK mRNA present at 4 or 10 h post-HSV-2 infection. Triplicate cultures of untreated, PAA (150 μg/ml), B106-96 (5 μM), B106-96 (5 μM) plus PAA (150 μg/ml) or ACV (44 μM) treated HSAV-2-infected Vero cells are shown. (B) Ribonuclease protection assay gels (using RNA extracted 4 h postinfection) were quantitated using a Betascope blot analyzer; RNA levels are expressed as a percent of the infected but untreated control cell RNA.

A.


TK (4 hr)
virus
PAA
B-106-96
B-106-96
+
PAA
ACV
IE63 (4 hr)
virus
PAA
B-106-96
B-106-96
+
PAA
ACV
TK (10 hr)
virus
PAA
B-106-96
ACV

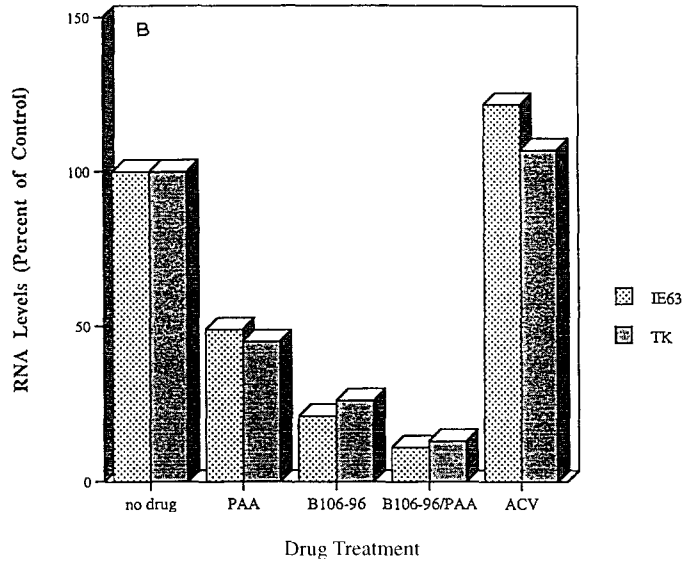

B
150
100
50
0
RNA Levels (Percent of Control)
no drug
PAA
B106-96
B106-96/PAA
ACV
Drug Treatment
IE63
TK

Table 4
Analysis of antiviral activity of G-rich and non-G-rich ODNs using the single-cycle viral yield assay (VYA)

ODN	Backbone [a]	Length [b]	%G [c]	VYA ID_{50} (μM) [d]
B106-62	PO	36	66.6	> 5.0
B106-96	PS	36	66.6	0.05–0.50
B106-140	PS	26	57.7	0.05–0.50
B106-97	PS	36	66.6	0.05–0.50
I100-12	PS	26	65.3	0.05–0.50
I100-21	PS	45	66.6	0.05–0.50
G106-57	PS	27	77.7	0.05–0.50
1162	PS	27	29.6	0.50–5.00
1207	PS	15	100	0.50–5.00
1205	PS	15	0	> 5.0

[a] PO = phosphodiester backbone and PS = phosphorothioate backbone.
[b] Length indicates the length in nucleotides of each oligodeoxynucleotide.
[c] The percent of the ODN composed of deoxyguanosine nucleotides.
[d] Viral yield assay results obtained from intracellular virus obtained 14 h postinfection. The values given are the ranges obtained from multiple experiments performed in triplicate.

at reducing HSV-2 titers as GT-rich PS-ODNs consisting of 36 (B106-96, B106-97) or 45 (I100-21) nt (Table 4). It is interesting to note that a shorter ODN (15 nt) consisting of only deoxyguanosine (1207) was less active than the longer GT-rich ODNs and the short ODN consisting of only deoxycytosine (1205) had no activity at the highest concentration tested (Table 4). In addition, the PS-ODN 1162 (27 nt in length and composed of all 4 deoxynucleotides) was also less active in this assay system than GT-rich ODNs of equal length.

4. Discussion

In this report we describe how a class of ODNs which contain only G and T, synthesized with either PO or PS backbones, were able to inhibit HSV in culture assays. Our studies reflect a continuation of many reports on the antiviral adsorption blocking ability of PS-containing ODNs. For example, in the study by Gao et al. (1990a) poly-SdC_{28}, but not poly-dC_{28}, was able to block the adsorption of HSV-2 to cells. In addition, Stein et al. (1993) postulate that some of the anti-HIV-1 activity observed for poly-SdC_{28} was by the binding of this molecule to the v3 loop of HIV-1 gp120. In the present study, we were able to demonstrate inhibition of HSV-2 adsorption even with PO-containing ODNs, possibly via a mechanism attributable to the unusual GT-rich nature of the ODNs used. The difference in results obtained in this study may be due to the increased stability of GT-rich PO-ODNs in tissue culture conditions relative to PO-ODNs composed of a more even representation of deoxynucleotides (Ojwang et al., 1994a). The antiviral activity of a GT-rich PO-ODNs was increased dramatically when the PO was changed to a PS backbone.

It is evident from the adsorption blocking data (Table 2) that when evaluating an ODN as an antiviral agent one should consider the ODN's effects on the external interactions of the virus with the cell. It is not possible at this time to predict with certainty which viruses will be influenced in their cellular binding by ODNs. For example, HSV-1 was relatively resistant to blocking by ODNs, even PS-containing ODNs, while HSV-2 was extremely sensitive (Table 2). The sensitivity of a virus to the adsorption blocking ability of PS-ODNs may correlate with their susceptibility to sulfated polysaccharides such as dextran sulfate. HSV, human cytomegalovirus (HCMV) and HIV-1 are all susceptible to blockage by both dextran sulfate and sulfur-modified ODNs (Babba et al., 1988; Milligan et al., 1993; Stein and Cheng, 1993). In addition to HSV, CMV and HIV, sulfated polysaccharides have been shown to be potent inhibitors of vesicular stomatitis virus and Sindbis virus, moderately inhibitory to vaccinia virus, but not inhibitory to adenovirus, coxsackievirus, poliovirus, hepatitis virus, parainfluenza virus or reovirus (Babba et al., 1988; Offensperger et al., 1991). It will be interesting to see in the future if other lipid-enveloped viruses, sensitive to sulfated polysaccharides, react similarly to PS-ODNs.

When the PS-containing GT-rich ODNs were evaluated using the single-cycle assay, the ID_{50} values were approximately 10-fold higher than the inhibitory values obtained in the CPE assay. The observed antiviral activity of the PO-ODNs was also diminished in the single-cycle assay (Fig. 2, Table 3). Any assay, therefore, in which ODNs are present at the time of virus infection, or in which the virus infection is allowed to progress beyond the first cycle of infection with ODN in the medium, could be influenced by the ability of ODNs to block virus/cell interactions. For example, Hoke et al. (1991) reported HSV-1 inhibition with PS-containing ODNs, though the assay conditions included a 48-h postinfection incubation with the ODN, thereby including at least one additional round of replication in the presence of ODN. In addition, Kmetz et al. (1991) and Draper et al. (1990), used PS-containing ODNs against HSV-1 for 24–48 h, thereby allowing the adsorption blocking effect of the ODNs to contribute to the overall antiviral profile.

Gao et al. (1990a,b) attributed a great deal of the antiviral activity of poly-SdC_{28} to the ability of the ODN to block viral adsorption, while at the same time speculating that other mechanisms of action, such as inhibition of herpes virus-induced DNA polymerase, were at work (Gao et al., 1989, 1990a). In addition, an increase in oligonucleotide uptake by the cell with virus infection would increase any proposed intracellular mechanisms. The dose-dependent decrease in viral DNA found in Vero cells treated with the PS-ODN B106-96, 14 h postinfection, supports the notion of alternative mechanisms of action (Fig. 4). It is not known at this time, however, whether the observed decrease in newly synthesized viral DNA in this single-cycle assay was due to a direct inhibition of the viral DNA polymerase. Both the timing of drug addition study and the analysis of the viral RNA and DNA indicate that B106-96 was capable of inhibiting immediate early and even early viral function (events leading up to and possibly including viral DNA synthesis). At this time it is difficult to assign a mechanism of action for the inhibition of the α-genes so that it is not known how much of the inhibition of these genes translates temporally into an inhibition of viral DNA synthesis. In these experiments, we have attempted to minimize the influence of the

ODN on extracellular virus by stringent washing; however, we cannot be certain that the reduction in early viral RNA levels (4-h time point) is due to a mechanism unrelated to adsorption blocking.

Previous studies on ODN inhibition of herpes viruses used only mixed sequences or short homopolymers of bases other than G (Gao et al., 1990a; Milligan et al., 1993; Stein and Cheng, 1993). Studies conducted on poly-SdC do not adequately predict the differences in activity of GT-rich ODNs observed in this study. For example the adsorption blocking ability of PO-containing ODNs for HSV-2, and of PS-containing molecules for HSV-1 was not detectable for poly-dC_{28} (Gao et al., 1990a), but is measurable for the GT-rich ODNs (Table 2). In addition, the ability of poly-SdC_{15} to inhibit HSV-2 in a CPE assay was minimal (Gao et al., 1990a) whereas poly-SdG_{15} (ODN 1207) significantly reduced HSV-2 titers in the single-cycle assay (Table 4).

All of the mechanisms by which this class of GT-rich ODNs inhibit viruses are not known. These molecules were not designed as antisense ODNs to a specific target, and it is unknown if they act as antisense molecules at unknown viral or cellular targets (though an alignment search of the known HSV-1 and HSV-2 genomic sequences failed to identify any significant matches with the B106-96 sequence). They do possess a relatively high G content and may form duplexes with unintended targets. Additionally, they may be capable of binding to duplex DNA to form triplex DNA structures at unknown sites. However, the fact that 4 different GT-rich sequences (Table 4) were able to inhibit HSV-2 with relatively the same efficacy suggests that these molecules do not specifically interact with viral mRNA or DNA targets. Experiments designed to help elucidate the potential mechanism(s) of action are underway. At the same time the good therapeutic index obtained for the GT-rich PS-ODNs offers encouragement for the continued development of this class of ODNs as antiviral agents.

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