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Antiviral effect of oligo(nucleoside methylphosphonates) complementary to the herpes simplex virus type 1 immediate early mRNAs 4 and 5

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Summary

We have previously shown that an oligo(nucleoside methylphosphonate) (deoxynucleoside methylphosphonate residues in italics) complementary to the acceptor splice junction of herpes simplex virus type 1 (HSV-1) immediate-early (IE) pre-mRNAs 4,5 [d(Tp*TCCTCCTGCGG*)], causes sequence-specific inhibition of virus growth in infected cell cultures (Smith et al., 1986; Kulka et al., 1989). Here we report a similar inhibition of HSV-1 growth by oligo(nucleoside methylphosphonates) complementary to the splice donor site of HSV-1 IE pre-mRNAs 4,5 [d(Gp*CTTACCCGTGC*)] and to the translation initiation site of IE4 mRNA [d(Ap*ATGTCGGCCAT*)]. An oligomer complementary to the translation initiation site of IE5 mRNA [d(Gp*GCCCACGACAT*)] or an unrelated oligomer [d(Gp*CGGGAAGGCAC*)] did not inhibit virus growth. IC_{50} values were 20, 25 and 20 μM for d(Tp*TCCTCCTGCGG*), d(Gp*CTTACCCGTGC*) and d(Ap*ATGTCGGCCAT*) respectively. In infected BALB/c mice d(Tp*TCCTCCTGCGG*) caused a significant decrease in HSV-1 growth (82% inhibition at 500 μM). A psoralen-derivative of d(Tp*TCCTCCTGCGG*) that binds covalently to complementary sequences after exposure to 365 nm irradiation, inhibited HSV-1 growth (86–91%) at a 10-fold lower concentration than the non-derivatized oligomer. The inhibition was sequence-specific and significantly

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lower (27%) for HSV-2 that differs from HSV-1 in 7 of the 12 bases targeted by d(Tp*TCCTCCTGCGG*). Virus growth was not inhibited by d(Gp*GCCCACGACAT*). The data suggest that oligo(nucleoside methylphosphonates) may be effective antiviral agents.

Antiviral; Antisense; HSV-1; Oligo(nucleoside methylphosphonates)

Introduction

Antisense RNA and DNA that selectively inhibit gene expression at the level of mRNA processing or translation can be used to study the regulatory aspects of gene expression, and has been proposed as a genetic approach for the prevention and treatment of diseases (reviewed in Helene and Toulme, 1990; Cohen, 1991). The limitations of the native oligodeoxynucleotides are their relatively short half-life and low cellular penetration. Additionally, the precise mechanism(s) by which antisense molecules inhibit their targets in eukaryotic cells are still poorly understood. To address some of these problems we have developed sequence-specific non-ionic nucleic acid analogues that contain a 3′-5′ methylphosphonate group in place of the negatively charged phosphodiester group normally found in oligonucleotides (Miller et al., 1986). These oligomers are resistant to nuclease hydrolysis, penetrate mammalian cells (Miller et al., 1981) and specifically inhibit the expression of several target genes (Agris et al., 1986; Blake et al., 1985).

To explore the possibility that control of viral gene expression by the methylphosphonates could be an effective antiviral modality we focused on their ability to inhibit HSV-1 growth. The strategy is based on the finding that HSV gene expression is regulated in a cascade fashion that includes three classes of genes: immediate-early (IE; alpha), delayed-early (DE; beta) and late (L; gamma). The products of the IE1, IE2 and IE3 genes are required for the regulation and expression of DE and late genes (reviewed in Everett, 1987), but controversy remains as to the function of the IE4 and IE5 genes. Genetic studies using deletion mutants suggested that IE5 is not required for HSV growth (Mavromara-Nazos et al., 1986). IE4 is required (Jacquemont et al., 1984) but its regulatory function can be complemented by host-encoded proteins (Sears et al., 1985). Whatever the exact roles of these genes in HSV-1 growth, we found that an oligo(nucleoside methylphosphonate) complementary to the splice acceptor junction of HSV-1 IE pre-mRNAs 4,5 [d(Tp*TCCTCCTGCGG*)] inhibits splicing of IE pre-mRNA 4,5 as well as viral protein and DNA syntheses and it reduces virus growth in culture (Smith et al., 1986; Kulka et al., 1989). However, because the IE4 and IE5 pre-mRNAs are identical at their splice junctions (McGeoch et al., 1986; McGeoch et al., 1985), the relative contribution, if any, of IE4 or IE5 towards inhibition of HSV-1 growth remained unclear. Furthermore, the antiviral activity of

d(Tp*TCCTCCTGCGG*) in HSV-1 infected animals is unknown.

The studies described in this report sought to delineate the importance of the IE4 and IE5 genes as targets for HSV-1 growth inhibition by oligo(nucleoside methylphosphonates) in culture, and determine the antiviral activity of the oligomers in HSV-1-infected animals.

Materials and Methods

Cells and virus

Vero (African green monkey kidney) cells were grown in Eagle's minimal medium (MEM) supplemented with 25 mM Hepes and 10% (vol/vol) fetal bovine serum. MRC-5 (human diploid lung) cells were grown in MEM with 10% heat-inactivated fetal bovine serum. HEp-2 (human epidermoid carcinoma no. 2) cells were grown in medium 199 with 10% calf serum. Cells were infected with the F strain of HSV-1 as described (Kulka et al., 1989). The mouse LD_{50} for this virus stock is 1.0×10^6 pfu.

Synthesis of oligo(nucleoside methylphosphonates)

The binding sites and sequences of the methylphosphonate-modified oligomers used in this study are shown in Fig. 1. They were synthesized by solid-phase techniques on a controlled pore glass support and purified and characterized as previously described (Miller et al., 1992). The oligomer, aeAMTd(pTp*TCCTCCTGCGG*) contains 4′-(aminoethyl) aminomethyl-4,5″,8-trimethylpsoralen (aeAMT) attached at the 5′-end by a nuclease-resistant phosphoramidate linkage. Its synthesis and purification were as described (Lee et al., 1988).

Oligo(nucleoside methylphosphonate) inhibition of HSV-1 growth in vitro

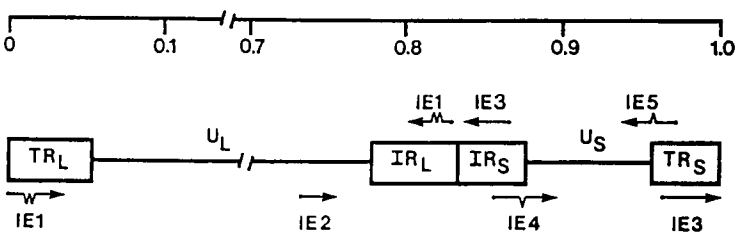
Vero cells were infected with HSV-1 (F) at a multiplicity of infection (MOI) of 0.1 or 10 PFU/cell. This typically represents 10^5 and 10^7 PFU/ml respectively. Following 1 h of adsorption (0 h post-infection), cells were treated with the respective oligomers (0–200 μM). After 24 h of incubation at 37°C, the infected cells were washed with phosphate-buffered saline (PBS; pH 7.2), scraped into fresh MEM (without oligomers) and extracts were prepared by seven consecutive cycles of freezing and thawing. Extracts were cleared of cell debris by centrifugation (10 min, 2000 $\times$ *g*) and virus titers were determined (in the absence of oligomers) by plaque assay on HEp-2 cells (Kulka et al., 1989).

*Effect of d(Tp*TCCTCCTGCGG*) on HSV-1 growth in infected BALB/c mice*

Antiviral activity was studied in the mouse ear or footpad models of HSV infection. In both models infection is intradermal (i.d.) and maximal virus titers at the infected site are observed on days 4 p.i. (Hill et al., 1975; Field et al., 1979; Wachsman et al., 1988). Briefly, male Balb/c mice were injected i.d. in the

ear pinna with a 40-μl solution containing 2×10^3 pfu of HSV-1 (F) and 100–500 μM of d(Tp*TCCTCCTGCGG*) or saline. This was followed by subsequent topical applications of the oligomer (100–500 μM) in a modified aqueous polyethylene glycol (PEG) (50%) cream (Spruance et al., 1986; Collins and Oliver, 1982) for 3 or 5 additional days. Saline in a modified aqueous PEG cream was used as control. For aeAMTd(pTp*TCCTCCTGCGG*) treatment, mice injected i.d. (40 μl) with 5×10^4 pfu of HSV-1 (F) (in the ear pinna or the footpad) were given topical applications of 50 μM of the derivatized oligomer (or saline control) in modified aqueous PEG cream and exposed to UV light (365 nm; 10 min) immediately after treatment in order to induce the cross-linking reaction. In order to assay for the presence of residual HSV-1, mice were sacrificed on days 4 or 6 post-infection (p.i.). The ear pinnae or the soft tissues of the footpads were aseptically removed and homogenized and the virus titers were determined by plaque assay on MRC-5 cells as described (Wachsman et al., 1988).

A.



B.

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                          SPLICE DONOR          SPLICE ACCEPTOR
HSV-1 IE mRNA 4,5: 5' UUUUGCACGG]GUAAGCACCU..CUUCCCGCAG]GAGGAACGUC 3'
                     3'CGTGCC CATTCG 5'     3'GGCGTC CTCCTT 5'
                                          3'AGGGCGTC CTCCTTGC 5'
                                          3'AGGGCGCT TCCCTTGC 5' (mutant)

                          INITIATION
HSV-1 IE mRNA 4: 5' CTGGAUGGCCGACAUUUCCC 3'
                     3'TACCGGCTGTAA 5'

                          INITIATION
HSV-1 IE mRNA 5: 5' AAGCAUGUCGUGGGCCCUGG 3'
                     3'TACAGCACCCGG 5'
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Fig. 1. A: approximate map locations of HSV IE mRNAs. B: partial nucleotide sequence of HSV-1 IE pre-mRNAs 4,5 at the intron/exon boundary (]) of the splice donor and splice acceptor sites and of IE mRNAs 4 and 5 at the translation initiation sites. Complementary dodecamers are shown below the respective target sequences.

Results

HSV-1 growth inhibition by oligomers complementary to IE pre-mRNAs 4,5

We have previously shown that d(Tp*TCCTCCTGCGG*) inhibits HSV-1 growth in culture (Smith et al., 1986; Kulka et al., 1989). To determine whether inhibition is due to the effect of the oligomer on a regulatory viral or cellular gene other than IE4 and/or IE5 that shares the sequence targeted by the oligomer with IE pre-mRNAs 4,5 (Sears et al., 1985), we synthesized an oligomer that has a different sequence and is complementary to the IE pre-mRNAs 4,5 donor splice site [d(Gp*CTTACCCGTGC*)] (Fig. 1). Vero cells were exposed to increasing concentrations of d(Tp*TCCTCCTGCGG*) or d(Gp*CTTACCCGTGC*), at the time of infection with HSV-1 (F) (0.1 PFU/cell) and virus titers were determined 24 h later. The results shown in Fig. 2A represent the average of 2 independent experiments that did not differ by more than 10%. Both oligomers caused a similar dose-dependent inhibition of HSV-1 growth. Virus titers were significantly (90–99%) decreased in cultures exposed to 100–200 μM of either oligomer and the concentration causing a 50% decrease in virus titers (IC_{50}) was 20 and 25 μM for d(Tp*TCCTCCTGCGG*) and d(Gp*CTTACCCGTGC*) respectively. Inhibition was specific, as there was minimal (15–20%), if any, reduction in virus growth in cells treated with similar concentrations of an unrelated oligomer [d(Gp*CGGGAAGGCAC*)].

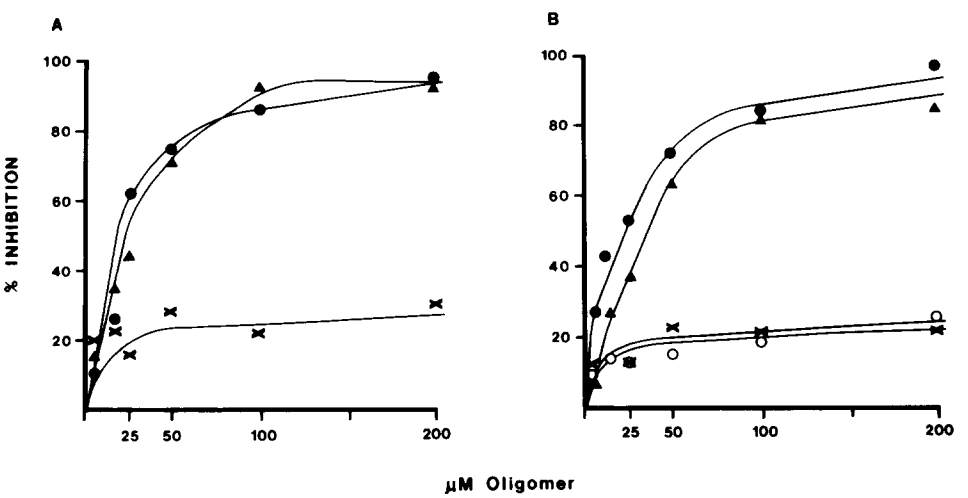

Fig. 2. The effect of virus MOI on the dose–response inhibition of HSV-1 growth by oligo(nucleoside methylphosphonates) complementary to the IE mRNA 4,5 splice donor [d(Gp*CTTACCCGTGC*)] or splice acceptor [d(Tp*TCCTCCTGCGG*)] site. Vero cells were infected with 0.1 PFU (panel A) or 10 PFU (panel B) per cell of HSV-1 (F) and treated (0 h post-infection) with increasing concentrations of oligomer to the splice donor (▲) or splice acceptor (●) site. An oligomer complementary to the splice acceptor site whose two central residues are inverted (○) or an unrelated oligomer [d(Gp*CGGGAAGGCAC*)] (×) were used as controls. Cell cultures were assayed for virus titers 24 h later. Results are expressed as the percent inhibition of virus titers compared to untreated cells.

To explore the role of the multiplicity of infection on the ability of these oligomers to inhibit virus growth, Vero cells were exposed to either d(Tp*TCCTCCTGCGG*) or d(Gp*CTTACCCGTGC*) at the time of infection with 10 PFU/cell of HSV-1 (F) and virus titers were determined 24 h later. As summarized in Fig. 2B, as an average of 3 independent experiments that did not differ by more than 12%, virus titers were significantly (85–98%) reduced by either oligomer in the concentration range of 100–200 μM. An IC_{50} value of 20–35 μM was estimated for each oligomer. Virus growth was not inhibited by the unrelated oligomer d(Gp*CGGGAAGGCAC*). Furthermore, as previously reported (Smith et al., 1986; Kulka et al., 1989), an oligomer in which the two central residues d(Tp*TCCTCCTGCGG*) were inverted, also failed to inhibit HSV-1 growth (data not shown).

To further explore the effect of oligomer length on its ability to inhibit HSV-1 growth, we studied a 16-base oligomer d(Cp*GTTCCTCCTGCGGGA*) that differs from d(Tp*TCCTCCTGCGG*) in that it is flanked by 2 additional bases on each side of the splice acceptor site (Fig. 1). An oligomer in which the 4 central bases were inverted d(Cp*GTTCCCTTCGCGGGA*) was used as control. Cells were exposed to the oligomers (100 μM) at the time of infection with 10 PFU/cell of HSV-1 (F) and virus titers were determined 24 h later. Results averaged from two independent experiments reveal that titers were significantly decreased (86%) in cells exposed to d(Cp*GTTCCTCCTGCGGGA*) but virus growth was not inhibited (0% inhibition) by a similar concentration of the mutated oligomer (data not shown). Together with our previous findings (Smith et al., 1986; Kulka et al., 1989), these data indicate that oligomers 8–16 bases in length that are complementary to the splice site (acceptor or donor) of IE pre-mRNAs 4,5 inhibit HSV-1 growth in a sequence-specific manner and suggest that the processing/expression of these mRNAs are important for optimal virus growth.

HSV-1 growth inhibition by oligomers complementary to IE4 and IE5 translation initiation sites

Due to the sequence homology (100%) of IE4 and IE5 mRNAs at their splice junctions (McGeoch et al., 1986), the individual contribution of IE4 or IE5 gene expression towards HSV-1 growth cannot be established with oligomers d(Tp*TCCTCCTGCGG*) and d(Gp*CTTACCCGTGC*). To address this question, we took advantage of the observation that there is relatively little (20%) homology between the IE4 and IE5 mRNAs at their translation initiation sites (McGeoch et al., 1985) and synthesized complementary oligomers (Fig. 1B). Vero cells infected with HSV-1 (10 PFU/cell) were exposed to d(Ap*ATGTCGGCCAT*) which is complementary to the translation initiation site of IE4 mRNA, or d(Gp*GCCCACGACAT*) which is complementary to the translation initiation site of IE5 mRNA, alone or in combination (0–200 μM). Exposure was at the time of infection. Virus titers were determined 24 h later. As shown in Fig. 3A, virus titers were 80–98% lower in cultures exposed to 50–200 μM of d(Ap*ATGTCGGCCAT*), the

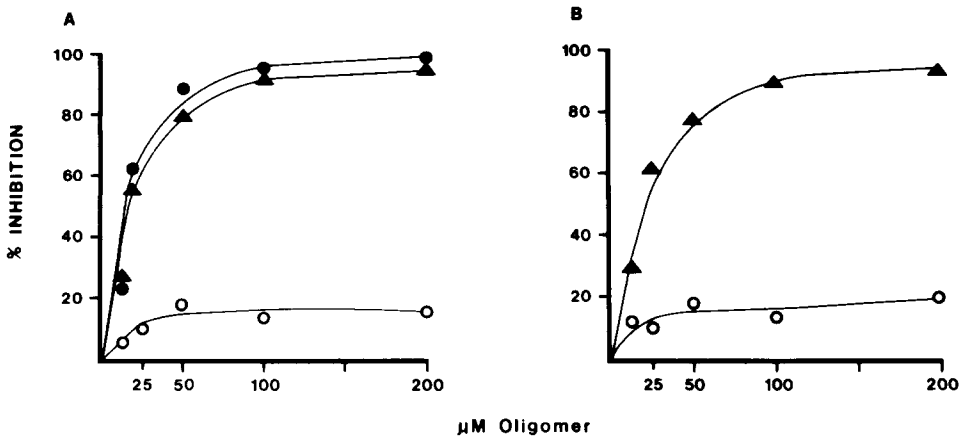

Fig. 3. Dose–response inhibition of HSV-1 growth by oligo(nucleoside methylphosphonates) complementary to the translation initiation site of IE mRNA 4 [d(Ap*ATGTCGGCCAT*)] or IE mRNA 5 [d(Gp*GCCCACGACAT*)]. Vero cells were infected with 10 PFU (panel A) or 0.1 PFU (panel B) per cell of HSV-1 (F) and treated (0 h post-infection) with increasing concentrations of oligomer to IE mRNA 4 (▲) or IE mRNA 5 (○) alone or in combination (●). Cell cultures were assayed for infectious virus 24 h later. Results are expressed as the percent inhibition of virus titers compared to untreated cells.

oligomer complementary to the translation initiation site of IE mRNA 4. The IC_{50} was estimated at 20 μM.

IE4 mRNA is colinear on the HSV-1 and HSV-2 genomes. However, there is a 2-base difference between HSV-1 and HSV-2 at the IE4 mRNA translation initiation site targeted by d(Ap*ATGTCGGCCAT*) (Table 1). Accordingly in order to determine the sequence specificity of the inhibition mediated by d(Ap*ATGTCGGCCAT*), Vero cells were infected with HSV-1 (F) or HSV-2 (G) (10 PFU/cell) and exposed to 25–100 μM of d(Ap*ATGTCGGCCAT*) at the time of infection. Virus titers were determined 24 h later. As shown in Table 1 for 2 experiments that did not differ by more than 2%, d(Ap*ATGTCGGCCAT*)

TABLE 1

Effect of d(Ap*ATGTCGGCCAT*) on HSV growth[a]

Oligomer concentration (μM)	% Inhibition		Fold difference[c] (%)
	HSV-1[b]	HSV-2[b]	
100	78 ± 10	52 ± 12	1.5
50	64 ± 8	37 ± 14	1.7
25	56 ± 12	7 ± 18	8.0

[a]Vero cells were infected with 10 PFU per cell of HSV-1 (F) or HSV-2 (G) and treated at 0 h p.i. with oligomer complementary to the HSV-1 IE4 mRNA translation initiation site [d(Ap*ATGTCGGCCAT*)]. Virus titers were assayed 24 h later. Results from 2 independent experiments are expressed as the mean percent inhibition ± S.E.M. of virus titers as compared to untreated controls.

[b]Nucleotide sequences at the IE4 mRNA translation initiation site targeted by the oligomer are: AUGGCCGACAUU for HSV-1 and AUGGCAGACAUC for HSV-2.

[c]Represents % inhibition for HSV-1/% inhibition for HSV-2.

caused a dose-dependent inhibition in the growth of both HSV-1 and HSV-2. However, the effect of d(Ap*ATGTCGGCCAT*) over the concentration range 25–100 μM was significantly lower for HSV-2 than HSV-1. The major difference between inhibition of HSV-1 and HSV-2 seen at 25 μM concentration (8-fold) supports the interpretation that inhibition is sequence-specific, at least at the lower concentrations. This conclusion is supported by the observation that inhibition mediated by 1-(2-deoxy-2 fluoro-β-D-arabino-furanosyl)-5-iodocytosine (FIAC) is similar for both HSV-1 and HSV-2 (ID_{50} = 0.012 and 0.017 μg/ml respectively), as previously described (Machida et al., 1984).

Treatment of the infected cells with the oligomer d(Gp*GCCCACGACAT*), complementary to the translation initiation site of IE mRNA 5, caused minimal, if any, inhibition of HSV-1 or HSV-2 growth (5–20%) over the concentration range examined. When HSV-1-infected cells were treated with a mixture containing equal concentrations of both oligomers, the inhibition was essentially similar to that seen for d(Ap*ATGTCGGCCAT*) alone, and the effective concentration range for virus inhibition was not decreased (90–99% at 50–200 μM; IC_{50} = 15 μM). These results suggest that inhibition of IE4, but not IE5, gene expression is associated with the inhibition of virus growth.

*Effect of MOI on HSV-1 inhibition by d(Gp*GCCCACGACAT*)*

Previous studies had shown that at high MOI other IE genes may complement the loss of the IE1 (ICPO) function (Sachs and Schaffer, 1987; Stow and Stow, 1986). To address this question with respect to IE5, Vero cells were infected with HSV-1 (F) at a low MOI (0.1 PFU/cell) and exposed to increasing concentrations of d(Gp*GCCCACGACAT*) (complementary to IE5 mRNA translation initiation site) at the time of infection. d(Ap*ATGTCGGCCAT*), the oligomer complementary to the IE4 mRNA translation initiation site was used as control. As previously found in cells infected at high MOI (10 PFU/cell), virus titers were decreased (80–95%) in cultures treated with d(Ap*ATGTCGGCCAT*) in the concentration range of 50–200 μM. The calculated IC_{50} was 20–25 μM (Fig. 3B). However, treatment with the oligomer d(Gp*GCCCACGACAT*) that is complementary to the IE5 mRNA translation initiation site, had no effect on virus growth (18–20% inhibition at 50–200 μM) even at low MOI (Fig. 3B).

*Effect of d(Tp*TCCTCCTGCGG*) on HSV-1 growth in infected BALB/c mice*

We first used the mouse ear pinna model of HSV-1 to study the effect of d(Tp*TCCTCCTGCGG*) on HSV-1 replication in vivo. Four experiments were done and the results of two representative ones are shown in Table 2. Consistent with previous findings, maximal virus titers in the ear were seen as early as day 4 p.i. and they did not significantly increase on day 6 p.i. Residual virus (on day 6 p.i.) in ears treated with a single dose (500 μM) of the oligomer given as an i.d. injection concomitant with HSV-1 infection (day 0), was similar to that in the untreated ears even though the oligomer concentration was 5-fold

TABLE 2

Effect of d(Tp*TCCTCCTGCGG*) on HSV-1 growth in the mouse ear[a]

Group	Virus titer (PFU)/ear
Experiment 1 (n = 10)	
Saline days 0–5 p.i.	170 ± 10
d(Tp*TCCTCCTGCGG*) (500 μM) day 0 p.i.	124 ± 27
d(Tp*TCCTCCTGCGG*) (100 μM) days 0–5 p.i.	350 ± 35
d(Tp*TCCTCCTGCGG*) (500 μM) days 0–5 p.i.	30 ± 3*
Experiment 2 (n = 6)	
Saline days 0–3 p.i.	310 ± 77
d(Tp*TCCTCCTGCGG*) (500 μM) days 0–3 p.i.	4.6 ± 1.4*
d(Gp*GCCCACGACAT*) (500 μM) days 0–3 p.i.	275 ± 45

[a]BALB/c mice were infected in both ears with 2×10^3 PFU of HSV-1 (F). Oligomer (500 μM in 40 μl) was administered by i.d. injection in the right ear at the time of infection (day 0 p.i.) and topically (in a PEG ointment) once daily for 3–5 days. The left ear was used as control. It received a saline injection (40 μl) at the time of infection (day 0 p.i.) and was topically treated with saline in PEG ointment once daily for 3–5 days. Ears were assayed for virus growth on day 4 (experiment 2) or 6 p.i. (experiment 1). n = number of animals. Results are expressed as mean ± S.E.M.
*P <0.005 as compared to saline control by Student's t-test.

higher than that (100 μM) causing maximal inhibition of virus growth in vitro.

Since multiple exposures may be required in order to achieve a sufficient concentration of the oligomer at the site of infection (skin) and for the time required in order to inhibit virus growth, subsequent experiments used a different treatment protocol. Specifically, animals were given one injection of 500 μM d(Tp*TCCTCCTGCGG*) i.d. at the time of infection (to insure skin penetration). This was followed by daily topical applications of the oligomer (500 μM) in a vehicle (PEG) that facilitates skin penetration (Spruance et al., 1986; Collins and Oliver, 1982) which is a more acceptable treatment regimen than injection. Virus titers were determined on days 4 or 6 p.i. Consistent with the finding that maximal virus titers are achieved as early as day 4 p.i., maximal inhibition (82–98%; P <0.005) was also seen on day 4 p.i. in the ears treated with multiple doses of d(Tp*TCCTCCTGCGG*). Similar inhibition was also seen on day 6 p.i. (Table 2). Treatment with 500 μM of the oligomer d(Gp*GCCCACGACAT*) complementary to the translation initiation site of IE5 mRNA, or with 100 μM of d(Tp*TCCTCCTGCGG*) did not decrease virus titers in the ear (Table 2).

*Effect of psoralen derivatized d(Tp*TCCTCCTGCGG*) on HSV-1 growth in vivo*

When bound to its complementary target sequence on mRNA, the 3,4 double bond of the pyrone ring of aeAMT can undergo a photocycloaddition reaction with the 5,6 double bond of deoxycytidine in the target to form a covalent cross-link between the oligomer and mRNA (Lee et al., 1988). We have previously shown that a psoralen derivative of d(Tp*TCCTCCTGCGG*) can covalently bind to complementary sequences after exposure to 365 nm irradiation and causes 90–98% inhibition of virus growth in vitro at a significantly lower concentration than the underivatized oligomer (Kulka et al., 1989). To determine whether this derivatized oligomer inhibits virus growth in vivo at concentrations lower than those required by the underivatized oligomer, animals were infected with HSV-1 in both ears and given three topical applications of aeAMTd(pTp*TCCTCCTGCGG*) (50 μM in PEG cream) at 0, 4 and 8 h p.i. in the right ear. The left ear was similarly treated with saline containing PEG modified cream. Both ears were exposed to UV light (365 nm) for 10 min immediately thereafter. For 5 days following this treatment animals were given one daily application of the derivatized oligomer (or saline) in the modified aqueous PEG cream followed by 10 min of irradiation. Virus titers were determined on day 6 p.i. The sequence specificity of the antiviral effect was determined in duplicate experiments done in parallel but with HSV-2 (G) instead of HSV-1 (F).

As shown in Table 3, HSV-1 titers in ears treated with 50 μM of aeAMTd(pTp*TCCTCCTGCGG*) were 91% lower than those in the saline-treated ears. Notably, despite the use of a 25-fold higher dose of infecting virus and a 10-fold lower concentration of oligomer, the inhibition of HSV-1 with the psoralen derivatized oligomer was similar to that achieved with the underivatized methylphosphonate (91% and 82% respectively). HSV-2 titers in these animals were reduced only 27%, consistent with the conclusion that virus growth inhibition is sequence-specific and is not due to the overall non-specific

TABLE 3

Effect of aeAMTd(pTp*TCCTCCTGCGG*) on HSV growth in the mouse ear[a]

Group ($n = 26$)	Virus titer (PFU)/ear	
	HSV-1	HSV-2
Saline	1.7×10^3	1.5×10^3
aeAMTd(pTp*TCCTCCTGCGG*)	1.6×10^2	1.1×10^3
Inhibition	91%	27%

[a]BALB/c mice were infected in both ears with 5×10^4 PFU of HSV-1 (F) or HSV-2 (G) (in 40 μl). They were given topical applications of 50 μM aeAMTd(pTp*TCCTCCTGCGG*) in PEG ointment in the right ear at 0, 4 and 8 h p.i. and exposed to UV light (365 nm) for 10 min immediately thereafter. This was followed by one daily application/irradiation for 5 additional days. The left ears were simultaneously treated with a mixture of saline [instead of aeAMTd(pTp*TCCTCCTGCGG*)] in PEG ointment and irradiated. Ears were assayed for virus growth on day 6 p.i. and the results are expressed as mean values. Data represent the average of two independent experiments that did not differ by more than 8%. n = number of animals.

inhibitory effect of psoralen. In vivo as well, FIAC inhibited both HSV-1 and HSV-2 equally well (data not shown).

To determine whether similar inhibition of HSV-1 growth is seen also under somewhat different conditions, we used the footpad model (Wachsman et al., 1988). Mice (groups of 5) were infected with HSV-1 in the footpad and given applications of 50 μM of aeAMTd(pTp*TCCTCCTGCGG*) followed by 10 min irradiation at 365 nm once daily beginning on day 0 and continuing up to day 3. Virus titers were determined on day 4 p.i. Two experiments were done using this protocol. The averaged results indicate that titers were significantly (86%) lower in animals treated with aeAMTd(pTp*TCCTCCTGCGG*) according to this protocol than in mice similarly treated with saline instead of aeAMTd(pTp*TCCTCCTGCGG*) (5.1×10^2 as compared to 3.6×10^3 PFU).

Discussion

The ability of antisense RNA (and oligonucleotides) to selectively inhibit the expression of specific genes has led to the suggestion that antisense molecules may be used in antiviral chemotherapy. As cellular uptake and survival of oligodeoxynucleotides may be limiting factors in their therapeutic use, recent efforts have focused on the development of chemically modified oligonucleotides, such as oligo(nucleoside methylphosphonates) developed in our laboratories (reviewed in Miller et al., 1992), or phosphorothioates (reviewed in Cohen, 1991). These antisense molecules successfully inhibit a number of viruses including HSV (Smith et al., 1986; Kulka et al., 1989; Gao et al., 1989, 1990a,b; Hoke et al., 1991). However, the antiherpetic activity was not always sequence-specific and the mechanism of action is unclear. Thus, while HSV-1 inhibition by an oligo(nucleoside methylphosphonate) complementary to the acceptor splice junction of IE mRNAs 4,5 was shown to be sequence-specific (Smith et al., 1986; Kulka et al., 1989) and presumably results from the inhibition of IE pre-mRNA 4,5 splicing (Kulka et al., 1989), the antiviral effect of the phosphorothioate oligomers [S-$(dC)_{28}$ and S$(dT)_{28}$] was not sequence-specific and their inhibitory activity was attributed to inhibition of viral DNA polymerase or interference with HSV uptake (Gao et al., 1989, 1990a,b). A degree of sequence specificity was described for HSV inhibition by phosphorothioates complementary to either the translation initiation AUG codon of Vmw65 or an internal AUG codon in UL13 mRNA (Draper et al., 1990; Hoke et al., 1991). However, the mechanism of virus growth inhibition was not established.

The studies described in this report sought to obtain a better understanding of the viral inhibitory effect of the oligo(nucleoside methylphosphonates) complementary to HSV-1 IE pre-mRNAs 4,5 and determine their ability to inhibit virus growth in infected animals. The following findings support the interpretation that processing of IE pre-mRNAs 4,5 and inhibition of IE4 gene expression are involved in antiviral activity. First, inhibition of IE4 pre-mRNA

splicing is mediated by the oligomer complementary to the splice acceptor site of IE pre-mRNA 4,5 [d(Tp*TCCTCCTGCGG*)] (Kulka et al., 1989). Second, oligomers complementary to the donor splice site of IE pre-mRNAs 4,5 or the translation initiation site of IE4 mRNA cause a similar inhibition of HSV-1 growth as that previously reported for d(Tp*TCCTCCTGCGG*) (Smith et al., 1986; Kulka et al., 1989) although they have distinct sequences. Third, inhibition is not mediated by an unrelated oligomer or by d(Gp*GCCCACG-ACAT*) that is complementary to the translation initiation site of IE5 mRNA. Fourth, an oligomer d(Cp*GTTCCTCCTGCGGGA*) that has 4 additional complementary bases gives rise to identical inhibitory patterns as those observed for d(Tp*TCCTCCTGCGG*), and fifth, inhibitory activity is sequence-specific. Thus: (1) antiviral function is abrogated by inversion of the two central residues in the oligomer complementary to the IE pre-mRNAs 4,5 acceptor splice site (Smith et al., 1986; Kulka et al., 1989) or 4 central residues in d(Cp*GTTCCTCCTGCGGGA*), and (2) d(Ap*ATGTCGGCCAT*) complementary to the translation initiation site of HSV-1 IE4 mRNA inhibits HSV-2 growth with a 8-fold lower efficacy than HSV-1, at least at a concentration of 25 μM. HSV isolates differ in their in vitro sensitivity to antiviral drugs reflecting distinct requirements for the target genes. While we cannot exclude the possibility that the function of the IE4 gene is less relevant for HSV-2 than HSV-1 growth, we believe that their different inhibition reflects sequence specificity since a similar difference was not observed with FIAC.

Our finding that oligomers complementary to the splice acceptor [d(Tp*TCCTCCTGCGG*)] or translation initiation [d(Ap*ATGTCGGCCAT*)] sites of IE4 mRNA inhibit HSV-1 growth in cells infected at low or high MOI has two significant implications regarding the role of the IE4 gene in HSV-1 growth. First, it argues that the function of the IE4 gene is not complemented by other viral genes at high MOI. This is unlike the situation for another HSV IE gene (IE1) and its product (Stow and Stow, 1986; Sacks and Schaffer, 1987). Second, there seems to be a minimal level of IE4 gene expression which provides the function necessary for virus growth. This implies that greater levels of IE4 gene product (produced under condition of high MOI) do not increase the efficiency of virus replication. Confirmation of the inhibition of HSV-1 IE4 gene expression would be possible with an appropriate, but currently unavailable anti-ICP22 antibody. However, this would not exclude the possibility that HSV growth inhibition by these oligomers is due to their interaction with a complementary host encoded gene product (Sears et al., 1985) whose regulatory function can substitute for the required IE4 gene product (Jacquemont et al., 1984). If the antiviral effect of d(Ap*ATGTCG-GCCAT*), d(Tp*TCCTCCTGCGG*), d(Cp*GTTCCTCCTGCGGGA*) and d(Gp*CTTACCCGTGC*) is due to inhibition of such a complementing cellular protein, the latter must closely resemble the viral protein at least at its translation initiation site and splicing regulatory controls. Additionally, since the inhibitory effect was observed both in Vero and HEp-2 cells (Smith et al., 1986), the IE4 complementing cellular protein must have a very similar

sequence in both cell species. Finally, since d(Tp*TCCTCCTGCGG*) is also effective in HSV-1 infected mice, IE4 mRNA must also share sequence similarity with a mouse gene that is involved in HSV-1 growth in vivo.

Our proposal that the IE5 gene product is not essential for HSV-1 growth agrees with previous conclusions using viable IE5 deletion mutants of HSV-1 (Mavromara-Nazos et al., 1986; Longnecker and Roizman, 1986). Indeed, it is unlikely that d(Gp*GCCCACGACAT*) (complementary to the translation initiation site of IE5 mRNA) was ineffective because it failed to bind its mRNA target, since: (1) as predicted by analysis of mRNA folding (Zucker and Steigler, 1981), the IE5 mRNA translation initiation site is contained within a secondary structure similar to that of IE4 mRNA translation initiation site, (2) translation initiation sites are valid targets for inhibition of HSV-1 growth as evidenced by the antiviral efficacy of the oligomer d(Ap*ATGTCGGCCAT*) that is complementary to the IE4 mRNA translation initiation site, and (3) a psoralen-derivatized oligomer complementary to the translation initiation site of IE1 mRNA specifically cross-links to its target mRNA in vitro (data not shown). However, we cannot exclude the possibility that IE5 mRNA is inaccessible due to unique intracellular localization of this transcript.

Much progress has been made in the design and analysis of antisense oligonucleotides that control gene expression in vitro. However, there is relatively little information on their in vivo efficacy, particularly as regards antiviral chemotherapy. In the present study we addressed the question of the antiviral activity of d(Tp*TCCTCCTGCGG*) in HSV-1 infected mice. The mouse model was chosen because it had been effectively used for the evaluation of antiviral therapy (Kern, 1990). One study of the disposition and metabolism of d(Tp*TCCTCCTGCGG*) indicated that it has a relatively short half-life (6–17 min) following a single i.v. injection (Chen et al., 1990). However, there is no published information on skin penetration, tissue distribution and metabolism subsequent to this administration route. Conclusions as to the percentage of delivered oligomer that actually penetrates the skin cannot be reached based on effective in vitro concentrations since molecular weight and original concentration do not accurately predict antiviral efficacy of topically applied agents (Spruance et al., 1986). To insure skin penetration we used at least one intradermal injection of the oligomers and for topical application the oligomers were delivered in a modified aqueous PEG cream that increases skin penetration (Spruance et al., 1986; Collin and Oliver, 1982). However, the most effective method of delivery remains unknown and the treatment protocol used in these experiments was perforce empirical.

Our data indicate that a single i.d. injection at a 5-fold higher concentration than that which causes significant inhibition in vitro (85% at 100 μM) has no antiviral activity in vivo. However, multiple treatments with this concentration of d(Tp*TCCTCCTGCGG*) including one i.d. injection at the time of infection and daily topical applications (in a modified aqueous PEG cream) reduced virus growth in the skin to the same extent (80–95%) as that achieved in culture. Similar to our findings in vitro (Kulka et al., 1989), the level of virus

inhibition (82%) achieved with underivatized oligomer (500 μM) was attained using a 10-fold lower concentration of psoralen-derivatized oligomer. This inhibition was obtained with an inoculum dose that was 25-fold higher than that used with the underivatized oligomer. We conclude that the effect of the psoralen-derivatized oligomer is sequence-specific since under identical conditions there was minimal inhibition of HSV-2. While similar experiments with a psoralen-derivatized oligomer which lacks antiviral activity in vitro will provide more definitive evidence in support of our conclusion, it is important to point out that psoralen derivatization is not essential for antiviral activity which was highly significant ($P < 0.005$) with the underivatized oligomer. The finding that equal levels of inhibition are achieved at higher MOI is particularly important as it confirms the potential for application of this antisense approach to antiviral chemotherapy.

While final conclusions pertaining to the chemotherapeutic potential of oligo(nucleoside methylphosphonates) must await the results of additional detailed in vivo studies, the antisense approach continues to be an important tool for the understanding of gene regulation and viral growth. As powerful alternatives to labor-intensive mutational analysis, they allow the investigation of viral gene regulation (Kulka et al., 1989) and expression under native conditions. Further optimization of antiviral activity may be accomplished by targeting additional genes and using alternative drug delivery systems/vehicles.

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