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Vmw65 phosphorothioate oligonucleotides inhibit HSV KOS replication and Vmw65 protein synthesis

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Summary

Phosphorothioate-modified oligomers have been shown to be more stable than natural oligomers to serum and cellular nucleases. For this reason we used these analogs to explore the utility of antisense molecules as potential antiviral agents. The oligomers we studied are complementary to the initiation region of the Vmw65 (α-TIF) gene of HSV-1 which is important both for its structural role and in the transactivation of the alpha genes of HSV. Our results demonstrate that Vmw65-specific oligomers inhibit HSV KOS replication in a dose-dependent manner from 25 μg/ml (4.3 μM) to 50 ng/ml (9 nM). Vmw65 protein synthesis is inhibited from 51 to 68% at 5 μg/ml (0.8 μM) using Vmw65-specific oligomers 293s and 432s respectively. A random AT-rich oligomer, 007s, inhibited HSV KOS replication in a non-dose-dependent manner. Inhibition was only observed at a concentration of 12.5 μg/ml (2.1 μM) or more, using an MOI (multiplicity of infection) of 0.05 PFU/cell and a 24-h post-infection harvest.

Phosphorothioate modification; Oligodeoxynucleotide; Protein synthesis inhibition; Replication inhibition

Introduction

The alteration of genetic expression by antisense RNA and DNA molecules has recently been reviewed (Van der Krol et al., 1988). Antisense regulation of gene expression occurs both naturally and in vivo in procaryotes and eucaryotes (Van

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der Krol et al., 1988). Zamecnik et al. (1976) showed oligodeoxynucleotides (ODNs) complementary to the tRNA primer binding site on HIV RNA donor or acceptor splice sites inhibited HIV replication in tissue culture and gene expression (virus-encoded proteins p15 and p24) by as much as 95%. The explosive growth in the application of antisense oligonucleotides has seen not only the synthesis of unmodified oligonucleotides but those with molecular substitutions such as methylphosphonates and phosphorothioates. Chemically modified nuclease-resistant ODNs should be more stable and therefore more efficiently inhibit viral replication and protein synthesis. Smith et al. (1986) reported that methylphosphonate analogs of ODNs complementary to the acceptor splice junction of HSV-1, i.e. pre-mRNAs 4 and 5 (ICP22 and ICP47), inhibited viral replication by 50 and 90% in cells treated with 25 and 75 μM respectively.

We have shown previously that an unmodified ODN complementary to the translation initiation region of Vmw65 (α-TIF) mRNA inhibited the expression of Vmw65 biological activity in a Vmw65-expressing cell line and reduced the yield of HSV KOS in Ltk– cells at 25 μg/ml (Draper et al., 1990). In this study, we expand upon our previous findings and determine if these modified sequence-specific oligomers (Vmw65) inhibited HSV KOS in a dose dependent manner. We then compare the effects of different MOIs (multiplicities of infection) and different times of harvest p.i. (post-infection) on the inhibitory levels of Vmw65 ODNs. Finally, we correlate decreased viral yield in the presence of oligomer with decreased amount of Vmw65 protein. We chose to use only the phosphorothioate-modified Vmw65 ODNs because these oligomers have been shown to be more stable than natural oligomers to serum and cellular nucleases (Ceruzzi and Draper, 1989; Van der Krol et al., 1988). These modified oligomers also have the same number of charges as *n*-ODNs, are soluble in aqueous media and exhibit more efficient hybridization with a complementary DNA sequence than corresponding methylphosphonate analogs (Matsukura et al., 1987). We observed higher and more reproducible levels of HSV KOS viral replication inhibition with the phosphorothioate modified ODNs than with the unmodified ODNs. The experimental conditions (MOI, time of harvest p.i. and amount of oligomer) which resulted in maximum inhibitory effect were established and used when radiolabelling infected cell lysates for immunoprecipitations.

Materials and Methods

Cells and virus

Ltk– cells, originally from the American Type Culture Collection, were maintained in Eagle's Minimal Essential Medium (EMEM) supplemented with glutamine, penicillin, streptomycin and 10% fetal calf serum. Vero cells were maintained in Medium 199 containing glutamine, penicillin, streptomycin, 10 mM Hepes (pH 8.0) and 5% fetal calf serum. HSV-1 (strain KOS) stocks were grown in Vero cells and harvested by standard procedures (Draper et al., 1990).

Oligodeoxyribonucleotides

Phosphorothioate-modified oligodeoxyribonucleotides were synthesized by Research Genetics, Huntsville, AL, using an automated DNA synthesizer and H-phosphonate chemistry. The final DMT group was not removed. Prior to deprotection the internucleotide phosphates were modified to form phosphorothioate linkages. Each oligo was purified by reverse-phase chromatography following deprotection in 30% NH_4OH. The DMT group was removed and each purified oligo was checked for a single band of the correct size by PAGE on a 20% acrylamide gel (Hudson, 1991). Phosphorothioate oligomers synthesized in this manner were purchased by us at different times in 1–10 mg batches. The sequence for oligomer 293 is 5′ GTC CGC GTC CAT GTC GGC 3′ and the sequence for 294 is 5′ CAA GAG GTC CAT TGG GTG 3′ (Ceruzzi and Draper, 1989). Oligomer 293s (s = phosphorothioate) is complementary to the HSV-1 mRNA sequence which encodes translation initiation codons for the Vmw65 protein and oligomer 294s is located 33 nucleotides upstream of oligomer 293s (Draper et al., 1990). The random 18-mer oligomer, 007s, is 72% AT rich. Its sequence is 5′ ATA GTT CAT GTT ATG AAT 3′. The sequence of oligomer 432s is the same as 293 but extended by 5 nucleotides (AC AAA) in the 3′ direction.

Oligodeoxyribonucleotide inhibition of HSV replication

Ltk– cell-viral lysates were prepared as previously described using a multiplicity of infection (MOI) of either 0.05 PFU/cell or 0.5 PFU/cell (Draper et al., 1990). Virus titers were determined by plaque assay on Vero cells as previously described (Draper et al., 1990).

Immunoprecipitation and quantitation of Vmw65 protein levels in the presence and absence of oligomer

Ltk– cells were seeded at 9.0×10^6 cells per 75-cm^2 T flask, five flasks in all. To each flask was added either oligomer 293s, 294s, 432s, 007s or no oligomer at a final concentration of either 5.0 or 0.5 μg/ml (15 ml of overlay, 2% FBS/EMEM, per T flask).

24 h later, monolayers (95% confluent) were washed with Hanks BSS and then infected with HSV KOS at 0.05 PFU/cell. After one hour adsorption of the virus at 36.5°C, the overlay was replaced, with or without the appropriate oligomer, and the infection allowed to proceed for 24 h.

Cell monolayers were then washed with methionine-free EMEM, fresh methionine-free EMEM was added and the cells incubated for 1 h at 36.5°C. The monolayers were then pulse-labelled with [^{35}S]methionine in methionine-free EMEM for 4 h. The monolayers were removed with cell scrapers (Costar) and lysates harvested as usual except that the final suspension/storage volume was reduced to approximately 300 μl per 75-cm^2 T flask (9×10^6 cells).

Immunoprecipitation of cell-viral lysates was performed according to the pro-

cedure of Costa et al. (1983) with the following modifications. Radiolabelled antigen was diluted 1:1 with 2 × lysis buffer (14 μl each) and incubated for 1 h on ice. Two μl (1:5 dilution) of monoclonal AB LP1 (kindly supplied by Tony Minson) made against purified Vmw65 was then added to each lysed sample and the samples incubated for another hour on ice. 150 μl of a 10% suspension of Pro-A Sepharose beads (Pharmacia) in the appropriate buffer were added and the suspension was incubated for 30 min longer on ice with frequent mixing. The Pro-A Sepharose beads with the adsorbed immune complexes were then pelleted by centrifuging for 5 min through a 1-ml pad of lysis buffer containing 1 M sucrose. The Sepharose was then washed by suspension in the appropriate buffer followed by recentrifugation. After five washes the Sepharose pellet was suspended in 40 μl of a buffer containing 0.075 M Tris-HCl (pH 6.8), 3% SDS and 10% β-mercaptoethanol. The suspension was heated to 95°C for 2 min, and the Pro-A Sepharose was pelleted by centrifugation. The supernatant was loaded onto a 12.5% SDS-acrylamide gel for size fractionation or stored at −80°C.

The amounts of Vmw65 protein made in the absence of oligomer or the presence of 293s, 294s, 432s or 007s were quantitated by densitometric analysis. AR XMAT (Kodak) film was used for the autoradiography and a 2–3 day exposure used for the densitometric scan.

[^{3}H]Leucine uptake

Ltk– cells were seeded in 96-well plates with or without oligomer in 10% FBS/EMEM at a density of 4×10^5 cells per ml. Twenty four hours later, the medium was replaced with fresh oligomer/EMEM and 10% FBS or just EMEM and 10% FBS, and after an additional 48 h cells were pulsed with [^{3}H]leucine for 1 h. Monolayers were washed twice with PBS, then lysed with 0.5% SDS/PBS. Lysates were transferred to Millipore filtration 96-well plates, and TCA-precipitated with 10% TCA. Filters were washed twice with 10% TCA, followed by two washes with 95% ETOH and then counted in a scintillation counter.

Results

Yield reduction followed by plaque reduction experiments were performed to see if sequence specific oligomers inhibited HSV KOS viral replication in a dose-dependent manner. Both oligomers 293s and 432s showed dose response levels of inhibition at 48 h p.i. using either 0.05 PFU/cell or 0.5 PFU/cell (Fig. 1a). Inhibitory levels ranged from 50–95% at 25 μg/ml to 8–18% at 50 ng/ml for oligomers 293s and 432s. The levels of inhibition were higher at each concentration at an MOI of 0.05 PFU/cell than at 0.5 PFU/cell with oligomer 432s. Under these conditions, oligomer 293s gave higher inhibition at 5 and 25 μg/ml. At both 0.05 and 0.5 PFU/cell, neither 432s or 293s showed inhibition of KOS viral replication below 10 ng/ml.

Oligomer 007s, an AT-rich, random 18-mer showed no inhibition at either MOI. Oligomer 294s showed a slight dose-dependent inhibition only at 0.05 PFU/cell,

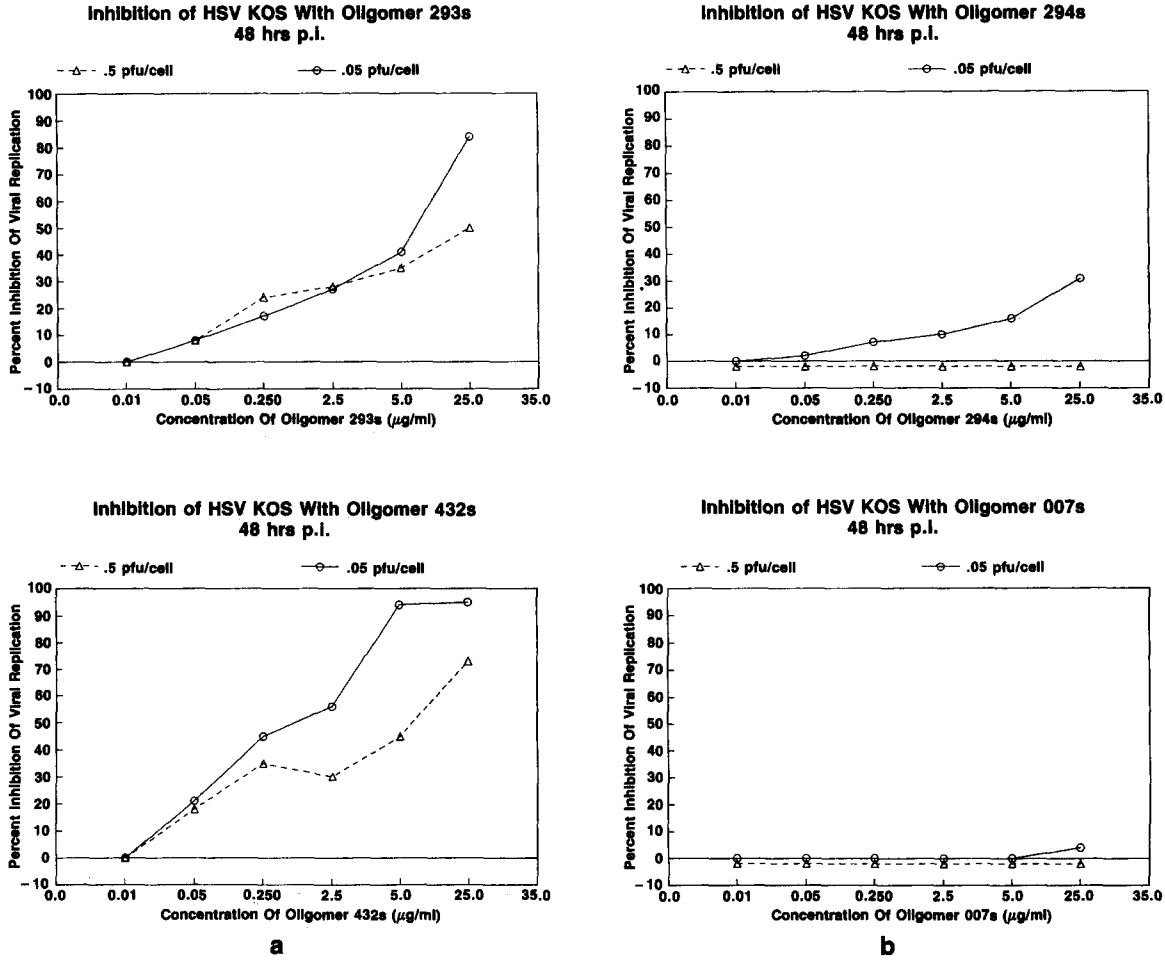

Fig. 1. A comparison of oligomer inhibition of HSV replication at an MOI of 0.05 and 0.5 PFU/cell. Viral lysates were harvested at 48 h p.i. Details of the analysis are described in the text. Each experiment was reproducible. A typical experimental graph is shown.

but much less than that of 293s and 432s (compare Figs. 1a and b).

The inhibitory activity of sequence-specific and random oligomers was also studied at different times p.i. (24 vs. 48 h p.i.). The inhibitory effect of oligomers 432s and 293s measured at 0.05 PFU/cell was essentially the same whether cell-viral lysates were harvested 24 or 48 h post-infection (Fig. 2a). For oligomer 293s, at 0.05 PFU/cell, inhibitory levels were higher at 24 h p.i. for all concentrations tested except 12.5 μg/ml at 48 h p.i. (Fig. 2a, top). Inhibitory levels of oligomer 432s were slightly higher at 48 h p.i. at all concentrations (Fig. 2a, bottom).

At an MOI of 0.05 PFU/cell, oligomers 294s and 007s showed no dose-dependent effect at either 24 or 48 h p.i. (Fig. 2b). The inhibition of both 294s and 007s at 12.5 μg/ml and above quickly dropped to negligible levels at lower concentra-

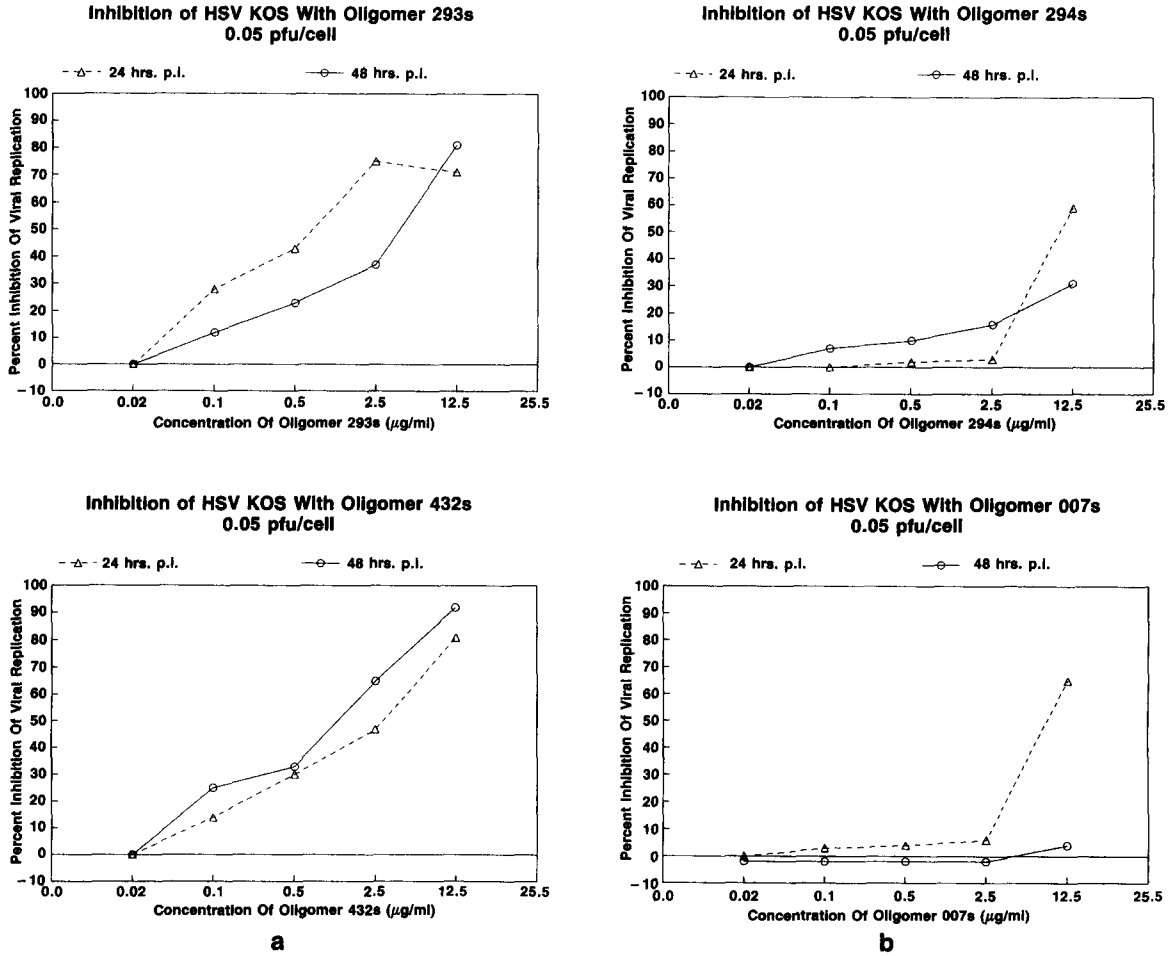

Fig. 2. The effect of oligomer inhibition was compared when cells were infected at an MOI of 0.05 PFU/cell and harvested at either 24 or 48 h p.i. Details of the analysis are described in the text. Experiments were reproducible and a typical experiment is shown.

tions and was present only at 24 h p.i., not 48 h p.i. (Fig. 2b).

We did not detect any adverse cellular effects by any of the oligomers as measured by [^{3}H]leucine incorporation. Incorporation of [^{3}H]leucine by oligomer-treated cells was 100% that of non-oligomer-treated cells (data not shown).

The dose-dependent response of oligomers 293s and 432s and the non-dose-dependent response of oligomers 294s and 007s at 0.05 PFU/cell and a 24 h harvest p.i. are also shown in two separate trials (Figs. 3a and b).

Immunoprecipitation of Vmw65 protein from oligomer- and non-oligomer-treated cells using LP1 monoclonal antibody (mAb), followed by SDS acrylamide gel electrophoresis and autoradiography, revealed discrete protein bands at 65 and 130 kDa (Fig. 4). The mouse mAb, LP1, is directed against the non-glycosylated

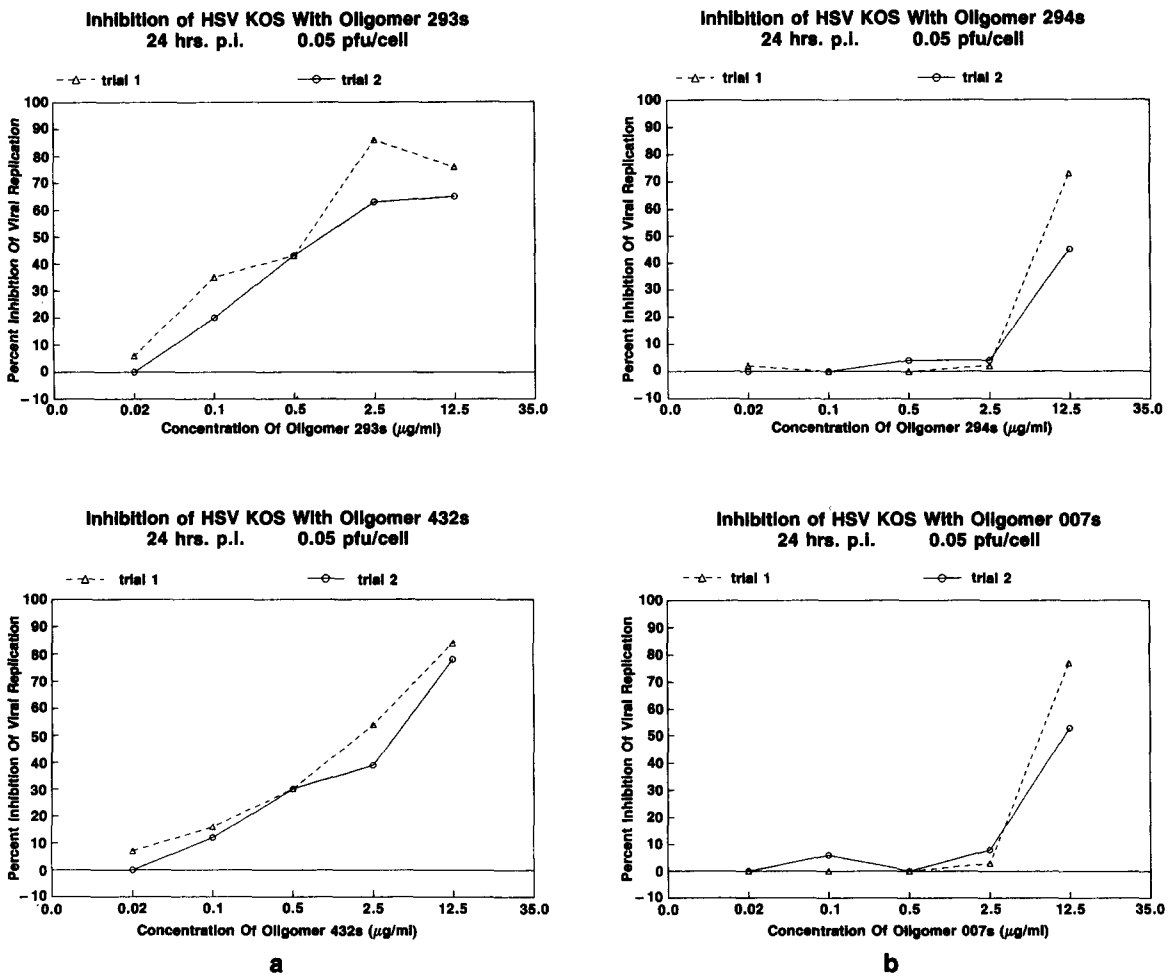

Fig. 3. Oligomers were added or not as described in the text. HSV-KOS-infected cell lysates were harvested after 24 h. Two separate experiments (trials) are shown at an MOI of 0.05 PFU/cell.

Vmw65 polypeptide of herpes simplex virus type 2; it is specific for polypeptide Vmw65 and cross-reacts with type HSV-1 Vmw65. This antibody does bind to protein A at neutral pH (McLean et al., 1982). The bands that migrated at about 130 kDa and higher were only seen in HSV-1-infected cells. As their intensity with the presence of specific oligos mimicked the intensity of Vmw65, we suspect that they may represent Vmw65 protein that was not fully denatured or modified. Immunoprecipitation analysis suggested that less Vmw65 protein was made in the presence of oligomers 432s and 293s when compared to the Vmw65 protein level of Ltk– KOS-infected cells with no oligomer (Fig. 4). Quantitation of Vmw65 protein was accomplished by densitometric scan. The results suggested that oligomers 293s and 432s reduced Vmw65 by 51 and 68%, respectively (Fig. 5). Oligomers 007s and 294s reduced Vmw65 by 15 and 6%, respectively (Fig. 5). HSV KOS viral repli-

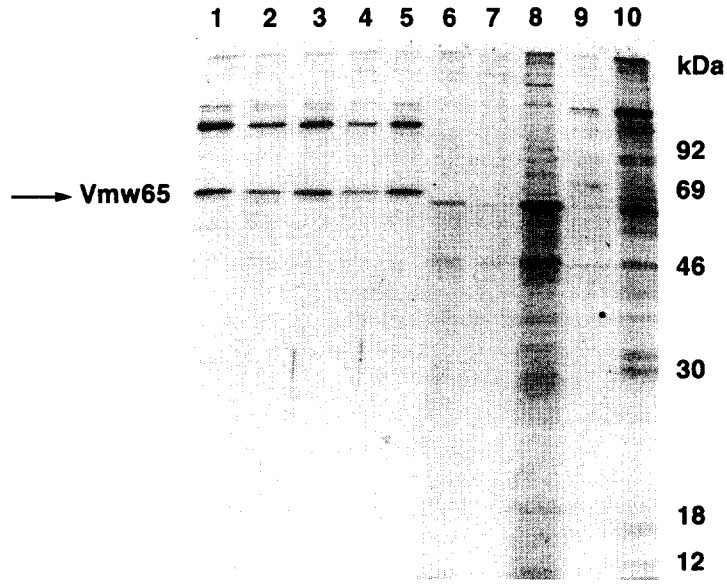

Fig. 4. Autoradiograph of a 12.5% SDS-PAGE gel showing an immunoprecipitation of viral-cell lysates after treatment with phosphorothioate oligomers. The antibody was against the Vmw65 protein. Lane 1: no oligo; Ltk−; KOS. Lane 2: 293s; Ltk−; KOS. Lane 3: 007s; Ltk−; KOS. Lane 4: 432s; Ltk−; KOS. Lane 5: 294s; Ltk−; KOS. Lane 6: AB; Ltk−; no KOS. Lane 7: preimmune serum; Ltk−; no KOS. Lane 8: no AB; Ltk−; no KOS. Lane 9: preimmune serum; Ltk−; KOS. Lane 10: no AB; Ltk−; KOS. 6×10^6 cpm per lysate was immunoprecipitated and the entire sample (supernatant) was loaded on gel. Exposure was for 72 h. Details describing the experiment can be found in Materials and Methods. Mobility of ^{14}C molecular size standards (NEN Research) is indicated on the right.

cation was also measured in this experiment by plaque reduction. Fig. 5 shows a comparison between the amount of protein and HSV KOS virus being made in the presence of oligomers 432s, 293s, 294s and 007s compared to the HSV KOS only control. Plaque reduction data correlated well with densitometric scan analysis (Fig. 5). ^{35}S-radiolabelled Ltk− lysates with oligomers 007s or 293s (at 5 μg/ml) or no oligomer were visualized by electrophoresis (Fig. 6). No inhibition of total cell protein synthesis by either oligomer 293s or 007s was evident.

Discussion

Our study of phosphorothioate-modified oligomers revealed significant (>50%) inhibition of HSV KOS replication at 25 μg/ml (4.3 μM) using two oligomers (293s and 432s) complementary to the initiation region of the Vmw65 gene which decreased in a dose-dependent manner to 0% inhibition at <50 ng/ml (9 nM) with both 0.5 and 0.05 PFU/cell. Lemaitre (1987) found that poly(L-lysine) conjugated with a 15-nucleotide-long sequence complementary to the initiation codon of VSV N protein reduced VSV yield in a dose-dependent fashion. The antiviral activity

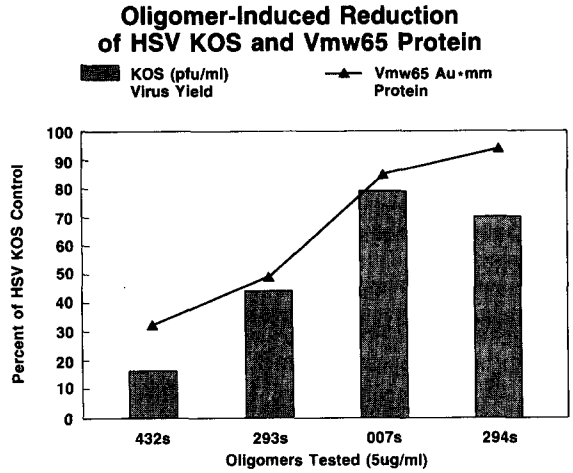

Fig. 5. Densitometric analysis showing the amount of Vmw65 protein generated during the presence of specific oligomers is shown as the line graph. The actual immune precipitation experiment used for this analysis is shown in Fig. 4. Intensities of the ^{35}S-labeled proteins were compared to control (KOS only, no oligo) considered 100%. The bar graph represents the virus yield from the same experiment obtained by plaque reduction assay.

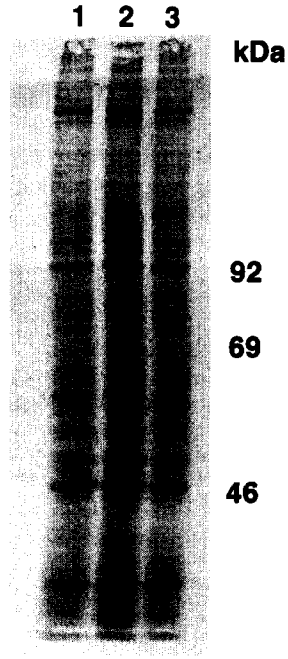

Fig 6. Ltk– cells with or without oligomer were pulse-labeled with [^{35}S]methionine. Cytoplasmic extracts (4×10^5 cpm/lane) were analyzed on a 12.5% SDS-PAGE gel. Exposure was for 24 h. Mobility of ^{14}C molecular size standards (NEN Research) is indicated on the right. Lane 1: Ltk– only. Lane 2: 007s; Ltk–. Lane 3: 293s; Ltk–.

was detectable with a concentration in the culture medium as low as 100 nM and reached >95% inhibition at 400 nM. This antiviral action of the 5′ end sequence conjugated to poly(L-lysine) was specific since no concentration of the conjugate significantly affected EMCV production (Lemaitre, 1987).

Another sequence-specific oligomer, 294s, which is complementary to a region 33 nucleotides 5′ from oligomer 293s, showed only a slight dose response (20% inhibition at 25 μg/ml decreasing to 2% at 50 ng/ml and this only at 0.05 PFU/cell at 48 h p.i.). It may be that under the conditions we use this oligomer does not hybridize as well with the mRNA (G+C content is 55% for 294s as compared to 72% for 293s). Matsukura et al. (1987) showed that G+C content of phosphorothioate ODNs influenced their ability to inhibit HIV viral cytopathic effect (CPE) even if they were not complementary to a specific mRNA. At 5 μM, with 14-mer non-antisense phosphorothioate ODNs, inhibition of HIV CPE was approximately linear with respect to the G+C content of the analog. It is not unexpected that this slight dose response with 294s was seen only using an MOI of 0.05 PFU/cell as the other sequence specific oligomers tested, in general, gave higher levels of inhibition using the lower MOI of 0.05 PFU/cell rather than 0.5 PFU/cell.

The random AT-rich oligomer did not inhibit HSV KOS replication in a dose-dependent manner under any conditions. Matsukura et al. (1989) demonstrated that phosphorothioate oligomers containing sense, random, or homopolymeric sequences did not have an inhibitory effect on HIV viral expression. Conversely, phosphorothioate oligomers complementary to the initiation sequence of human immunodeficiency virus type 1 (HIV-1) rev significantly suppressed viral expression (70%) in T cells chronically infected with HIV-1 from 2.5–10 μM and no cellular toxicity was observed. These investigators also found that a complementary oligomer with unmodified phosphodiester linkage did not inhibit HIV viral expression and concluded that sequence specificity and nuclease resistance were critical for the anti-viral-gene regulatory effect of the antisense molecules (Matsukura et al., 1989).

Immunoprecipitations of HSV-KOS-infected cell lysates using a Vmw65-specific mAb correlated with the yield reduction followed by plaque reduction data of specific oligomer inhibition of HSV KOS replication. Analysis by densitometry revealed a 51–68% reduction of Vmw65 protein synthesis in HSV-KOS-infected cells with oligomers 293s and 432s respectively. Slight (<20%) reduction in viral replication and Vmw65 protein was seen with oligomers 007s and 294s. This reduction correlated well with the dose response results using 5 μg/ml of 007s and 294s, HSV KOS at an MOI of 0.05 PFU/cell and a 24-h harvest p.i. (Figs. 2b and 3b). These experiments confirmed an approximate 20% reduction in HSV KOS yield with both oligomers 007s and 294s under those conditions.

The dramatic reduction of Vmw65 protein synthesis (51 and 68% for 293s and 432s respectively) gave further proof that the reduction in viral yield was due to the sequence specificity of the oligomers and not to the s-moiety. This was further confirmed by our [^{3}H]leucine data and our autoradiographic visualization of Ltk– cell proteins with and without oligomer by electrophoresis (Fig. 5) which indicated no overall inhibition of cellular protein synthesis at ODN concentrations of 4.3 μM or

less. Wickstrom (1987) showed inhibition of human c-myc p65 protein expression by the c-myc-complementary oligomer but not by the oligomer complementary to VSV matrix protein mRNA or complementary to nucleotides 5399–5413 of HIV *tat* gene mRNA indicating a sequence-specific dose-dependent inhibition of protein expression. It would be informative to use Northern blot analysis to determine whether these specific oligomers affect transcription or result in a degradation of mRNAs in tissue culture. Studies with natural oligomers suggest that they bind to mRNA and may be able to affect translation (Draper et al., 1990).

A high level (>50%) of nonspecific inhibition of HSV KOS replication was seen with oligomers 294s and 007s at 12.5 μg/ml (2.1 μM) or higher using an infectivity of 0.05 PFU/cell and harvesting 24 h p.i. This inhibition disappeared immediately below 2.1 μM. Matsukura et al. (1987) demonstrated that ODNs complementary to HIV sequences as well as homo-ODNs exhibited potent antiviral activity at 1 μM but failed to inhibit *gag* expression in chronically infected T cells up to 25 μM. Gao et al. (1990) found that phosphorothioate homo-oligodeoxynucleotides (nonspecific) inhibited HSV-2 growth by 90% from 1–3 μM and there was no apparent toxicity up to 10 μM. The effect on specific protein expression was not included in this study. This correlates with the nonspecific inhibition we saw with oligomers 294s and 007s at 2.1 μM (12.5 μg/ml).

In this study no correlation was shown between time of harvest (24 or 48 h p.i.) and inhibition levels of oligomers 432s and 293s.

Herpes virus replicates in a cascade fashion and one part of this mechanism is the upregulation of alpha genes by Vmw65. It would be interesting to determine if a specific oligomer can indirectly influence the expression of an alpha gene by decreasing the production of Vmw65. Work is in progress to examine this potential effect.

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