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Synergistic Antiviral Activities of Oligonucleoside Methylphosphonates Complementary to Herpes Simplex Virus Type 1 Immediate-Early mRNAs 4, 5, and 1

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An oligonucleoside methylphosphonate (ONMP) complementary to the splice acceptor site of immediate-early (IE) pre-mRNAs 4 and 5 (IE4,5SA) inhibits herpes simplex virus type 1 (HSV-1) growth in vitro and in infected animals. The antiviral effect appears to be due to inhibition of IE pre-mRNA 4 and 5 splicing and/or IE4 gene expression (M. Kulka, M. Wachsman, S. Miura, R. Fishelevich, P. S. Miller, P. O. P. Ts'o, and L. Aurelian, Antiviral Res. 20:115–130, 1993). We describe the potentiation of antiviral activity when we targeted two IE genes with different ONMPs. A *psoralen* derivative of an ONMP complementary to the IE mRNA 1 (IE1) translation initiation site (IE1TI) covalently bound a 2.8-kb transcript that hybridized with a 20-base oligonucleotide complementary to the 5′ leader sequence of IE1 but not a 20-base oligonucleotide complementary to the first intron of IE1. IE1TI inhibited IE1 gene expression and virus replication in cells infected with HSV-1 in vitro. Inhibition was specific because it was not observed with oligomers mutated in two (IE1TI*mu*1) or four (IE1TI*mu*2) central residues or in cells infected with an IE1 deletion mutant (HSV-1 *dl*1403). IE1TI potentiated the antiviral activity of IE4,5SA (synergistic effect), while potentiation was not observed when IE4,5SA was mixed with IE1TI*mu*1. A similar synergistic effect was seen when IE1TI was mixed with an ONMP complementary to the translation initiation site of IE mRNA 4 but not with an ONMP complementary to the translation initiation site of IE mRNA 5. These findings suggest that synergistic antiviral activity is mediated by targeting at least two IE genes (IE1 and IE4).

Antisense RNA that selectively inhibits gene expression has been proposed as a genetic approach for the prevention and treatment of diseases (7). The limitations of the native oligodeoxynucleotides are their relatively short half-lives and low degrees of cellular penetration. To address these limitations, we developed sequence-specific nonionic nucleic acid analogs (oligonucleoside methylphosphonates [ONMPs]) that contain a 3′-5′-methylphosphonate group in place of the negatively charged phosphodiester group (25). ONMPs penetrate mammalian cells in culture, are resistant to nuclease hydrolysis (15), and specifically inhibit the expression of several target genes (1, 4).

To explore the possibility that control of gene expression by ONMPs could be an effective antiviral modality, we focused on the ability of ONMPs complementary to the herpes simplex virus type 1 (HSV-1) immediate-early (IE) genes to inhibit virus growth. We showed that an ONMP complementary to the splice acceptor junction of HSV-1 IE pre-mRNAs 4 and 5 (IE4,5SA) inhibits splicing of the targeted mRNA as well as HSV-1 protein and DNA syntheses, and it significantly reduces viral growth in vitro and in infected animals (10, 11, 23). Inhibition appears to involve faulty processing of IE mRNA 4 (IE4) or decreased synthesis of the encoded protein (IE68), since it was also seen with an ONMP targeted to the translation initiation site of IE4 (IE4TI) but not with an ONMP targeted to the translation initiation site of IE mRNA 5 (IE5TI) (11). In the studies described in this report, we sought to determine the antiviral effect resulting from the simultaneous targeting of multiple IE genes. The data indicate that targeting of at least two IE genes, i.e., IE4 and IE1 (which were previously shown to be involved in HSV-1 growth [8, 21, 24]), results in synergistic inhibition of HSV-1 replication.

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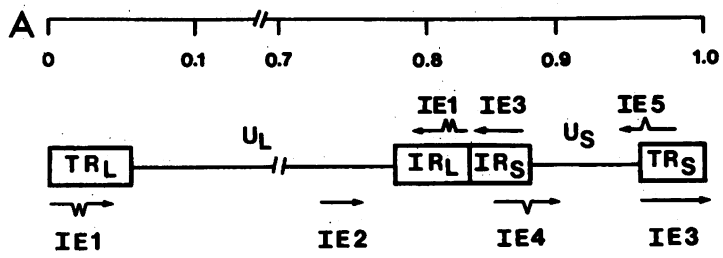
MATERIALS AND METHODS

Cells and virus. Vero cells were grown in Eagle's minimal essential medium with 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) and 10% fetal bovine serum. HEp-2 cells were grown in medium 199 with 10% calf serum. They were infected with the F strain of HSV-1 or with the IE110 deletion mutant *dl*1403 (24) as described previously (10).

Synthesis of ONMPs. The binding sites and the sequences of the ONMPs used in the present study are shown in Fig. 1. They were synthesized by solid-phase techniques and were purified and characterized as described previously (14). The oligomer aeAMT-[^{32}P]d(GpCGGGGCTCCAT) contains 4′-(aminoethyl)aminomethyl-4,5′,8-trimethyl*psoralen* (aeAMT) attached at the 5′ end by a nuclease-resistant phosphoramidate linkage (*psoralen*-[^{32}P]IE1TI). Its synthesis and purification were described previously (3). Prior to derivatization, the oligomer was radiolabeled (specific activity, 1 Ci/mmol) at the 5′ position with T4 polynucleotide kinase and [γ-^{32}P]ATP.

ONMP inhibition of HSV-1 growth in vitro. Vero cells were infected with HSV-1 F at a multiplicity of infection (MOI) of 1 PFU per cell or with the *dl*1403 mutant at an MOI of 0.05 and 5 PFU per cell and were treated with the respective oligomers (0 to 200 μM) at various times relative to the time of



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NAME	TARGET	SEQUENCE
		INITIATION
	HSV-1 IE mRNA 1:	5' CCCCAUGGAGCCCCGCCCCG 3'
IE1TI		3'TACCTCGGGGCG 5'
IE1TImu1		3'TACCTGCGGGCG 5' (2 base mutant)
IE1TImu2		3'TACCGGCTGGCG 5' (4 base mutant)
		INITIATION
	HSV-1 IE mRNA 4:	5' CUGGAUGGCCGACAUUUCCC 3'
IE4TI		3'TACCGGCTGTAA 5'
IE4TImu1		3'TACCGCGTGTAA 5' (2 base mutant)
		INITIATION
	HSV-1 IE mRNA 5:	5' AAGCAUGUCGUGGGCCCUGG 3'
IE5TI		3'TACAGCACCCGG 5'
IE5TImu1		3'TACAGACCCCGG 5' (2 base mutant)
		SPLICE ACCEPTOR
	HSV-1 IE pre-mRNA 4,5:	5' CUUCCCGCAG]GAGGAACGUC 3'
IE4,5SA		3'GGCGTC CTCCTT 5'
IE4,5SAmu1		3'GGCGTC TCCCTT 5' (2 base mutant)
Scrambled	None	3'CACGGAAGGGCG 5'

FIG. 1. (A) Approximate map locations of HSV IE mRNAs. (B) Nucleotide sequence of IE1, IE4, and IE5 at their translation initiation sites and the intron-exon boundary of IE4 and IE5.

infection. The progeny were harvested at 24 h postinfection, and virus titers were determined by plaque assay on HEp-2 cells as described previously (10). Consistent with previous reports (24), *dl*1403 replicates and forms plaques on non-complementing cells. However, virus titers are 3 to 4 $\log_{10}$ units lower than those seen for wild-type HSV-1.

In vitro cross-linking of aeAMT-[^{32}P]d(GpCGGGGCTC CAT) to IE1. Vero cells were infected with 50 PFU of HSV-1 per cell or were mock infected with phosphate-buffered saline pH 7.4 in the presence of 50 μg of cycloheximide per ml to enhance IE mRNA synthesis (16). At 6 h postinfection, the mRNA was isolated with a Mini RiboSep kit (Collaborative Research) according to the manufacturer's instructions. Aliquots of HSV-1 (2 μg) or mock-infected (4 μg) cellular mRNA were resuspended in 10 μl of TMK buffer (30 mM Tris-Cl [pH 7.5], 20 mM $MgCl_2$, 300 mM KCl), denatured at 65°C for 5 min, and cooled (25°C, 2 h). They were incubated (37°C, 10 min) with 100 pmol of *psoralen*-[^{32}P]IE1TI (final concentration, 10 μM) and then for 1 h at 4 or 15°C and were UV irradiated (365 nm, 15°C, 16 min). Irradiated mixtures were diluted and ethanol precipitated. Following centrifugation (4°C), the pellets were resuspended in 10 μl of 1× TBE buffer (90 mM Tris-Cl [pH 8.0], 90 mM boric acid, 1 mM EDTA) containing 90% formamide, 0.2% bromphenol blue, and 0.2% xylene cyanol. They were heated (65°C, 7 min) and electrophoresed on a 5% polyacrylamide–7 M urea gel in 1× TBE for 6 h. Radiolabeled products were visualized by autoradiography overnight at −80°C on Kodak X-Omat-S film. Similarly cross-linked VSV mRNAs served as size markers (20).

Northern blot hybridization. Northern blot hybridization

was performed as described previously (12). Two oligodeoxynucleotide probes were used. They were complementary to the HSV-1 IE1 5′ leader sequence (5′-CCGAGTGCCGAG CTGCAAAT-3′) and to sequences located within the first intron (5′-CCGGTTCCAGTGTAAGGGTC-3′). They were synthesized by the phosphoramidite method and were purified by high-pressure liquid chromatography and 5′ end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP.

Synergy analysis. To characterize ONMP interactions, a three-dimensional analytical method was performed with the three-dimensional model MacSynergy II (18). Theoretical additive interactions were calculated from the dose-response curves of the individual ONMPs. The additive surface was subtracted from the experimental surface to reveal regions of greater than expected interaction (synergy). For each point in the plot, the synergy was considered to be significant if the lower 95% confidence limit of the experimental data was greater than that for the calculated additive surface. The plots and values presented here are at the level of 95% confidence.

Cytotoxicity determinations. Cytotoxicity assays were done in 96-well plates by a rapid method for quantitating cells. Cell growth was quantitated as described previously (18) by staining the cell sheet with crystal violet and measuring the bound dye by eluting with 1% (vol/vol) HCl in 95% ethanol. The optical densities were determined at 570 and 405 nm in a microplate biokinetics reader (Biotek, Winooski, Vt.). The data were analyzed as described above.

RESULTS

IE1TI ONMP specifically binds its mRNA target. The studies described here were designed to determine whether the ONMP complementary to the IE1 translation initiation site (IE1TI) can specifically recognize its target sequence in vitro. They are based on previous findings indicating that *psoralen*-derivatized oligomers bind their targets after exposure to 365-nm UV irradiation (9). A 2.8-kb band consistent with IE1 (17) was observed in extracts from HSV-1-infected cells (Fig. 2A, lanes 1 and 2) but not in extracts from mock-infected cells. The oligomer complexed to its mRNA target with a high degree of specificity and affinity, since the band was detected equally well when the hybridization temperature was increased from 4 to 15°C (Fig. 2A, lanes 1 and 2).

The 2.8-kb transcript was observed when the mRNA extracted from HSV-1-infected cells was hybridized with the oligodeoxynucleotide probe complementary to the IE1 5′ leader sequence (Fig. 2C, lane 2). It was not observed in uninfected cell extracts hybridized with this probe (Fig. 2C, lane 1) or in the HSV-1-infected (Fig. 2B, lane 2) or mock-infected (Fig. 2B, lane 1) cell extracts probed with the oligodeoxynucleotide complementary to the first intron of IE1. These findings suggest that *psoralen*-IE1TI specifically cross-links to its mRNA target. IE1 gene expression in cells infected for 3 h was inhibited by IE1TI but not by IE1TI*mu*1, as determined by immunohistochemistry with antibody to IE1 protein (Advanced Biotechnologies, Inc.) as described previously (2) (data not shown).

ONMPs complementary to IE1 inhibit the growth of HSV-1 but not *dl*1403. Previous studies with IE1 deletion mutants have shown that IE1 expression is required for optimal HSV-1 growth in cells infected at a low MOI (<10 PFU per cell) (21, 24). Accordingly, we sought to determine whether IE1 is a valid target for antisense inhibition of virus growth in cultures infected with wild-type HSV-1 at an MOI of 1 PFU per cell. Exposure to IE1TI was initiated at the time of infection and was continued for the duration of infection (24 h). The

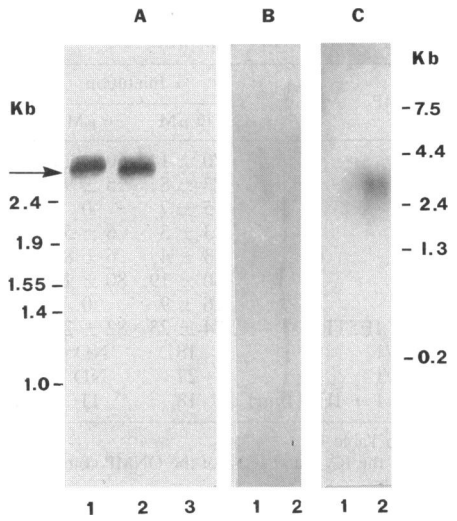

FIG. 2. Autoradiogram of cross-linked *psoralen*-[^{32}P]IE1TI (A). mRNA from HSV-1-infected (lanes 1 and 2) or mock-infected (lane 3) cells (in the presence of cycloheximide) was hybridized (1 h) with 10 μM *psoralen*-[^{32}P]IE1TI at 4°C (lanes 1 and 3) or 15°C (lane 2). After UV irradiation (365 nm, 16 min), cross-linked products were analyzed by electrophoresis on a 7 M urea–5% polyacrylamide gel. The *psoralen*-[^{32}P]IE1TI–IE1 complex is identified (arrow). mRNA extracted from Vero cells infected with HSV-1 (lane 2) or mock infected (lane 1) in the presence of cycloheximide was electrophoresed on a formaldehyde agarose gel, transferred to nylon membranes, and hybridized with oligodeoxynucleotide probes complementary to the 5′ leader (C) or first intron (B) of IE1. Molecular size markers are shown.

concentrations that caused a 50% (IC_{50}) and a 90% (IC_{90}) decrease in virus titers were 17 and 110 μM, respectively (Table 1). Inhibition was sequence specific. Thus, there was minimal (8 to 20%), if any, reduction in virus growth with a scrambled oligomer or with ONMPs that were mutated in two or four central residues (IE1TI*mu*1 and IE1TI*mu*2, respectively).

To further determine whether IE1 is a valid target for HSV-1 growth inhibition, we sought to verify the target gene specificity of IE1TI by using the *dl*1403 mutant that is deleted in the IE1 gene (24). Consistent with the absence of a functional IE1 target in *dl*1403, the results indicated that

TABLE 1. Effect of treatment with ONMP combinations on HSV-1 growth[a]

ONMP	% Inhibition		IC_{50} (μM)	IC_{90} (μM)
	12 μM	6 μM		
IE4,5SA	25 ± 7	12.5 ± 4	20	95
IE4,5SA*mu*1	15 ± 6	8 ± 5	NA[b]	NA
IE1TI	28 ± 12	9 ± 12	17	110
IE1TI*mu*1	13 ± 7	11 ± 6	NA	NA
IE1TI + IE4,5SA	70 ± 14	60 ± 14	4[c]	17[c]
IE4,5SA + IE1TI*mu*1	27 ± 11	22 ± 3	19[c]	95[c]

[a] HSV-1 F-infected Vero cells (1 PFU per cell) were treated (0 to 24 h postinfection) with ONMPs complementary to the indicated HSV-1 mRNA target alone or in combination. Virus titers were assayed 24 h after infection. Results are expressed as mean percent inhibition of virus titers compared with those of untreated controls for two to three experiments. Inhibition ranging between 0 and 20% was not considered significant (10, 11). The IC_{50}s and IC_{90}s were estimated from dose-response curves.

[b] NA, not applicable.

[c] Values represent the IC_{50}s and IC_{90}s of the ONMP combinations.

TABLE 2. Effect of treatment with ONMP combinations on HSV-1 growth[a]

ONMP	% Inhibition		IC_{50} (μM)	IC_{90} (μM)
	12 μM	6 μM		
IE1TI + IE4,5SA	70 ± 14	60 ± 14	4[b]	17[b]
IE4TI	23 ± 8	5 ± 9	20	98
IE4TI*mu*1	5 ± 7	0	NA[c]	NA
IE5TI	3 ± 3	6 ± 5	NA	NA
IE5TI*mu*1	3 ± 4	6 ± 8	NA	NA
IE1TI + IE4TI	80 ± 19	80 ± 11	3[b]	15[b]
IE1TI + IE5TI	16 ± 9	0	20[b]	10[b]
IE1TI + IE4TI + IE5TI	74 ± 23	82 ± 23	4[b]	17[b]
IE1TI + IE4TI*mu*1	18	ND[d]	ND	ND
IE1TI + IE5TI*mu*1	27	ND	ND	ND
IE1TI + IE4TI*mu*1 + IE5TI*mu*1	18	11	18	112

[a] See footnote *a* to Table 1.
[b] Values represent the IC_{50}s and IC_{90}s of the ONMP combinations.
[c] NA, not applicable.
[d] ND, not determined.

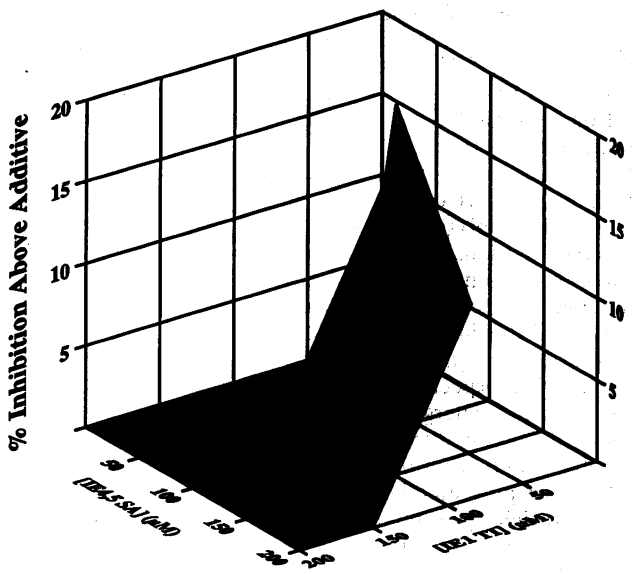

FIG. 3. Synergistic inhibition of HSV-1 replication by IE1TI and IE4,5SA. The synergy plot in three-dimensional form reveals the synergy at the 95% level of confidence (volume, 4.04 μM^2 %). Maximal synergy was observed at 12 μM E1TI and IE4,5SA.

ONMP concentrations up to 100 μM did not inhibit virus growth in cells infected with this mutant at an MOI of 0.5 or 5 PFU per cell (0 to 19% inhibition).

Potentiation of antiviral activity of IE4,5SA by IE1TI. To determine whether simultaneous targeting of the IE genes involved in the regulation of HSV-1 growth enhances the antiviral activities of the ONMPs, we used combinations of IE1TI and oligomers that target the IE4 and/or IE5 genes. These ONMPs were previously shown to bind their respective viral mRNA targets and inhibit HSV-1 growth in a sequence-specific manner (10, 11).

Two series of experiments were done. In the first series, Vero cells were infected with HSV-1 in the presence of mixtures of IE4,5SA and IE1TI, and virus titers were determined 24 h later. At 6 μM, these ONMPs had minimal, if any, inhibitory effect. However, in combinations in which the ONMPs were used at 6-μM concentrations, inhibition was greater than 50% (Tables 1 and 2). The combination of the two oligomers caused a fivefold greater inhibition of virus growth than that by each ONMP alone and a threefold greater inhibition than the calculated sum of effects of the individual ONMPs.

An isobologram (5, 18) of the data was constructed for the ONMP concentrations that inhibited plaque formation by 60%. This conventional plot indicated the existence of synergistic interactions between the two ONMPs (data not shown). When the data were analyzed by the three-dimensional model MacSynergy II (18), the antiviral effects of the ONMPs were confirmed (Fig. 3). The maximal synergistic antiviral activity was seen at 12 μM (each) ONMP. The potentiation of antiviral activity was statistically significant, as determined by the 95% confidence intervals around the experimental surface. The antiviral activity was specific because the ONMPs did not inhibit cell proliferation when they were used alone or in combination (0.3 to 2% reduction at 10 to 100 μM). The analysis suggests that ONMPs targeted to pre-mRNA 4 and 5 and to IE1 can act synergistically to inhibit HSV-1 growth. Synergy is sequence specific, because it was not observed when IE4,5SA was combined with IE1TI*mu*1 (Table 1) or when IE1TI was mixed with IE4,5SA*mu*1 (data not shown).

Synergistic antiviral activity involves targeting of IE4 and IE1 mRNAs. IE4,5SA is complementary to the splice acceptor site sequence which is identical in both IE4 and IE5 (11). Previous studies with ONMPs complementary to the translation initiation sites of IE4 and IE5 suggested that targeting of the IE5 gene has no effect on virus growth inhibition (11). In the present study, we sought to investigate the individual contributions of the IE4 and IE5 genes in the antiviral potentiation of IE4,5SA by IE1TI. As shown in Table 2, the levels of inhibition (80% ± 11%) obtained with a combination of IE1TI and IE4TI at 6 μM each were similar to those seen with a combination of IE4,5SA and IE1TI at 6 μM concentrations (60% ± 14%). They were significantly higher than those seen for each ONMP alone and were also higher than the sum of effects of individual ONMPs. Potentiation was not observed when IE1TI was combined with IE5TI, and combinations that targeted all three genes were not superior to those that targeted only IE4 and IE1. Furthermore, potentiation was not observed in combinations in which any one of the ONMPs was substituted with its companion mutant. In these combinations, the levels of growth inhibition were in the range of those obtained for the individual ONMP (Tables 1 and 2).

DISCUSSION

The studies described in this report sought to determine whether targeting of multiple IE genes that are potentially involved in HSV-1 growth regulation (6) results in synergistic antiviral activity. In addition to IE4 and IE5, we focused on IE1 on the basis of previous findings indicating that expression of this gene, while not obligatory, is required for optimal HSV-1 growth in culture (21, 24). In support of the role of IE1 in optimal HSV-1 growth, an ONMP targeted to this mRNA inhibited HSV-1 growth in a sequence-specific and dose-dependent manner. Our data support the interpretation that viral growth inhibition by the ONMP that targets IE1 is directly related to its ability to bind to its target. Indeed, *psoralen*-[^{32}P]IE1TI cross-linked to a 2.8-kb transcript from HSV-1-infected cells. The following evidence supports the conclusion that this 2.8-kb transcript is IE1: (i) the transcript is present in HSV-1-infected but not uninfected cells and its size is consistent with that of IE1 (17), (ii) Northern blot hybridization of

comparable extracts with a probe complementary to the IE1 5′ leader sequence but not the intron identified the 2.8-kb transcript, and (iii) the length of this cross-linked product excludes any other HSV-1 IE mRNA. IE1 gene expression was inhibited by IE1TI, but not IE1TI*mu*1, and IE1TI did not further reduce the growth of the HSV-1 *dl*1403 mutant whose IE1 gene is deleted.

Our past (11) and present findings are consistent with the conclusion (8, 13) that IE4, but not IE5, is required for virus growth. However, since ONMP activity is abrogated by simple base substitutions, minor modifications in the location of the targeted sequence within the gene, and secondary mRNA structure (10), we do not exclude the possibility that IE5TI does not target the significant IE5 gene sequence. Because we do not have the cloned IE5 gene or antibody specific to this gene product, the question is as yet unanswered.

The role of IE4 in virus replication, however, remains a matter of controversy. Using IE4 deletion mutants, Sears et al. (22) and Purves et al. (19) concluded that IE4 is not required for virus growth in Vero cells, although this mutation affects virus replication in rodent cells. In order to explain this apparently contradictory finding, those investigators invoked the contribution of a Vero cell gene functionally homologous to IE4. There is no evidence in support of this hypothesis, nor do we have direct evidence for the specific inhibition of IE4. However, IE4,5SA and IE4TI ONMPs inhibit virus growth in Vero, HEp-2 (23), and rodent cells (11), suggesting that the ONMP also inhibits this putative cellular homolog whose target sequence is highly conserved among the species. The likelihood of such an interpretation is unclear.

Significantly, we found that modest concentrations of IE1TI reduced the IC_{50}s and IC_{90}s of IE4,5SA without significant toxicity to the host cells. The mechanism(s) by which IE1TI potentiates IE4,5SA presumably involve(s) the simultaneous inhibition of genes that play significant roles in the regulation of HSV-1 growth since potentiation was not seen when either ONMP in the combination was replaced with its mutant, nor when IE1TI was combined with IE5TI. We interpret these findings to indicate that the synergistic antiviral effects seen with IE4,5SA and IE1TI are due primarily, if not only, to the targeting of IE1 and IE4 genes and that coexpression of at least IE1 and IE4 is important for HSV-1 growth.

Final conclusions pertaining to the chemotherapeutic potential of ONMPs and their synergistic effects must await the results of in vivo studies with various HSV isolates that may show sequence variability in the targeted genes. However, as powerful alternatives to labor-intensive mutational analysis, the antisense approach continues to be an important tool for understanding gene regulation and viral growth.

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