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Inhibition of Herpes Simplex Virus Replication by Antisense Oligo-2′-*O*-methylribonucleoside Methylphosphonates†

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ABSTRACT: Antisense oligonucleoside methylphosphonates complementary to the 12 nucleotides found at the intron/exon junction of the splice acceptor site of herpes simplex virus type 1 (HSV-1) immediate early mRNAs 4 and 5 were synthesized. The methylphosphonate oligomers contained either 2′-deoxyribose nucleosides, d-OMPs, or 2′-*O*-methylribose nucleosides, mr-OMPs. At 37 °C, the affinity of the mr-OMP for a complementary 12-mer RNA target was approximately four times higher than that of the corresponding d-OMP as measured by a constant activity gel electrophoresis mobility shift assay. An mr-OMP whose sequence contained two mismatched bases did not bind to the RNA target under these conditions. The mr-OMP also showed improved ability to inhibit HSV-1 replication in HSV-1 infected Vero cells in culture. Thus the IC_{50} of the mr-OMP was five times less than that of the d-OMP. No inhibition was observed by the mismatched mr-OMP, and no inhibition of herpes simplex virus type 2 (HSV-2) replication was observed with any of the oligomers. These results demonstrate a direct correlation between oligomer binding affinity and antisense activity in cell culture and suggest that oligo-2′-*O*-methylribonucleoside methylphosphonates are promising candidates for development of effective antisense reagents.

Antisense oligodeoxyribonucleoside methylphosphonates, d-OMPs, complementary to various mRNAs of herpes simplex virus type 1 have been shown to be effective inhibitors of HSV-1 replication both in cell culture and in mouse model systems (Kulka *et al.*, 1993, 1994). The common sequence found in the acceptor splice junction of immediate early mRNAs 4 and 5 has proven to be a particularly good target for these oligomers. Thus an octamer, d-TpCCTCCTG, which is complementary to the last five nucleotides of the intron and the first three nucleotides of the exon, was shown to inhibit HSV-1 replication and to a lesser extent HSV-2 replication in Vero cells in culture (Smith *et al.*, 1986). A dodecamer, d-TpTCCTCCTGCGG, **A**, which is complementary to the last six nucleotides of the intron and the first six nucleotides of the exon, selectively inhibited HSV-1 replication but not HSV-2 replication in cell culture and was also shown to inhibit splicing of immediate early mRNA 4 (Kulka *et al.*, 1989). In addition, oligomer **A** reduced virus titer when applied as a cream to mouse ear pinna that were infected

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[1] Abbreviations: d-OMP, oligo-2′-deoxyribonucleoside methylphosphonate; mr-OMP, oligo-2′-*O*-methylribonucleoside methylphosphonate; EDTA, ethylenediamine tetraacetate; EMEM, Eagle's minimal essential medium; TBE, 89 mM tris(hydroxymethyl)aminomethane (Tris), 89 mM boric acid, 0.2 mM ethylenediaminetetraacetate buffered at pH 8.0.

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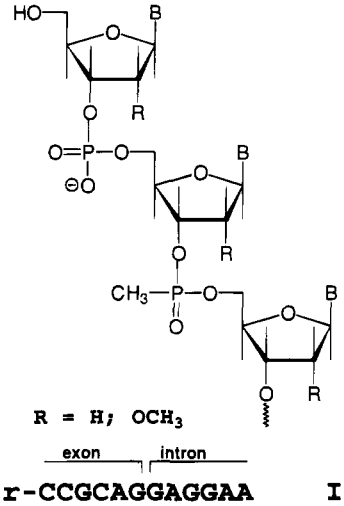

FIGURE 1: Structures of oligonucleoside methylphosphonates and sequence of the complementary RNA target.

with HSV-1 (Kulka *et al.*, 1993).

Modifications which enhance the affinity between the oligomer and its target would be expected to enhance antisense activity of the oligomer (Stull *et al.*, 1992). This may be particularly important for oligonucleoside methylphosphonates because, unlike oligodeoxyribonucleotide or oligonucleotide phosphorothioates, duplexes formed between d-OMPs and RNA are not substrates for RNase H (Agarwal *et al.*, 1990). Consequently the inhibitory action displayed by these oligomers most likely results from a "steric blocking" mechanism (Milligan *et al.*, 1993). This idea receives support from studies with psoralen-derivatized d-OMPs. These oligomers are capable of forming covalent adducts with targeted RNAs (Kean & Miller, 1994; Levis & Miller, 1994). Thus 100 μM oligomer **A** was found to inhibit HSV-1 replication ~95%, whereas similar levels of inhibition could be achieved by the psoralen derivative of **A** at a concentration of 5 μM (Kulka *et al.*, 1989).

Previous studies from our laboratory have demonstrated that like their phosphodiester counterparts, oligonucleoside methylphosphonates composed of 2′-*O*-methylribonucleosides have greater affinity for complementary RNA targets than do the corresponding oligomers composed of deoxyribonucleosides (Miller *et al.*, 1991a; Kean *et al.*, 1994). These oligo-2′-*O*-methylribonucleoside methylphosphonates, mr-OMPs, were found to be extraordinarily stable under physiological temperature and pH conditions and thus appear to be promising candidates as effective antisense compounds. To determine if enhanced binding affinity of mr-OMPs for RNA translates into enhanced biological efficacy, the 2′-*O*-methylribonucleoside analog of oligomer **A**, mr-UpUCCUCCUGCGG, was prepared and its anti-HSV-1 activity was determined in cell culture.

EXPERIMENTAL PROCEDURES

Synthesis of Oligoribonucleotide Target I. The oligoribonucleotide target **I**, shown in Figure 1, was prepared on a controlled pore glass support in an ABI model 392 DNA/RNA synthesizer using protected 5′-*O*-(dimethyoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)ribonucleoside-3′-*O*-*N*,*N*-diisopropylamino-β-cyanoethyl phosphoramidites purchased from Glen Research Inc. Upon completion of the synthesis, the support was treated for 18 h at 55 °C with 1 mL of absolute ethanol saturated with ammonia (Scaringe *et al.*, 1990). The support was filtered and washed with three 0.5 mL portions of 50% aqueous ethanol, and the combined filtrate and washings were evaporated. The solid residue was dissolved in 600 μL of a solution containing 1 M tetra-*n*-butylammonium fluoride in tetrahydrofuran, and the solution was incubated at room temperature for 48 h. The solution was loaded directly onto a 1.7 cm × 10 cm column of Bio-Gel P6, the column was eluted with RNase-free water, and 1 mL fractions were collected and monitored by UV. Fractions containing the RNA oligomer were diluted with an equal volume of 95% acetonitrile and stored at −20 °C. RNA stored in this manner is stable for at least 1 year. For use in the binding experiments described below, 0.8 nmol of the crude RNA was phosphorylated using 10 μCi of γ-[^{32}P]ATP and polynucleotide kinase. The [^{32}P]RNA was purified by electrophoresis on a 30 cm × 40 cm × 0.4 mm, 20% polyacrylamide gel containing 7 M urea. The running buffer, TBE, contained 89 mM tris(hydroxymethyl)aminomethane (Tris), 89 mM boric acid, and 0.2 mM ethylenediaminetetraacetate buffered at pH 8.0. The oligomer was extracted from the gel at 37 °C using a solution containing 0.1 M sodium chloride and 50 mM Tris, pH 7.6. The solution was desalted on a C-18 reversed-phase Sep Pak cartridge (Waters Chromatography Division) and stored as a solution in 50% aqueous acetonitrile at −20 °C.

Syntheses of Oligonucleoside Methylphosphonates (OMPs). Oligo-2′-deoxyribonucleoside- and oligo-2′-*O*-methylribonucleoside methylphosphonates were synthesized on controlled pore glass supports in an ABI model 392 DNA/RNA synthesizer using protected 5′-*O*-dimethoxytrityl-2′-deoxyribonucleoside-3′-*O*-*N*,*N*-(diisopropylamino)methylphosphonamidites supplied by JBL Scientific, Inc., and 5′-*O*-dimethoxytrityl-2′-*O*-methylribonucleoside-3′-*O*-*N*,*N*-(diisopropylamino)methylphosphonamidites supplied by Prime Synthesis Inc., respectively (Miller *et al.*, 1991b; Hogrefe *et al.*, 1993a; Kean *et al.*, 1994). The last coupling step was performed using the appropriate protected 5′-*O*-(dimethoxytrityl)nucleoside-3′-*O*-*N*,*N*-diisopropylamino-β-cyanoethyl phosphoramidite. The support was treated with 1 mL of a solution containing ethanol, acetonitrile, and concentrated ammonium hydroxide (45:45:10 v/v) for 30 min at room temperature, after which time 1 mL of ethylenediamine was added and incubation was continued for 6 h (Hogrefe *et al.*, 1993b). The solution was cooled on ice and neutralized by addition of 15 mL of 2 N hydrochloric acid. After being desalted on a C-18 reversed-phase cartridge, the oligomers were first purified by ion exchange chromatography on a DEAE cellulose column and then by C-18 reversed-phase HPLC (Miller *et al.*, 1991b). The oligomers were >97% pure as judged by analytical C-18 reversed-phase HPLC and by polyacrylamide gel electrophoresis.

Measurements of Oligomer Binding Affinities. The interactions between the OMPs and RNA target **I** were studied using a constant activity gel electrophoresis mobility shift assay (Trapane, 1993; Trapane & Ts'o, 1993). A Teflon template was used that allowed lanes of a 14 cm × 16 cm × 0.75 mm polyacrylamide gel to be cast individually. Each lane was cast using approximately 1.5 mL of polyacrylamide solution which in addition contained 0−100 μM OMP. The concentration of polyacrylamide in each lane was 16%, and the gel buffer was TBE. The [^{32}P]RNA target, 1 pmol, was dissolved in 4 μL of loading buffer containing 25% glycerol

Table 1: Apparent Dissociation Constants of Oligonucleoside Methylphosphonate/RNA Duplexes and Effects of Oligonucleoside Methylphosphonates on HSV Replication in Cell Culture

oligomer[a]		$K_{d_{app}}$ (M)[b]	IC_{50} (μM) HSV-1	IC_{50} (μM) HSV-2
d-TpTCCTCCTGCGG	**A**	2.0×10^{-6}	22	>100
mr-UpUCCUCCUGCGG	**B**	5.0×10^{-7}	4	>100
mr-UpUCCCUCUGCGG	**C**	$\gg 10^{-5}$	>100	>100

[a] Underlines indicate the position of the methylphosphonate linkages. [b] Apparent dissociation constant of the duplex as measured by CAGE at 37 °C in a buffer containing TBE.

in TBE and loaded into each lane of the gel which had previously been equilibrated at 37 °C. The gel was run at 37 °C at a constant voltage at 375 V for 1.5 h. The apparent dissociation constant was equated to the concentration of oligonucleoside methylphosphonate required to reduce the mobility of the RNA target halfway between that of the free target and the target/OMP duplex.

Effects of Oligonucleoside Methylphosphonates on HSV Replication in Cell Culture. Vero (African green monkey kidney) cells which were grown in Eagle's minimal essential medium, EMEM, supplemented with 10% fetal bovine serum were infected with HSV-1, strain F, or HSV-2, strain G, at a multiplicity of infection of 10 pfu/cell. The cells were treated 0−24 h postinfection with 0−50 μM oligomer. After 24 h, the infected cells were washed with phosphate-buffered saline and then scraped into fresh EMEM containing no oligomer. Cell extracts were prepared by seven consecutive cycles of freezing and thawing. The cell extracts were centrifuged at 2000*g* for 10 min to remove cell debris, and virus titers were determined by plaque assay on HEP-2 (human epidermoid no. 2) cells (Aurelian, 1992). Results were determined as the mean percent inhibition of virus titers compared to untreated controls for two experiments. Inhibition in the range 0%−20% was not considered significant (Kulka *et al.*, 1994).

RESULTS AND DISCUSSION

The general structures of the oligonucleoside methylphosphonates are shown in Figure 1. These oligomers contain either 2′-deoxyribonucleosides, d-OMP, or 2′-*O*-methylribonucleosides, mr-OMP, and racemic methylphosphonate groups at all positions except the 5′-terminal linkage, which is a phosphodiester. The sequences of the oligomers, which are complementary to the acceptor splice junctions of HSV-1 immediate early mRNAs 4 and 5, are shown in Table 1. Oligomers **A** and **B** are completely complementary to this region, whereas oligomer **C** contains two mismatched bases in the center of the sequence.

The interactions of oligomers **A**−**C** with the oligoribonucleotide target **I** were examined by a constant activity gel electrophoresis, CAGE, mobility shift assay (Trapane 1993; Trapane & Ts'o, 1993). In this procedure, individual lanes of a nondenaturing polyacrylamide gel are cast with increasing concentrations of OMP. The [^{32}P]-labeled target **I** is electrophoresed through each lane of the gel. Because the OMP has a single negative charge, it is essentially immobile during the electrophoresis and consequently the RNA target is subject to a constant concentration of OMP as it moves through the gel. Duplex formation is signaled by a decrease in mobility of the target. The concentration of OMP which causes the target to migrate halfway between the free target and the duplexed target corresponds to the apparent dissociation constant, $K_{d_{app}}$, of the OMP/RNA duplex. The thermodynamic parameters determined by measurement of apparent dissociation constants as a function of temperature by CAGE have been found to be in good agreement with those determined by shape analysis of UV melting curves (Trapane, 1993).

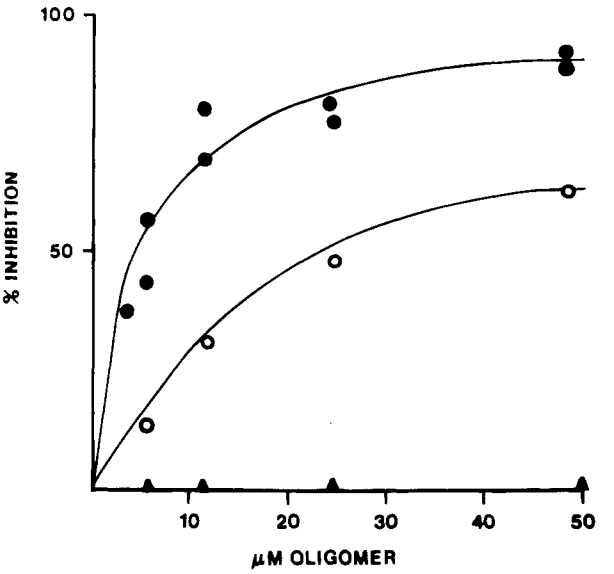

FIGURE 2: Dose−response inhibition of HSV-1 growth by oligomer **B** (●), **A** (○), or **C** (▲). Vero cells were infected with 10 pfu/cell of HSV-1, strain F, and treated 0−24 h postinfection with the indicated oligomer. Virus titers were determined 24 h postinfection. The results are expressed as the percent inhibition of virus titers compared to untreated controls.

Table 1 shows the apparent dissociation constants for duplexes formed between RNA target **I** and the OMPs at 37 °C. The $K_{d_{app}}$ for d-OMP **A** is approximately four times higher than that measured for mr-OMP **B**. This result is consistent with previous experiments which demonstrate that oligo-2′-*O*-methylribonucleoside methylphosphonates form more stable duplexes with RNA targets than do the corresponding oligodeoxyribonucleoside methylphosphonates (Miller *et al.*, 1991; Kean *et al.*, 1994). Although the binding constant of **B** is significantly increased, the specificity of binding is still maintained. Thus the $K_{d_{app}}$ of the mr-OMP **C**, which contains two mismatched bases, is at least 2 orders of magnitude higher than that of **B**.

The anti-HSV activity of oligomers **A** and **B** in cell culture was determined. Figure 2 shows the dose−response curves for HSV-1 replication in the presence of these oligomers. The IC_{50} values for these oligomers are tabulated in Table 1. There is a direct correlation between the $K_{d_{app}}$ of these oligomers and their IC_{50} values. Thus the IC_{50} of mr-OMP **B** is approximately five times lower than that of d-OMP **A**. Mismatched oligomer **C** showed no inhibitory activity even at 50 μM concentration. None of the oligomers inhibited HSV-2 replication, a virus whose splice acceptor sequence is similar to, but not identical with, that of HSV-1.

These results demonstrate that an mr-OMP binds selectively to its complementary RNA target and that the binding affinity of this oligomer is significantly higher than that of the corresponding d-OMP. The increased antiviral activity of the mr-OMP compared to that of the d-OMP suggests that enhanced binding correlates directly with increased

antisense efficacy of these methylphosphonate oligomers. Additional studies in our laboratories have shown that, depending upon the oligomer sequence involved, the binding affinities of racemic mr-OMPs for RNA targets can be as much as 100 times greater than those of d-OMPs of comparable sequence. It appears that oligo-2′-*O*-methylribonucleoside methylphosphonates may hold considerable promise as antisense reagents.

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REFERENCES

Agarwal, S., Mayrand, S. H., Zamecnik, P. C., & Pederson, T. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 1401−1405.

Aurelian, L. (1992) in *Clinical Virology Manual*, 2nd ed. (Spector, S., & Lancz, G., Eds.) pp 72−99, Elsevier, New York.

Hogrefe, R. I., Reynolds, M. A., Vaghefi, M. M., Young, K. M., Riley, T. A., Klem, R. E., & Arnold, L. J., Jr. (1993a) in *Methods in Molecular Biology, Vol. 20: Protocols for Oligonucleotides and Analogs* (Agrawal, S., Ed.) pp 143−164, Humana Press Inc, Totowa, NJ.

Hogrefe, R. I., Vaghefi, M. M., Reynolds, M. A., Young, K. M., & Arnold, L. J. (1993b) *Nucleic Acids Res. 21*, 2031−2038.

Kean, J. M., & Miller, P. S. (1994) *Biochemistry 33*, 9178−9186.

Kean, J. M., Cushman, C. D., Kang, H., Leonard, T. E., & Miller, P. S. (1994) *Nucleic Acids Res. 22*, 4497−4503.

Kulka, M., Smith, C. C., Aurelian, L., Fishelevich, R., Meade, K., Miller, P., & Ts'o, P. O. P. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 6868−6872.

Kulka, M., Wachsman, M., Miura, Fishelevich, R., Miller, P. S., Ts'o, P. O. P., & Aurelian, L. (1993) *Antiviral Res. 20*, 115−130.

Kulka, M., Smith, C. C., Levis, J., Fishelevich, R., Hunter, J. C. R., Cushman, C. D., Miller, P. S., Ts'o, P. O. P., & Aurelian, L. (1994) *Antimicrob. Agents Chemother. 38*, 675−680.

Levis, J. T., & Miller, P. S. (1994) *Antisense Res. Devel. 4*, 223−230.

Miller, P. S., Bhan, P., Cushman, C. D., Kean, J. M., & Levis, J. T. (1991a) *Nucleosides Nucleotides 10*, 37−46.

Miller, P. S., Cushman, C. D., & Levis, J. T. (1991b) in *Oligonucleotides and Analogues: A Practical Approach* (Eckstein, F., Ed.) pp 137−154, IRL Press, Oxford.

Milligan, J. F., Matteucci, M. D., & Martin, J. C. (1993) *J. Med. Chem. 36*, 1923−1937.

Scaringe, S. A., Francklyn, C., & Usman, N. (1990) *Nucleic Acids Res. 18*, 5433−5441.

Smith, C. C., Aurelian, L., Reddy, M. P., Miller, P. S., & Ts'o, P. O. P. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 2787−2791.

Stull, R. A., Taylor, L. A., & Szoka, F. C., Jr. (1992) *Nucleic Acids Res. 20*, 3501−3508.

Trapane, T. L. (1993) Ph.D. Thesis, The Johns Hopkins University, Baltimore, MD.

Trapane, T. L., & Ts'o, P. O. P. (1993) Biophysical Society 34th Annual Meeting, 14−18 February, 1993, Washington, DC, Abstract # W-Pos514.

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