In Vitro Toxicological Evaluation of ISIS 1082, a Phosphorothioate Oligonucleotide Inhibitor of Herpes Simplex Virus

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ISIS 1082, a phosphorothioate oligonucleotide targeted to a translation initiation codon of herpes simplex virus type 1 (HSV-1) and herpes simplex virus type 2 (HSV-2) virion capsid protein UL13 inhibits in vitro viral replication. To better understand the pharmacological properties of ISIS 1082, we examined its effects in nonviraly infected HeLa cells by using a number of cytotoxicity assays. Our data indicate that ISIS 1082 had no effect on HeLa cell viability as measured by cellular proliferation and clonogenic assays at concentrations as high as 100 μM. Additionally, DNA, RNA, and protein syntheses were only inhibited by 25% in cells treated with 100 μM ISIS 1082. The effects of ISIS 1082 on DNA synthesis were compared with those of acyclovir and trifluorothymidine, two clinically used antipherpetic agents. Acyclovir displayed effects similar to that of ISIS 1082. However, trifluorothymidine, which has been reported to be a potential mutagen and teratogen, significantly altered DNA replication at concentrations from 1 to 100 μM. Isolated HeLa DNA polymerases were inhibited by the compound, with a 50% inhibitory concentration of 2 μM. The in vitro antiviral (K. Draper and V. Brown-Driver, submitted for publication; K. G. Draper and V. Brown-Driver, Antiviral Res. Suppl. 1:106, 1991) and cytotoxicity studies suggest that ISIS 1082 is a selective, nontoxic, antipherpetic therapeutic agent.

Herpes simplex virus (HSV) is the etiologic agent responsible for a variety of diseases, including ocular herpetic keratitis, an illness that can lead to severe corneal scarring and blindness (16, 23). Patients with this condition may be treated with several drugs, including the nucleoside analogs acyclovir (ACV) and trifluorothymidine (TFT) (21, 31). Although each of the antiviral drugs has displayed clinical efficacy, the emergence of resistant strains of HSV and the occurrence of toxic and hypersensitivity reactions to the compounds underscore the need for more specific, potent, and less toxic antiviral therapy (25).

The theoretical selectivity of antisense oligonucleotides for specific genomic and viral RNA targets makes their use as therapeutic agents extremely attractive (17, 29, 32). Our laboratory has designed and tested several sequence-specific oligonucleotides targeted to the translation initiation regions of HSV mRNAs (9, 10). ISIS 1082, a phosphorothioate oligonucleotide 21 nucleotides in length (5'-GCCGAGGTC CATGTCGTACGC 3') is complementary to a translation initiation codon region within the UL13 open reading frame of HSV. The UL13 sequence is highly conserved within strains of HSV, and the target sites for ISIS 1082 are identical in HSV type 1 (HSV-1) and HSV-2, except for the 3'-terminal base of the target mRNAs. On the basis of sequence homology with other virus RNAs, the UL13 gene product is postulated to be a virion capsid protein with phosphotransferase activity (7, 24). ISIS 1082 inhibited HSV-1 replication in cell culture in a dose-dependent manner, with 50% inhibitory concentrations (IC50s) ranging from 0.4 to 1 μM, and displayed potencies similar to that of ACV in infectious yield assays (9, 10).

While it is assumed that ISIS 1082, like other antiviral oligonucleotides, selectively binds to mRNA and blocks translation of specific messages vital to viral replication, several groups have suggested that the mechanism of action of certain antisense oligonucleotides is more complex than simple translational arrest (6, 22). Alternate mechanisms of antiviral activity include nonspecific inhibition of protein translation (6) and direct inhibition of viral DNA polymerases (14, 15, 22). Antiviral activity could also result from general cellular toxicity, as is the case for many cytotoxic, chemotherapeutic agents (4). To better understand the pharmacological properties of ISIS 1082 and to demonstrate that its in vitro antiviral activity is not due to nonspecific cytotoxicity, we examined the in vitro toxicity of the compound in nonviraly infected HeLa cells.

MATERIALS AND METHODS

Preparation of drugs. ISIS 1082 was synthesized at Applied Biosystems, Inc. (Foster City, Calif.) on an automated DNA synthesizer (Applied Biosystems model 380B) by the phosphoramidite method. Sulfurization of the oligonucleotide was performed as described by Iyer et al. (19). The compound used in these experiments was shown to be greater than 95% pure by a variety of methods, including polyacrylamide gel electrophoresis, high-performance liquid chromatography, and capillary electrophoresis, was greater than 98% pure phosphorothioate versus phosphodiester linkages, and contained less than 1% (wt/wt) organic solvent impurities. Prior to use in cytotoxicity assays, ISIS 1082 was prepared by first incubating stock solutions at 37°C for 1 h and diluting prewarmed drug in tissue culture medium to specified concentrations. Diluted compound was filter sterilized by centrifugation through 0.2-μm-pore-size Centrex filters (Schleicher and Schuell) at 2,060 × g for 10 min.

ACV (Burroughs-Wellcome) was obtained from Sigma (catalog number A4669; lot 117F-0756). The compound was resuspended in dimethyl sulfoxide to a final concentration of 1 mg/ml (4.4 mM). ACV, like ISIS 1082, was filter sterilized by centrifugation through Centrex filters. TFT (Burroughs-Wellcome) was obtained from PCR Research Chemicals (catalog number 12138-4). TFT was diluted to a 4% stock
solution in sodium acetate, pH 7.0 (135 mM), and was also filtered sterilized prior to addition to HeLa cells.

**Cell line maintenance.** HeLa cells (ATCC CCL2) and normal human dermal fibroblasts (NHDF; Clonetics CC2010), a primary human cell line, were maintained as monolayer cultures in low-glucose Dulbecco’s modified Eagle medium (Eagle Scientific) supplemented with 100 U of penicillin G, 100 μg of streptomycin sulfate per ml, and 4 mM l-glutamine (DMEM) and in 10% heat-inactivated fetal bovine serum in a 5% CO2-humidified incubator at 37°C.

In asynchronous, logarithmically growing medium 528 sterile medium was harvested, washed with PBS, and pelleted at 1,000 x g at 4°C for 5 min, and the final pellet was resuspended in 5 ml of cell membrane lysis buffer (20 mM Tris-Cl, pH 7.2, 100 mM NaCl, 1% Nonidet-P-40) for 20 min at 4°C. Nuclei were pelleted from the resulting suspension, and the final nuclear pellet was resuspended in 1× PBS. The nuclei were ruptured following homogenization in a 2-ml Dounce homogenizer by 20 strokes each with a loose and tight pestle. Final protein concentration was determined by using the Bio-Rad protein assay (Bio-Rad, Richland, Calif.) and bovine serum albumin as a standard.

DNA polymerase assays were performed by essentially the procedure described by Edenberg et al. (12). The incorporation of [3H]dUTP into activated DNA was measured in a 0.225-ml reaction mixture consisting of 10 mM Tris-HCl, pH 8.0, 10 mM Mg-acetate, 0.67 mg of bovine serum albumin per ml, 5 mM dithothreitol (DTT), 50 μM (each) dATP, dCTP, dGTP, and TTP, 0.5 mg of calf thymus DNA (sonicated, heated to 95°C, and placed on ice) per ml, and 7.3 μCi of [3H]dUTP. Nuclear extract (25 μl) was added (10 μg of total nuclear protein), and the reactions were incubated for 30 min at 37°C. Samples contained either ISIS 1082 or ACV at 100, 10, 1, 0.1, or 0.01 μM in drug-treated reactions. After incubation at 37°C, samples were frozen at −20°C until the filter assay was performed. Samples were thawed, and the DNA was precipitated by addition of 0.2 ml of 2 N HCl containing 10% sodium P, and placed at 4°C for 15 min. Samples were applied to wetted (2 N HCl, 10% Na P,) GF/c filters by using a vacuum filter manifold. Precipitates on filters were washed with 2 ml of 2× 2 N HCl and then with 10 ml of 95% ethanol. Filters were filtered and counted by liquid scintillation.

Total DNA polymerase (alpha and beta) activity was determined in the presence of DTT. For determination of beta DNA polymerase activity, reactions without DTT supplemented with 10 mM N-ethylmaleimide (NEM) were carried out. Alpha DNA polymerase activity was calculated as activity in the presence of DTT minus activity in the presence of N-ethylmaleimide. In the presence of N-ethylmaleimide, beta DNA polymerase activity represented less than 10% of the total activity.

**Cellular uptake of 35S-ISIS 1082. (i) Preparation of 35S-ISIS 1082.** ISIS 1082 was radiolabelled to high specific activity (2 × 10⁹ to 5 × 10⁸ cpm/μmol) by synthetic incorporation of elemental 35S by using hydrogen phosphonate chemistry as described by Stein et al. (28). Breakdown products and overall quality of the radiopure ISIS 1082 were determined by running a 1-μl aliquot of the compound on a 20% acrylamide–7 M urea gel.

35S-ISIS 1082 (10⁸ cpm/μmol) was prepared in tissue culture plates without fetal bovine serum. The integrity and purity of cold ISIS 1082 used to adjust the radiolabelled solution to the correct concentration was assessed as described above.

(ii) Cellular uptake of 35S-ISIS 1082. The cellular uptake of 35S-ISIS 1082 in nonviral infected HeLa cells was determined. Logarithmically growing HeLa cells were harvested from T-175 flasks by trypsinization, combined, and washed...
twice with DME+ and 11% heat-inactivated fetal bovine serum. After the second wash, the cells were resuspended in medium containing 11% serum and diluted to a final concentration of 1.12 × 10^6 cells per ml. Cells (450 μl) were then transferred to 15-ml Corning screw-cap centrifuge tubes and placed on ice prior to uptake experiments.

A 50-μl aliquot of radiolabelled ISIS 1082 was added to cells, and experiments were performed by placing the reaction mixture at 37°C in a shaking water bath (or on ice) for predetermined times. Reactions were stopped by placing the tubes on ice and centrifuging the reaction mixture at 4°C in a Beckman Tabletop centrifuge for 5 min at 1,750 × g. Prior to the initial centrifugation, viability of cells was determined by removing a 10-μl aliquot of reaction mix and counting the cells in the presence of trypan blue. After centrifugation, the supernatant was removed from the tubes and saved for counting, and the cell pellet was washed twice with ice-cold PBS to remove nonspecific cell-associated radioactivity. Integrity of 35S-ISIS 1082 in the supernatant fluid was determined by gel chromatography. Aliquots of the supernatant fluid, PBS washes, and the entire final cell pellet were placed in vials, and radioactivity was determined by counting in a Beckman LS5000TD scintillation counter.

RESULTS

Effects of ISIS 1082 on cellular proliferation. The effects of ISIS 1082 on the cellular proliferation of nonvirally infected HeLa and NHDF cells after 1, 4, 12, and 24 h of treatment with increasing concentrations of the compound were studied as described in Materials and Methods. Essentially no change in cell growth was observed at any concentration tested (1 to 100 μM) over a 24-h period (data not shown). Viability of control and treated cells over the entire course of the experiment, as assessed by the ability of cells to exclude the dye trypan blue, was >95%. Other studies examining ISIS 1082 effects on cellular proliferation after a 3-day treatment demonstrate that NHDF cells were more sensitive to the effects of the compound than HeLa cells, with 100 μM ISIS 1082 inhibiting NHDF cell growth by approximately 60%, while HeLa cell proliferation was inhibited by approximately 45% of control values (data not shown).

Effect of ISIS 1082 on the clonogenic capacity of HeLa cells. Our laboratory has demonstrated the anti-HSV activity of ISIS 1082 by using an infectious yield assay with a 3-day drug treatment protocol (9, 10). The compound inhibited viral replication in a dose-dependent manner with 90% inhibition of virus yield achieved at 2 μM. The effect of the compound on cell growth after a similar exposure was evaluated by determining the clonogenic capacity of HeLa cells. Figure 1 shows that treatment of cells with up to 50 μM compound had no statistically significant effect on HeLa clonogenic capacity. The effect of ACV on HeLa cell growth was compared with that of ISIS 1082. ACV, like ISIS 1082, had essentially no effect on the clonogenic capacity of the cells at the concentrations tested.

Effect on DNA, RNA, and protein synthesis. Inhibition of macromolecular syntheses was evaluated by determining the effects of ISIS 1082 on the incorporation of 3H-precursors into DNA, RNA, and protein of HeLa cells. Table 1 demonstrates the effect of increasing concentrations of ISIS 1082 on 3H-thymidine incorporation in nonvirally infected HeLa cells. The compound had very little effect on the incorporation of 3H-thymidine at the 1- and 4-h incubation periods at any concentration (data not shown). A 15 to 20% reduction in precursor incorporation was seen at the 12- and 24-h times but only after treatment with 100 μM ISIS 1082. In one experiment, HeLa cells were treated for 24 h with drug at up to 500 μM (data not shown). Even after incubation with approximately 500 times more compound than that needed to achieve an effective anti-HSV effect, i.e., 50% inhibition of viral replication, 3H-thymidine incorporation was inhibited by only 37%.

The effect of ACV and TTF on 3H-thymidine incorporation over a 24-h period in HeLa cells was also examined. ACV, like ISIS 1082, had minor effects on precursor incorporation (Table 1). TTF displayed a different toxicological profile from those of both ISIS 1082 and ACV, in that TTF stimulated DNA synthesis at low concentrations (1 μM) while severely inhibiting thymidine incorporation after treatment of cells with 100 μM compound after 1 h of treatment (data not shown).

The effects of ISIS 1082 on the incorporation of 3H-uridine and 3H-leucine (Table 2) were also studied. As was seen with 3H-thymidine, ISIS 1082 had very little effect, if any, on precursor incorporation after 1 and 4 h (data not shown). In treatment, while at 24 h, precursor uptake was inhibited by approximately 15 to 25% at all concentrations tested (1 to 100 μM).

Effect on HeLa cellular DNA polymerase. It had been previously reported that antisense oligonucleotides may directly inhibit cellular and viral DNA polymerases (14, 15, 22). To better understand the mechanism of inhibition of DNA synthesis observed in our macromolecular syntheses assay, we examined the effects of ISIS 1082 on alpha DNA polymerase from extracts of HeLa nuclei. Alpha DNA polymerase is a key enzyme involved in normal chromosomal replication (30). As shown in Fig. 2, alpha polymerase activity was significantly inhibited by ISIS 1082, with an IC50 of 2 μM. ACV essentially had little or no effect on enzyme activity (data not shown).

Cellular uptake of 35S-ISIS 1082. The uptake of 5 μM 35S-ISIS 1082 in nonvirally infected HeLa cells was both time and temperature dependent (Fig. 3). 35S-ISIS 1082 cell
association increased as a function of time over the 12-h experimental period at 37°C with a rapid initial phase between 0 and 2 h, and plateauing from 8 to 12 h. By 12 h, approximately 8% of the total input radioactivity, which corresponds to an intracellular concentration of approximately 400 μM, associated with cells. A constant amount of [35S]-ISIS 1082, representing only 2% of total input radiolabel, associated with cells over the incubation period at 4°C. Viability of cells was greater than 90% during the course of the experiment.

**DISCUSSION**

ISIS 1082 was evaluated in nonvirally infected HeLa cells by using a variety of in vitro toxicity assays. A 24-h exposure of those cells as well as NHDF cells, a primary cell line, to the compound had no effect on either cellular proliferation or viability, as measured by trypan blue exclusion (data not shown). The clonogenic capacity of HeLa cells (Fig. 1) was also unaffected by a 3-day treatment with ISIS 1082. The clonogenic assay, a more sensitive measure of cytotoxicity than vital dye exclusion, assesses reproductive capacity of cells after rather than during drug challenge (11). The major macromolecular synthetic pathways of HeLa cells were minimally and nonspecifically affected by incubation with ISIS 1082. (Tables 1 and 2). Minor inhibition of [3H]-precursor incorporation into DNA, RNA, and protein occurred only after exposure to concentrations of drug 100 to 500 times that necessary for in vitro antiviral activity. The results of these in vitro assays suggest that ISIS 1082 is minimally toxic to HeLa cells.

The pharmacological activity of ISIS 1082, as well as any other therapeutics, depends on a number of factors that influence the effective concentration of these agents at specific intracellular targets. The minimal toxicity of the compound could be explained by lack of uptake into or metabolism of the oligonucleotide within HeLa cells. However, experiments performed in our laboratory with [35S]-ISIS 1082 indicate that the oligonucleotide was taken up into HeLa cells in a time-, temperature (Fig. 3), and concentration-dependent manner (data not shown) (8). Furthermore, the activity of ISIS 1082 against HSV requires uptake into HeLa cells. Additionally, intracellular stability studies show that the compound was stable within cells for at least 3 days (data not shown). These data are consistent with reports from our laboratory and others suggesting the resistance of phosphorothioate oligonucleotides to enzymatic degradation in serum and within cells and the demonstration of anti-HSV activity in infected HeLa cells (5, 9, 18).

Studies on the toxicity of some antisense oligonucleotides have been reported. Many of these compounds have been tested in numerous in vitro cytotoxicity assays, including vital dye exclusion, macromolecular syntheses, cell proliferation, and clonogenic assays (1, 2, 26). The use of multiple classes of oligonucleotides of various lengths targeted to specific viral sequences in different experimental systems complicates data interpretation. General conclusions concerning the toxicity of oligonucleotides as a class of drugs are, therefore, difficult to draw. Nonetheless, cytotoxicity

![FIG. 2. Effects of ISIS 1082 on cellular alpha DNA polymerase activity. Values for each drug concentration represent means and standard deviations from seven separate HeLa cell nuclear preparations.](http://aac.asm.org/)

### TABLE 1. Effects of drugs on [3H]thymidine incorporation into HeLa cells

<table>
<thead>
<tr>
<th>Conc (μM)</th>
<th>ISIS 1082</th>
<th>ACV</th>
<th>TFT</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>12 h</td>
<td>24 h</td>
<td>12 h</td>
</tr>
<tr>
<td>1</td>
<td>92 ± 10</td>
<td>99 ± 23</td>
<td>121 ± 10</td>
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<td>10</td>
<td>88 ± 8</td>
<td>101 ± 17</td>
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</tr>
<tr>
<td>50</td>
<td>94 ± 5</td>
<td>93 ± 2</td>
<td>126 ± 5</td>
</tr>
<tr>
<td>100</td>
<td>81 ± 7</td>
<td>86 ± 7</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Inhibition of thymidine uptake into DNA was measured as a percentage of control samples (cells incubated with [3H]thymidine in the absence of drugs). The values shown are means and standard deviations from two experiments performed in triplicate. ND, not determined.*

### TABLE 2. Effects of ISIS 1082 on [3H]uridine and [3H]leucine incorporation into HeLa cells

<table>
<thead>
<tr>
<th>Conc (μM)</th>
<th>12 h</th>
<th>24 h</th>
<th>12 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[3H]uridine</td>
<td>[3H]leucine</td>
<td>[3H]uridine</td>
<td>[3H]leucine</td>
</tr>
<tr>
<td>1</td>
<td>93 ± 5</td>
<td>102 ± 4</td>
<td>81 ± 16</td>
<td>84 ± 7</td>
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<td>10</td>
<td>94 ± 4</td>
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<td>96 ± 16</td>
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<td>75 ± 6</td>
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<tr>
<td>100</td>
<td>102 ± 6</td>
<td>78 ± 5</td>
<td>75 ± 3</td>
<td>79 ± 6</td>
</tr>
</tbody>
</table>

*Inhibition of uridine uptake into RNA and leucine incorporation into protein was measured as a percentage of control samples (cells incubated with [3H]-precursor in the absence of drugs). The values shown are means and standard deviations from two experiments performed in triplicate.*
was usually seen at concentrations much higher than those required for specific, antiviral, antisense effects.

Although the potential mechanisms of toxicities of oligonucleotides have not been addressed, it has been hypothesized that phosphorothioates, because of their chemical structure, may bind nonspecifically to cellular proteins and interfere with metabolic processes (6, 27). One class of cellular and viral proteins that may be nonspecifically affected by phosphorothioates in this manner is DNA polymerase (14, 15, 22). Results showing inhibition of alpha DNA polymerase in HeLa nuclear extracts by ISIS 1082 (Fig. 2) are consistent with these reports. However, the data argue that inhibition of the enzyme by ISIS 1082 may only be a minor mechanism of toxicity and unrelated to the pharmacology of the drug. First, the IC<sub>50</sub> of ISIS 1082 towards alpha DNA polymerase (2 μM) was greater than that required for the antiviral effects (430 nM). Second, as seen in Table 1, only minor effects of ISIS 1082 on intact HeLa cell DNA replicative machinery were seen when cells were exposed to 100 μM drug, 50 times higher than the DNA polymerase IC<sub>50</sub> value. Third, since another 21-mer phosphorothioate, dC<sub>21</sub> also inhibited the enzyme at similar concentrations (data not shown), inhibition of alpha DNA polymerase by ISIS 1082 more than likely results from nonspecific interactions with the enzyme.

The effects of ISIS 1082 in HeLa cells were compared with those obtained by using ACV and TFF, two of the several nucleoside analogs used for the treatment of ocular herpetic keratitis (21, 31). ACV and ISIS 1082 had little effect on the clonogenic and DNA synthetic capacity of HeLa cells (Fig. 1; Table 1). The therapeutic indices of the compounds (50% lethal concentration/50% effective dose), as determined by antiviral data (9, 10) and the data described in this paper, were equivalent and in excess of 50. While the antiviral activities of the two compounds are similar, ISIS 1082, whose mechanism of action appears to be antisense, is effective against ACV-resistant strains of HSV (9, 10). TFF, unlike ISIS 1082 and ACV, significantly affected incorporation of [3H]thymidine into HeLa cells (Table 1). The apparent biphasic effect of the compound, i.e., stimulation of precursor incorporation at low concentrations of TFF, which more than likely represents activation of the DNA repair synthetic machinery, and the severe inhibition of DNA synthesis at 100 μM, are consistent with reports indicating the potential mutagenic and teratogenic effects of the compound (13, 20). These data and preliminary in vivo data demonstrating that ISIS 1082 was effective in reducing the severity of HSV-induced stromal keratitis in mice (3) suggest that this compound may be a highly selective, minimally toxic therapeutic agent for the treatment of ocular herpetic keratitis.

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