Antiviral Activity and Ocular Kinetics of Antisense Oligonucleotides Designed to Inhibit CMV Replication

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PURPOSE. To compare the antiviral activity and ocular distribution of first- and second-generation antisense oligonucleotides intended for the treatment of cytomegalovirus (CMV) retinitis.

METHODS. The antiviral activity of ISIS 13312 and ISIS 2922 (Isis Pharmaceuticals, Inc., Carlsbad, CA) against 10 clinical CMV isolates was compared with a plaque-reduction assay. The ocular pharmacokinetics were compared after intravitreal injection in rabbits (36–90 μg) and monkeys (125–500 μg). Vitreous and/or retina were collected after single and multiple injections to characterize ocular distribution, clearance, and accumulation. Oligonucleotide concentrations were measured by capillary gel electrophoresis and immunohistochemical techniques.

RESULTS. ISIS 13312 and ISIS 2922 demonstrated comparable antiviral activity that was consistent among the 10 clinical isolates examined (50% inhibitory concentration [IC50], <1 μM). Activity was independent of the resistance of CMV isolates to DNA polymerase inhibitors. After intravitreal injection, the kinetics of ISIS 2922 and ISIS 13312 were characterized by clearance from vitreous and distribution to the retina; however, ISIS 2922 was cleared more quickly from the retina than ISIS 13312. The half-life of ISIS 13312 in the monkey retina was approximately 2 months. Retinal concentrations of ISIS 13312 were dose dependent, with approximately a twofold increase in concentration after once-monthly doses compared with single-dose concentrations. Immunohistochemical analysis indicated that both oligonucleotides were efficiently distributed to numerous ocular tissues, including retina, ciliary body, and optic nerve.

CONCLUSIONS. ISIS 13312 possesses antiviral activity and pharmacokinetic properties that favor its use as a therapeutic agent in treatment of CMV retinitis. The half-life of ISIS 13312 in retina is longer than that of ISIS 2922, potentially allowing for less frequent administration. (Invest Ophthalmol Vis Sci. 2001; 42:2646–2651)

Infection of the retina by cytomegalovirus (CMV) is a sight-threatening disease that occurs in patients with advanced AIDS. 1 The most common treatments to control CMV infection include systemic treatment with DNA polymerase inhibitors, such as ganciclovir (GCV), foscarnet (PFA), and cidovir (CDV). 2–4 Limitations in the use of these drugs to treat CMV infection include poor delivery to the retina from systemic administration and the high degree of virus resistant to DNA polymerase inhibitors. 5 DNA polymerase cross-resistance to two or more of these drugs is also a problem. 6,7 Furthermore, these drugs are not highly selective for the viral DNA polymerase and therefore are associated with significant systemic toxicities, including neutropenia and renal toxicity.

Recently, an antisense inhibitor of human CMV fomiviren (Vitravene; also known as ISIS 2922; Isis Pharmaceuticals, Inc., Carlsbad, CA) was approved for treatment of CMV retinitis. ISIS 2922 is administered once monthly by intravitreal injection, providing local therapy for retinal infection. 8,9 This therapy has been demonstrated to be efficacious and safe in patients who undergo repeated intravitreal injection. Examination of ocular pharmacokinetics of ISIS 2922 in rabbits and monkeys has revealed a favorable tissue distribution pattern and clearance rate after intravitreal injection. 10,11 The major advantages are that local administration prevents systemic toxicity, and the novel antisense mechanism of action potently inhibits antiviral-resistant strains of CMV.

A new class of antisense oligonucleotides is now being characterized with the objective of improving overall therapeutic utility in the treatment of ocular diseases. These so-called second-generation antisense oligonucleotides contain modifications of the 2′ position on the sugar backbone with either methyl or methoxymethyl moieties, in addition to phosphorothioate linkages. These 2′-Oalkyl substituents have been reported to improve pharmacologic potency and tolerability through decreased immune stimulation. 12–14 From a pharmacokinetic perspective, addition of 2′-O-methyl or 2′-O-methoxyethyl (2′-MOE) substituents increases the resistance to nuclease degradation, which is the primary route of clearance for the first-generation oligonucleotides. 15 The increased durability of second-generation oligonucleotides may be exploited to lower the dose or further extend the treatment interval relative to the first generation of oligonucleotides.

The second-generation antisense inhibitor of human CMV (ISIS 13312; Isis Pharmaceuticals, Inc.) has the same nucleotide sequence as ISIS 2922, but has 2′-O-methoxymethyl (MOE) modification on seven residues on the 5′ end of the oligonucleotide and six residues on the 3′ end. This oligonucleotide has been shown to have antiviral potency comparable to that of ISIS 2922 against a laboratory strain of CMV in fibroblast and retinal pigment epithelial cells. 16 In this study, we examined the consistency of antiviral activity against a number of clinical isolates of human CMV and the ocular kinetics of a 2′-MOE-modified oligonucleotide (ISIS 13312) after single or repeated intravitreal injection. The properties of this compound were compared with the published literature on the first-generation compound, ISIS 2922 (Vitravene). 10,11 Immunohistochemical staining was also used to determine localization of representative first- (ISIS 2505; Isis Pharmaceuticals, Inc.) and second-generation (ISIS 13920; Isis Pharmaceuticals, Inc.) oligonucleotides in ocular tissues.

MATERIALS AND METHODS

Oligonucleotides

The sequence of compounds used in this study and the respective chemical modifications are detailed in Table 1. Oligonucleotides were...
synthesized at Isis Pharmaceuticals, Inc. by DNA synthesizer (model 8800; Milligen, Burlington, MA), using solid-phase triester chemistry, as described in the literature. The 2'-O-deoxy phosphoramidites were purchased from commercial sources (Amersham Pharmacia Biotech, Piscataway, NJ); the 2'-O-methoxymethyl phosphoramidites were manufactured specifically for Isis Pharmaceuticals under contract. The phosphorothioate internucleotide linkage was generated by use of Beaucage reagent (Clauson-Kaas Chemical Research Company, Farum, Denmark) as the P(III)—P(V) oxidant. The test compounds were purified by reversed-phase HPLC to a final purity of more than 92% full-length oligomer (determined by capillary electrophoresis and strong-anion-exchange HPLC). There were no detectable levels of endotoxin in the final lyophilized product as determined by Limulus amoebocyte lysate (LAL) analysis (Associates of Cape Cod, Woods Hole, MA). Lyophilized bulk drug substance was formulated in phosphate-buffered saline (pH 7.4) at the concentrations used in this study (0.4, 2, and 10 mg/ml).

Plaque-Reduction Assay

Human foreskin fibroblast (MRHF) cells were obtained from BioWhittaker (Walkersville, MD) and propagated at 37°C in a 5% carbon dioxide atmosphere. Earl minimal essential medium (MEM; Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) was used for propagation of cells and production of virus stocks.

Human DMV strain AD169 obtained from the American Type Culture Collection (Rockville, MD) was used as a standard for comparison purposes. Clinical isolates were obtained by two of the authors (WLD, YS) from an Illinois public health agency and propagated at 37°C in a 5% carbon dioxide atmosphere. Earl minimal essential medium (MEM; Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) was used for propagation of cells and production of virus stocks.

For plaque-reduction assays, 24-well culture plates were seeded with MRHF cells at a density of 200,000 cells per well in MEM supplemented with 10% fetal bovine serum (FBS) and incubated until cells exhibited uniform cytopathology (days 7–9). After mild agitation, culture supernatant and infected cells were harvested and frozen in 1-ml aliquots at −150°C. Titers of virus stocks were between 1 and 3 × 10⁶ pfu/ml.

For plaque-reduction assays, 24-well culture plates were seeded with MRHF cells at a density of 200,000 cells per well in MEM supplemented with 10% fetal bovine serum (FBS). Subconfluent monolayers were pretreated with indicated concentrations of test compound for 1 hour and then rinsed three times to remove residual oligonucleotide before virus infection. CMV in MEM-0.2% FBS was added to cells at a dilution sufficient to result in the formation of approximately 50 to 100 plaques per well in untreated cells. After a 3-hour adsorption, virus was removed, and cells were overlaid with Earl MEM containing 0.3% agarose, 0.2% FBS, and the appropriate dilution of test compound. Seven days after infection, monolayers were fixed with 0.2% formaldehyde, permeabilized with 0.1% Triton X-100, and stained with Wright-Giemsa stain. Duplicate samples were counted using an inverted microscope and the mean expressed as the percentage of plaques or the percentage of inhibition that developed in treated compared with untreated cells.

Animal Studies

Rabbits and monkeys in this study were obtained and cared for in accordance with all applicable federal and state guidelines for animal care. The use of animals adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Rabbits were treated with either ISIS 2922 (35 or 82 μg/eye) or ISIS 13312 (56 or 90 μg/eye) by single intravitreal injection (0.05 ml) to compare ocular kinetics. Intravitreal injections were performed using a 30-gauge needle placed 2 to 3 mm behind the limbus after a povidone-iodine rinse of the eye. The doses were chosen to yield initial estimated vitreal concentrations of 4 or 10 μM oligonucleotide at the high and low doses, respectively, assuming a 1.0-ml vitreal volume. The greater amount of ISIS 13312 administered reflected the slightly greater molecular weight of this oligonucleotide. Animals were killed and eyes collected at the indicated time point for measurement of either vitreal or retinal concentrations of oligonucleotide.

Another group of rabbits was treated with ISIS 2503 (82 μg/eye) or ISIS 13920 (90 μg/eye) by single intravitreal injection (0.05 ml) to compare ocular tissue distribution. The targeted initial vitreal concentration in these animals was 10 μM, and injections were performed as for ISIS 2922 and ISIS 13312. At 2, 4, 10, or 30 days after injections, animals were killed and eyes collected for immunohistochemical analysis. The lens was removed to facilitate fixation and eyes were placed in formalin-parafomaldehyde solution. After fixation, eyes were embedded in paraffin and cut to 4-μm sections. Sections were deparaffinized, blocked with normal donkey serum, incubated for 1 hour with the anti-oligonucleotide monoclonal antibody (diluted at 1:2000), rinsed, and incubated with donkey anti-mouse IgG-peroxidase conjugate diluted 1:100 for 1 hour. Peroxidase activity was visualized using 3,3’-diaminobenzidine substrate, and slides were counterstained with hematoxylin and mounted in a permanent mounting medium. It was necessary to use ISIS 2503 and ISIS 13920 for immunolocalization after intravitreal injection, because the antibody used in this study does not bind to ISIS 2922 or ISIS 13312.

Young adult cynomolgus monkeys were treated with 125, 250, or 500 μg/eye ISIS 13312 by single and repeated intravitreal injections (0.05 ml) to study accumulation over time and with increasing dose. Intravitreal injections were performed using a 30-gauge needle placed 2 to 3 mm behind the limbus after a povidone-iodine rinse of the eye. The doses were chosen to yield initial estimated vitreal concentrations of 10, 20, or 40 μM oligonucleotide, assuming a 1.1-ml vitreal volume. Animals were killed and eyes collected at the indicated time point for measurement of either vitreal or retinal concentrations of oligonucleotide.

Doses are reported in micrograms, but were administered to achieve initial vitreal concentrations of oligonucleotide (i.e., 4–40 μM, as indicated). Based on differences in molecular weight, a slightly greater amount of the 2′-MOE second-generation oligonucleotides was administered.

Oligonucleotide Measurement

Oligonucleotides were extracted from fresh-frozen retinal tissue using a procedure developed at Isis Pharmaceuticals, Inc. Retina samples were weighed and homogenized in 20 mM Tris-HCl (pH 8.0), 20 mM EDTA, and 100 mM NaCl buffer solution. A known amount of internal standard was added to each sample to facilitate measurement. The tissue was homogenized using a tissue disrupter (Bio101, Inc., Carlsbad, CA) for 25 seconds at a speed setting of 5. Proteinase K (2 mg/ml) was added to the sample after homogenization, and the mixture incubated overnight at 37°C. The samples were then further purified by phenol-chloroform extraction and vacuum dried (Savant, Farmingdale, New York, NY) and reconstituted with 100 μl of 100 mM Tris-Cl (pH 8.0), 100 mM NaCl buffer solution.

Table 1. Oligonucleotide Sequence and Modifications

<table>
<thead>
<tr>
<th>Compound</th>
<th>Target</th>
<th>Sequence (5’ → 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISIS 2922</td>
<td>Human CMV</td>
<td>GcGsCsTsTsCsTsCsTsCsTsCsTsCsTsCsTsCsTsCsGsGsG</td>
</tr>
<tr>
<td>ISIS 13312</td>
<td>Human CMV</td>
<td>GcGsCsTsTsCsTsCsTsCsTsCsTsCsTsCsTsCsTsCsGsGsG</td>
</tr>
<tr>
<td>ISIS 2503</td>
<td>Human H-raf</td>
<td>TsCsCsGsTsGsTsCsGsCsTsGsCsGsGsGsG</td>
</tr>
<tr>
<td>ISIS 13920</td>
<td>Human H-raf</td>
<td>TsCsCsGsTsGsTsCsGsGsGsG</td>
</tr>
</tbody>
</table>

T, deoxynucleotide; T, 2′-O-methoxymethyl modified nucleotide; s, phosphorothioate linkage.

Antiviral Activity and Ocular Kinetics of ISIS 13312

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were extracted using a solid-phase technique. Samples were concentrated with ammonium hydroxide and incubated at 55°C overnight. The ability to inhibit replication of CMV clinical isolates was investigated using the plaque reduction assay. Eight of 10 clinical isolates examined were resistant to one, two, or three of the available DNA polymerase inhibitors. Thus, the ability to inhibit replication of CMV clinical isolates was compared in Table 2.

Both ISIS 13312 and ISIS 2922 produced consistent antiviral activity in all clinical isolates examined regardless of the drug resistance profiles. The 50% inhibitory concentration (IC50) for both ISIS 13312 and ISIS 2922 were reached between 0.1 and 1.0 μM for all 10 clinical isolates, irrespective of other drug-resistant properties. At a concentration of 1 μM ISIS 13312, greater than 50% percent inhibition of plaque formation was observed in all 10 isolates, and greater than 70% inhibition was observed in 6 of 10 isolates (Table 2). Similarly, a concentration of 1 μM ISIS 2922 produced greater than 50% inhibition of plaque formation in all 10 of the clinical isolates, and greater than 70% inhibition in 5 of 10 isolates. Estimated IC50 for ISIS 13312 ranged from 0.15 to 0.96 μM, whereas estimated IC50 for ISIS 2922 ranged from 0.35 to 0.92 μM. The IC50 values were all within one SD of the mean. Thus, there were no clear differences in the potencies of ISIS 13312 or ISIS 2922 against various clinical strains of CMV. Similarly, all clinical isolates examined in this study appeared to be equally sensitive to inhibition by either ISIS 13312 or ISIS 2922, regardless of other drug-resistance properties.

In contrast, the concentrations required for 50% inhibition of plaque formation varied greatly for the DNA polymerase inhibitors and was dependent on the resistance profile of the isolates. For example, median effective concentrations (EC50) of GCV for GCV-resistant virus ranged from 4 to 20 times that required to inhibit sensitive virus. Similarly, EC50 of PFA for ISIS 2922 ranged from 0.35 to 0.92 μM. The IC50 values were all within one SD of the mean. Thus, there were no clear differences in the potencies of ISIS 13312 or ISIS 2922 against various clinical strains of CMV. Similarly, all clinical isolates examined in this study appeared to be equally sensitive to inhibition by either ISIS 13312 or ISIS 2922, regardless of other drug-resistance properties.

Comparison of Antiviral Activity against CMV Clinical Isolates

The ability of ISIS 13312 and ISIS 2922 to inhibit replication of CMV clinical isolates was investigated using the plaque reduction assay. Eight of 10 clinical isolates examined were resistant to one, two, or three of the available DNA polymerase inhibitors (i.e., GCV, PFA, and/or CDV). Thus, the ability to inhibit CMV clinical isolates both sensitive and resistant to DNA polymerase inhibitors was determined. The anti-CMV activity for the two antisense inhibitors is compared in Table 2.

Both ISIS 13312 and ISIS 2922 produced consistent antiviral activity in all clinical isolates examined regardless of the drug resistance profiles. The 50% inhibitory concentration (IC50) for both ISIS 13312 and ISIS 2922 were reached between 0.1 and 1.0 μM for all 10 clinical isolates, irrespective of other drug-resistant properties. At a concentration of 1 μM ISIS 13312, greater than 50% percent inhibition of plaque formation was observed in all 10 isolates, and greater than 70% inhibition was observed in 6 of 10 isolates (Table 2). Similarly, a concentration of 1 μM ISIS 2922 produced greater than 50% inhibition of plaque formation in all 10 of the clinical isolates, and greater than 70% inhibition in 5 of 10 isolates. Estimated IC50 for ISIS 13312 ranged from 0.15 to 0.96 μM, whereas estimated IC50 for ISIS 2922 ranged from 0.35 to 0.92 μM. The IC50 values were all within one SD of the mean. Thus, there were no clear differences in the potencies of ISIS 13312 or ISIS 2922 against various clinical strains of CMV. Similarly, all clinical isolates examined in this study appeared to be equally sensitive to inhibition by either ISIS 13312 or ISIS 2922, regardless of other drug-resistance properties.

In contrast, the concentrations required for 50% inhibition of plaque formation varied greatly for the DNA polymerase inhibitors and was dependent on the resistance profile of the isolates. For example, median effective concentrations (EC50) of GCV for GCV-resistant virus ranged from 4 to 20 times that required to inhibit sensitive virus. Similarly, EC50 of PFA for PFA-resistant virus were 3 to 6 times or more higher than EC50 for sensitive virus. EC50 of CDV for CDV-resistant virus were 4 to 10 times higher than concentrations required to inhibit sensitive virus. Thus, the antisense inhibitors are equally active in strains of CMV either sensitive or resistant to DNA polymerase inhibitors.

Comparison of Oligonucleotide Concentration in Rabbit Eye

Concentrations of ISIS 2922 or ISIS 13312 were measured in vitreous of rabbits after single intravitreal injection. Consistent with intravitreal injection, concentrations were highest at the first time point examined (3 days) and declined thereafter. The number of time points at which data were recorded was insufficient to determine a half-life, but both compounds were

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**Table 2. ISIS 13312 and ISIS 2922 Inhibition of Plaque Formation by a Panel of CMV Clinical Isolates**

<table>
<thead>
<tr>
<th>HCMV Isolate</th>
<th>GCV IC50 (μM)</th>
<th>PFA IC50 (μM)</th>
<th>CDV IC50 (μM)</th>
<th>0.1 μM (%)</th>
<th>1 μM (%)</th>
<th>0.1 μM (%)</th>
<th>1 μM (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD169</td>
<td>4</td>
<td>240</td>
<td>0.2</td>
<td>0</td>
<td>90.5</td>
<td>7.8</td>
<td>93.9</td>
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<tr>
<td>MMBt</td>
<td>2.6</td>
<td>155</td>
<td>NT</td>
<td>0</td>
<td>75</td>
<td>33</td>
<td>54</td>
</tr>
<tr>
<td>COND16725⁺</td>
<td>20</td>
<td>110</td>
<td>NT</td>
<td>0</td>
<td>51</td>
<td>12</td>
<td>53</td>
</tr>
<tr>
<td>MS15954§</td>
<td>12</td>
<td>120</td>
<td>0.3</td>
<td>15</td>
<td>81</td>
<td>48</td>
<td>80</td>
</tr>
<tr>
<td>MCG8741‡</td>
<td>47</td>
<td>&gt;1000</td>
<td>NT</td>
<td>18</td>
<td>60</td>
<td>23</td>
<td>71</td>
</tr>
<tr>
<td>JWR16715 §§</td>
<td>47</td>
<td>&gt;1000</td>
<td>NT</td>
<td>18</td>
<td>60</td>
<td>23</td>
<td>71</td>
</tr>
<tr>
<td>RM1651§</td>
<td>44</td>
<td>470</td>
<td>NT</td>
<td>18</td>
<td>60</td>
<td>23</td>
<td>71</td>
</tr>
<tr>
<td>MZ6304§</td>
<td>45</td>
<td>700</td>
<td>0.8</td>
<td>18</td>
<td>55</td>
<td>19</td>
<td>56</td>
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<tr>
<td>MS15953 ‡</td>
<td>9.3</td>
<td>&gt;1000</td>
<td>NT</td>
<td>18</td>
<td>55</td>
<td>19</td>
<td>56</td>
</tr>
<tr>
<td>DH11962‡</td>
<td>0.9</td>
<td>830</td>
<td>0.14</td>
<td>14</td>
<td>70</td>
<td>31</td>
<td>61</td>
</tr>
<tr>
<td>SWC16253‡</td>
<td>44</td>
<td>125</td>
<td>2.7</td>
<td>20</td>
<td>71</td>
<td>35</td>
<td>99</td>
</tr>
</tbody>
</table>

NT, not tested; HCMV, human CMV.
* As reported by Mt. Zion Medical Center of the University of California San Francisco.
† Plaque reduction assays were performed as detailed in the Methods section.
‡ HCMV resistant to GCV; IC50 greater than 6.0 μM.
§ HCMV resistant to PFA; IC50 greater than 400 μM.
¶ HCMV resistant to CDV; IC50 greater than 2.0 μM.
cleared from the vitreous by 15 days after administration of a dose that achieved an initial vitreal concentration of either 4 or 10 μM (Fig. 1A). Clearance from the vitreous is consistent with a reported 62-hour half-life of ISIS 2922 in rabbit vitreous. The half-life for total oligonucleotide (ISIS 13312 plus oligonucleotide metabolites) was even longer—estimated to be approximately 4 to 10 months (Fig. 2), considerably longer than the half-life of ISIS 2922 in the monkey retina, which is reported to be 3 days. The half-life for ISIS 13312 in the retina was approximately 29 days, with detectable oligonucleotide present in only two of three eyes treated with the high dose. However, ISIS 13312 was still present at both 29 and 44 days in eyes at both dose levels with tissue concentrations ranging from approximately 4 to 10 μM (Fig. 1B). This indicates much longer residence time of ISIS 13312 in rabbit retina than of ISIS 2922.

Kinetics of ISIS 13312 in Monkey Retina

Monkeys received a single intravitreal injection of 125 μg ISIS 13312 (10 μM initial vitreal concentration), and eyes were collected 43, 98, 190, and 255 days after injection. Eyes were dissected and the retinas were removed for concentration analysis. The retinas contained concentrations of oligonucleotide at all the time points examined. The concentrations of ISIS 13312 and total oligonucleotide metabolites are listed in Table 3. The half-life for ISIS 13312 in the retina was approximately 2 months (Fig. 2), considerably longer than the half-life of ISIS 2922 in the monkey retina, which is reported to be 3 days. The half-life for total oligonucleotide (ISIS 13312 plus oligonucleotide metabolites) was even longer—estimated to be approximately 5 months (Fig. 2).

TABLE 3. Concentration and Percentage of Intact ISIS 13312 in Monkey Retina after a 125-μg Intravitreal Injection

<table>
<thead>
<tr>
<th>Time Point (d)</th>
<th>Total Oligo* (μM)</th>
<th>% Intact†</th>
<th>ISIS 13312‡ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>43</td>
<td>5.0 ± 0.41</td>
<td>72</td>
<td>3.6</td>
</tr>
<tr>
<td>89</td>
<td>3.7 ± 1.6</td>
<td>59</td>
<td>2.2</td>
</tr>
<tr>
<td>180</td>
<td>2.6 ± 0.23</td>
<td>32</td>
<td>0.83</td>
</tr>
<tr>
<td>255</td>
<td>2.0 ± 0.56</td>
<td>&lt;15</td>
<td>&lt;0.3</td>
</tr>
</tbody>
</table>

* Total oligonucleotide concentration determined from CGE analysis.
† Percentage of intact oligonucleotide was determined from LC/MS.
‡ ISIS 13312 concentration was determined by multiplying the concentration of total oligonucleotide by the percentage of intact oligonucleotide.

The longer half-life of ISIS 13312 in the retina was attributed to increased resistance to nuclease-mediated metabolism. Only intact ISIS 13312 and oligonucleotide shortened by 1 nucleotide residue were detected, even at the later time points. This is in marked contrast to ISIS 2922 for which metabolism is a continuous process resulting in progressively lower concentrations of each successive shortened oligonucleotide metabolite until the nucleotide is completely degraded. This difference in durability accounts for the longer residence time of 2′MOE-modified oligonucleotides. The only reason an N-1 metabolite of ISIS 13312 is seen is because the 3′ terminal residue does not contain a 2′MOE modification, and therefore, is more easily removed by exonuclease (Table 1). As a result, the half-life of total oligonucleotide is a close approximation of an oligonucleotide that is fully 2′MOE modified on both the 3′ and 5′ termini.

Multiple-Dose Ocular Exposure to ISIS 13312 in Monkey Retina

Multiple doses of ISIS 13312 were administered to cynomolgus monkeys by intravitreal injection once monthly for a total of four doses. Retinal concentrations of ISIS 13312 were measured over the course of treatment after doses of 125, 250, and 500 μg (initial vitreal concentrations of 10, 20, and 40 μM, respectively). The concentrations of ISIS 13312 in retina was dose dependent. When they were measured 25 days after the last of the four doses, concentrations of ISIS 13312 had increased from approximately 18 μM at the 125-μg dose level to approximately 45 μM at the 500-μg dose level (Fig. 3). Exposure was also dose dependent when measured 4 days after the second dose.

Accumulation in retina was evident with repeated administration once a month for 3 months (four doses). Mean concentrations of ISIS 13312 in the retina after a dose of 125 μg increased from a single-dose concentration of approximately 9 μM, to 15 μM after two doses, with little additional increase in concentration after four doses. Greater accumulation was ob-
RPE cell layer, indicating that oligonucleotide was distributed throughout the full thickness of the retina. There was some localized staining in the outer nuclear layer at this time point, as was evident with focal staining, but overall there was considerably less staining than in other cell layers (Fig. 4). Both ISIS 2503 and ISIS 13920 were also localized to optic nerve and ciliary body (data not shown). Patches of vitreous that remained after histologic processing also showed significant staining for both oligonucleotides at this time point. Because staining patterns were similar for both ISIS 2503 and ISIS 13920, there were no differences in the distribution pattern between first- and second-generation oligonucleotides.

**DISCUSSION**

An antisense oligonucleotide targeting inhibition of virus replication represents a new paradigm in the currently available treatment for CMV retinitis. The antiviral activity of ISIS 2922 (Vitravene; Isis Pharmaceuticals, Inc.) against laboratory strains of human CMV is comparable to that for classic DNA polymerase inhibitors, and this compound has been shown to be efficacious in the treatment of CMV retinitis. ISIS 2922 is distinguished from other available treatments for CMV retinitis by the unique antiviral mechanism. In vitro experiments have demonstrated that the antiviral activity of this antisense oligonucleotide is independent of the resistance of CMV to DNA polymerase inhibitors. Furthermore, the ocular kinetics of intravitreal ISIS 2922 have been characterized in rabbits and monkeys and shown to be favorable for treatment of local infection, allowing once-monthly treatment of patients.

Results from experiments using ISIS 13312 indicate that the second-generation antisense CMV inhibitor has antiviral activity comparable to that of ISIS 2922 and improved pharmacokinetic properties for the treatment of CMV retinitis. Mean IC50 for both ISIS 13312 and ISIS 2922 for the inhibition of CMV replication was approximately 0.6 μM. It is important to note that the potency of ISIS 13312 and ISIS 2922 was comparable in the 10 clinical isolates examined and was independent of the resistance to DNA polymerase inhibitors.

Immunohistochemical localization of representative first- and second-generation oligonucleotides in the eye indicate that both compounds are distributed to most of the major ocular tissues. High concentrations of oligonucleotide were observed in the outer plexiform layer, outer limiting membrane, inner plexiform layer, ganglion cells, ciliary body, RPE, and optic nerve. Lesser amounts of oligonucleotide appeared to be distributed in the inner and outer nuclear layers at the 4-day time point. The subtissue distribution of ISIS 2922 and ISIS 13312 is expected to be very similar because the pharmacokinetic and distribution properties of oligonucleotides are independent of sequence. A subtle difference in uptake or tissue concentration is theoretically possible based on the presence of sequence-specific nucleases, but a significant difference is regarded as a remote possibility.
The most notable difference between the first- and second-generation oligonucleotides was the half-life in the retina. ISIS 13512 was cleared very slowly from retina, with a half-life of 2 months compared with a half-life of 3 days reported for ISIS 2922 in monkey retina.\textsuperscript{11} Slower clearance from the site of virus replication (i.e., retina) may afford some advantage to ISIS 13512 in the treatment of chronic ocular disease, in that the dose interval may be prolonged relative to the first generation of antisense oligonucleotides. In combination with the potential for an improved tolerability profile relative to ISIS 2922,\textsuperscript{13,14,22} this 2'-MOE modification represents an overall improvement in the therapeutic profile of antisense oligonucleotides.

These data suggest that intravitreal injection of antisense oligonucleotides provides a potentially effective and convenient route of administration for treatment of ocular diseases. The procedure was well tolerated in the clinical trials of ISIS 2922.\textsuperscript{23} A long half-life provides the opportunity to administer the drug at times that coincide with infrequent office visits. There are also considerable advantages to this local route of administration for treatment of ocular diseases, including optimal delivery to the site of interest and limitation of systemic exposure and, therefore, of the potential for systemic toxicity. However, it should be noted that local administration promotes antiviral activity only in the treated eye and does not provide treatment for systemic or contralateral ocular infection.

References


