PHARMACOKINETICS AND METABOLISM IN MICE OF A PHOSPHOROTHIOATE OLIGONUCLEOTIDE ANTISENSE INHIBITOR OF C-RAF-1 KINASE EXPRESSION

RICHARD S. GEARY, JANET M. LEEDS, JON FITCHETT, TODD BURCKIN, LOANNE TRUONG, CHARLES SPAINHOUR, MOIRE CREEK, AND ARTHUR A. LEVIN

Isis Pharmaceuticals (R.S.G., J.M.L., J.F., T.B., L.T., A.A.L.), Chrysalis (C.S.), and SRI International (M.C.)

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ABSTRACT:
The plasma and tissue disposition of CGP 69846A (ISIS 5132) was characterized in male CD-1 mice following iv bolus injections administered every other day for 28 days (total of 15 doses). The doses ranged from 0.8 mg/kg to 100 mg/kg. Urinary excretion of oligonucleotide was also monitored over a 24-hr period following single dose administration over the same dose range. Pharmacokinetic plasma profiles were determined following single dose administration (dose 1) and after multiple doses (dose 15) at doses of 4 and 20 mg/kg. Concentrations in kidney, liver, spleen, heart, lung, and lymph nodes were characterized following dosing 1, 8, and 15 for all doses. Capillary gel electrophoresis was used to quantitate intact (full-length) oligonucleotide and its metabolites (down to N – 11 base deletions) in both plasma and tissue at all time points. The plasma and tissue disposition of CGP 69846A was characterized by a rapid distribution into all tissues analyzed. Rapid plasma clearance of the parent oligonucleotide (9.3–14.3 ml/min/kg) was predominantly the result of distribution to tissue and, to a lesser extent, metabolism. Appearance and pattern of chain-shortened metabolites seen in plasma and tissue were consistent with predominantly exonuclease-mediated base deletion. No measurable accumulation of oligonucleotide was observed in plasma following multiple-dose administration, but both the liver and the kidney exhibited 2–3-fold accumulations. In general, the tissues exhibited half-lives for the elimination of parent oligonucleotide of 16–60 hr, compared with plasma half-lives of 30–45 min. After repeated administrations, significant decreases in plasma clearance and volume of distribution at steady state (Vss) were observed following dose 15 at the dose of 20 mg/kg but not at the dose of 4 mg/kg. Changes in tissue accumulation and evidence for saturation of tissue distribution at the high doses may explain the plasma disposition changes observed in the absence of alteration of metabolism or plasma accumulation. Urinary excretion was a minor pathway for elimination of oligonucleotide over the 24-hr period immediately following iv administration. However, the amount of oligonucleotide excreted in the urine increased as a function of dose from less than 1% to approximately 13% of the administered dose over a dose range of 0.8 mg/kg to 100 mg/kg.

CGP 69846A (ISIS 5132) is an antisense oligonucleotide complementary to the 3′-untranslated region of the mRNA of C-raf-1 kinase. This oligonucleotide molecule has been shown to specifically inhibit C-raf-1 kinase gene expression by an antisense mechanism and tumor cell growth in culture and in vivo tumor growth using xenograft models in nude mice (1). The pharmacokinetics of phosphorothioate oligonucleotides, as a class of compounds, have been well characterized in a number of species (2–4), including man (5, 6). In general, radiolabel associated with phosphorothioate oligonucleotides is rapidly distributed, exhibits a prolonged elimination half-life in plasma (30–60 hr), and is distributed broadly to tissues with kidney and liver showing the highest concentrations (2, 4, 7–11). Phosphorothioate oligonucleotides are highly bound to plasma proteins (12–14), and their clearance from plasma results from a combination of extravascular distribution and metabolism with very little to no urinary or fecal excretion of intact full-length oligonucleotide at doses below 10 mg/kg (9, 15).

There is virtually no information in the literature concerning the dose dependency of the pharmacokinetics of phosphorothioate oligonucleotides because almost all published studies report results for a single dose level. The effect of multiple doses on the pharmacokinetics and metabolism of oligonucleotides is also lacking. Because published accounts describing the disposition of phosphorothioate oligonucleotides have relied primarily on total radioactivity to characterize their pharmacokinetics, little to no detailed information on the rate and identity of metabolites in plasma and tissue has been published. This study addresses these issues and is the first study to report the pharmacokinetics of intact oligonucleotide and its metabolites in tissue using capillary gel electrophoresis.

The objectives of the study were, therefore, to evaluate pharmacokinetics and metabolism of the intact oligonucleotide, CGP 69846A, as a function of dose and repeat administration in mice.

Materials and Methods

Synthesis and Purity of CGP 69846A (ISIS 5132). CGP 69846A is a 20-base phosphorothioate oligonucleotide of the sequence TCC CGC CTG TGA CAT GCA TT. CGP 69846A was chemically synthesized via conventional phosphoramidite coupling chemistry (16). The phosphorothioate linkage was generated by oxidation (17). The crude synthetic oligonucleotide was purified by HPLC; and the purified compound used in this study was found to be 94.4% full length (by capillary gel electrophoresis; approximately 5%ให้คุณกำลังอ่านเนื้อหาลงในรูปแบบที่สมบูรณ์แบบได้ ไม่ต้องการคำนึงถึงการบอกรายละเอียด financial support
N – 1 single base deletion sequence) and was approximately 87% fully thiated (9–12% monophosphodiester, by anion exchange HPLC). CGP 69846A was supplied by ISIS Pharmaceuticals, Inc. (Carlsbad, CA) as 0.08, 0.4, 2, or 10 mg/ml solutions in sterile phosphate-buffered saline.

**Animals and Treatment.** C57BL/6 mice were purchased from Charles River Laboratories (Raleigh, NC), and, at treatment initiation, were 8–9 weeks old and weighed 28–35 g. The mice were housed under a 12-hr light/dark cycle, and temperature and humidity were maintained at 19 to 25°C and 40 to 70%, respectively. Mice were randomly assigned by weight into each of four dose groups as detailed in table 1. CGP 69846A was administered every other day for up to 28 days at (15 doses) a volume of 10 ml/kg/injection by iv injection via the lateral tail vein. Dose volume was based upon the individual animal body weights obtained just before initiation of dosing.

Separate groups of CD-1 mice were placed in metabolism cages for collection of urine following single dose administration of CGP 69846A at doses of 0.8, 4, 20, and 100 mg/kg. These animals were allowed access to food ad libitum during the 24-hr urine collection period. Ad libitum access to water was withdrawn during the 24-hr urine collection period. Animals in these groups were hydrated with 1 ml of water by oral gavage at approximately 0–6, 6–12, and 12–24 hr after administration of drug.

**Sample Collection, Preparation, and Extraction.** Blood and tissues were collected from animals on days 0, 14, and 28 to characterize the pharmacokinetics of CGP 69846A (table 1). Three animals were sacrificed at each time point. Blood was collected after CO2 anesthesia by percutaneous cardiocentesis and transferred to tubes containing EDTA and centrifuged to separate plasma. Plasma and tissue samples were frozen and stored at −80°C until analyzed. Urine was collected in metabolism cages that allowed for separate collection of urine and feces. Urine samples were collected into 0.5 ml of urine and feces. Urine samples were collected into 0.5 ml of digestion buffer consisting of 0.5% Nonidet P-40 with 20 mM Tris-HCl, pH 8.0, 20 mM EDTA, and 100 mM NaCl, the tissues were homogenized in a Savant Bio 101 tissue disruptor. An aliquot of proteinase K was added so that the final concentration was 2.0 mg/ml, and the samples were incubated overnight at 37°C. The samples were then extracted with isoaamylalcohol layer was back-extracted with 500 ml of distilled H2O, and the aqueous phases were pooled. Samples were then evaporated to dryness, resuspended in 200 ml of concentrated ammonium hydroxide, and incubated at 55°C overnight, re-evaporated to dryness, resuspended, and then processed identically to plasma (18). Urine was processed identically to plasma.

**Capillary Gel Electrophoresis Analysis.** Capillary gel electrophoresis separations for plasma and urine samples were accomplished with a Bio-Rad (BioFocus 3000) capillary electrophoresis instrument using a 27-cm column with an effective length of 20 cm. Capillary gel electrophoresis separations for tissue samples were accomplished with a Beckman P/ACE (model 5010) using the same capillary column for separation. The gel-filled capillary column contained 12% polyacrylamide with 8.3 M urea, and the running buffer was 100 mM Tris-Borate, pH 8.5. Separation was achieved by running the gel at 50°C and 550 V per cm. Oligomers eluting from the column were detected by ultraviolet absorption at a wavelength of 260 nm.

**Pharmacokinetic Analysis.** CGP 69846A plasma clearance was estimated by dividing dose by area under the plasma concentration-time curve (AUC). AUC was calculated using the linear trapezoidal rule and extrapolated to infinity by dividing the last plasma concentration (C last) by the terminal elimination rate constant unless otherwise specified. Volume of distribution at steady state (V ss ) was estimated using the following equation: V ss = (Dose/AUC)² (AUC), where AUC is the area under the first moment curve (integral of dC dt). Log-linear regression of the CGP 69846A concentrations in tissue vs. time from 90 min to 96 hr were used for calculation of terminal half-lives. Plasma disposition rate constants and nonlinear regression of plasma concentration-time data were determined using PCNONLIN 4.2 (Statistical Consultants, Inc., Apex, NC). Selection of the most appropriate model was determined using Akaike criteria in combination with standard statistical error assessment.

**Results**

**Effect of Dose and Repeat Dosing on Pharmacokinetics.** Plasma concentrations of intact CGP 69846A rapidly decreased following iv administration at all doses administered. The CGP 69846A plasma concentrations showed a clear biphasic pattern over time and were best described using two or three exponential equations (fig. 1 and table 2). An initial rapid distribution phase was best seen in the results from the 20 mg/kg iv dose group because of the greater number of early time points sampled. The initial rapid phase was not characterized at the 100 mg/kg dose due to a lack of early sampling points (no samples between 2 and 90 min), but, in contrast to the lower doses, a longer elimination phase (t1/2, approximately 6 hr) was observed. The longer elimination phase was not detected at the lower doses likely due to the limited sensitivity of the assay. Intact oligonucleotide measured at 24 hr (0.12 µg/ml) was approximately 0.03% of the plasma concentration at 2 min (412 µg/ml) following a dose of 100 mg/kg. These results demonstrate that the potential for accumulation in plasma when CGP 69846A is dosed once every other day is small.

**TABLE 1**

<table>
<thead>
<tr>
<th>Group</th>
<th>No. Mice</th>
<th>Dose Level (mg/kg/m)</th>
<th>Sampling Times</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Day 0 (Dose 1)</td>
</tr>
<tr>
<td>1</td>
<td>36</td>
<td>0.8</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>72</td>
<td>4</td>
<td>2, 5, 10, 20, 40, 90, 180, 300 min; 24, 48, 96 hr</td>
</tr>
<tr>
<td>3</td>
<td>72</td>
<td>20</td>
<td>2, 5, 10, 20, 40, 90, 180, 300 min; 24, 48, 96 hr</td>
</tr>
<tr>
<td>4</td>
<td>62</td>
<td>100</td>
<td>2, 90, 180, 300 min; 24, 48, 96 hr</td>
</tr>
</tbody>
</table>

**Toxicokinetic experimental design**

**Pharmacokinetic Analysis.** CGP 69846A plasma clearance was estimated by dividing dose by area under the CGP 69846A plasma concentration-time curve (AUC). AUC was calculated using the linear trapezoidal rule and extrapolated to infinity by dividing the last plasma concentration (C last) by the terminal elimination rate constant unless otherwise specified. Volume of distribution at steady state (V ss ) was estimated using the following equation: V ss = (Dose/AUC)² (AUC), where AUC is the area under the first moment curve (integral of dC dt). Log-linear regression of the CGP 69846A concentrations in tissue vs. time from 90 min to 96 hr were used for calculation of terminal half-lives. Plasma disposition rate constants and nonlinear regression of plasma concentration-time data were determined using PCNONLIN 4.2 (Statistical Consultants, Inc., Apex, NC). Selection of the most appropriate model was determined using Akaike criteria in combination with standard statistical error assessment.

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A fraction of the CGP 69846A in plasma was rapidly metabolized to shorter oligonucleotides in a progressive fashion that resulted in decreasing concentrations of shortened metabolites (full-length oligonucleotide minus one, two, and three nucleotides) as shown in fig. 6. The per cent of total oligonucleotide detected as full length decreased from approximately 85% of total at 2 min after iv injection to approximately 65% at 10 min [initial apparent degradation half-life (\( \alpha \)) estimated to be 1–5 min] and remained in the range of 30–60% from 20 to 180 min [second apparent degradation phase half-life (\( \beta \)) of approximately 3 hr]. The pattern of metabolites did not change from the first to the last (15th) dose. Thus, the overall rate and pattern of metabolism in plasma does not seem to be altered after 15 repeated doses over the entire dose range of 0.8 to 100 mg/kg (fig. 7).

Intact CGP 69846A in tissue was metabolized to progressively shorter oligonucleotides. In tissues, the full-length oligonucleotide was typically the predominant oligonucleotide species at the early time points following both single and repeated administration of CGP 69846A. By 24 hr after iv administration of CGP 69846A, the metabolites shortened by one or two nucleotides (N-1 and N-2 oligomers) were at concentrations approximately equivalent to the parent in all tissues analyzed. Additionally, slower migrating metabolites were observed in tissues that were not observed in plasma (fig. 8). Similar metabolites of phosphorothioate oligodeoxynucleotides have been reported previously (7), and it has been reported that they correspond to the parent compound with ribonucleotide adducts (19).

In all tissues examined, the full-length oligonucleotide of CGP 69846A represented between 40 and 90% of the total detected oligonucleotide at 2 min after administration. Overall, the fraction of total oligonucleotide represented by the intact oligonucleotide in tissues did not change appreciably at equivalent time points after repeated injection through dose 15 (day 28) but consistently decreased over time to approximately 9 to 20% of total detected oligonucleotide at 96 hr after administration. At this dose regimen and over the dose range studied, the amount and pattern of metabolites seen in the tissues remained constant and predictable.

**Elimination and Urine Excretion.** Excretion of CGP 69846A in urine seems to be a minor route of elimination for this compound in measured in plasma at all time points analyzed after 2 min. The rapid initial plasma disposition half-lives were consistent with this initial rapid extravascular distribution of oligonucleotide. At 24 hr after administration, the kidney concentrations were the highest observed among the tissues tested at all dose levels following both single and repeated injection. The tissues with the next highest concentrations were the liver followed by spleen followed by lung. Some accumulation was observed in liver and kidney at all dose levels. This accumulation ranged from approximately two to three times the single dose concentrations in these tissues at 24 hr after injection of the 15th dose on day 28. However, CGP 69846A did not seem to accumulate in the other organs analyzed with the exception of the lung and lymph node at the high dose only (fig. 4). Retention and subsequent accumulation of oligonucleotide in the liver and kidney are predicted based on the rate of elimination of the parent compound from the organs following a single dose (fig. 5 and table 3). CGP 69846A was slowly cleared from all organs with estimated half-lives of elimination ranging from 16 to 60 hr following a single iv injection. In general, the liver and kidney exhibited the slowest clearance of CGP 69846A as compared with the other organs analyzed. The concentration of CGP 69846A and its metabolites in all organs analyzed increased in a dose-dependent and dose-proportional manner over the dose range studied.

**Metabolism.** Analysis of plasma for metabolites indicated that intact CGP 69846A was the predominant species at all time points following iv injection. A fraction of the CGP 69846A in plasma was rapidly metabolized to shorter oligonucleotides in a progressive fashion that resulted in decreasing concentrations of shortened metabolites (full-length oligonucleotide minus one, two, and three nucleotides) as shown in fig. 6. The per cent of total oligonucleotide detected as full length decreased from approximately 85% of total at 2 min after iv injection to approximately 65% at 10 min [initial apparent degradation half-life (\( \alpha \)) estimated to be 1–5 min] and remained in the range of 30–60% from 20 to 180 min [second apparent degradation phase half-life (\( \beta \)) of approximately 3 hr]. The pattern of metabolites did not change from the first to the last (15th) dose. Thus, the overall rate and pattern of metabolism in plasma does not seem to be altered after 15 repeated doses over the entire dose range of 0.8 to 100 mg/kg (fig. 7).

Intact CGP 69846A in tissue was metabolized to progressively shorter oligonucleotides. In tissues, the full-length oligonucleotide was typically the predominant oligonucleotide species at the early time points following both single and repeated administration of CGP 69846A. By 24 hr after iv administration of CGP 69846A, the metabolites shortened by one or two nucleotides (N-1 and N-2 oligomers) were at concentrations approximately equivalent to the parent in all tissues analyzed. Additionally, slower migrating metabolites were observed in tissues that were not observed in plasma (fig. 8). Similar metabolites of phosphorothioate oligodeoxynucleotides have been reported previously (7), and it has been reported that they correspond to the parent compound with ribonucleotide adducts (19).
mice (Table 4). Less than 3% of the total administered dose was recovered as measurable oligonucleotide in urine collected over a 24-hr period following dosing of 0.8 and 4 mg/kg. At doses of 20 and 100 mg/kg, the per cent of total oligonucleotide increased to 6.5% and 15%, respectively. Indeed, the amount of full-length oligonucleotide and total measurable oligonucleotide in urine increased greater than would be predicted by dose alone. For example, excretion of total measurable oligonucleotide increased from approximately 1.8 mg to 419 mg, over a 25-fold increase in dose (4 mg/kg to 100 mg/kg). Also, the fraction of total excreted oligonucleotide that was accounted as

### Table 2

Summary of estimated pharmacokinetic parameters for CGP 69846A in mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Dose (mg/kg)</th>
<th>4 (Dose 1)</th>
<th>4 (Dose 15)</th>
<th>20 (Dose 1)</th>
<th>20 (Dose 15)</th>
<th>100 (Dose 1)</th>
<th>100 (Dose 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (µg/ml)</td>
<td></td>
<td>39.1</td>
<td>19.1</td>
<td>362</td>
<td>577</td>
<td>542</td>
<td>—</td>
</tr>
<tr>
<td>B (µg/ml)</td>
<td></td>
<td>3.13</td>
<td>0.78</td>
<td>65.9</td>
<td>134</td>
<td>17.8</td>
<td>—</td>
</tr>
<tr>
<td>C (µg/ml)</td>
<td></td>
<td>—</td>
<td>8.81</td>
<td>15.1</td>
<td>2.1</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>$t_{1/2,1}$ (min)</td>
<td></td>
<td>2.3</td>
<td>5.1</td>
<td>0.9</td>
<td>2.6</td>
<td>4.4</td>
<td>—</td>
</tr>
<tr>
<td>$t_{1/2,2}$ (min)</td>
<td></td>
<td>29.1</td>
<td>51.7</td>
<td>11.4</td>
<td>7.5</td>
<td>64.3</td>
<td>—</td>
</tr>
<tr>
<td>$t_{1/2,3}$ (min)</td>
<td></td>
<td>—</td>
<td>29.7</td>
<td>60.8</td>
<td>380</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Cp-2 min (µg/ml)</td>
<td></td>
<td>24.4</td>
<td>15.1</td>
<td>168</td>
<td>274</td>
<td>413</td>
<td>501</td>
</tr>
<tr>
<td>Cp-24 hr (µg/ml)</td>
<td></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>bql</td>
<td>0.12</td>
<td>0.32</td>
</tr>
<tr>
<td>AUC (µg*min/ml)</td>
<td></td>
<td>278.9</td>
<td>246.8</td>
<td>1540.1</td>
<td>6874.6</td>
<td>10722</td>
<td>—</td>
</tr>
<tr>
<td>C1 (ml/min/kg)</td>
<td></td>
<td>14.3</td>
<td>16.2</td>
<td>13.0</td>
<td>2.9</td>
<td>9.3</td>
<td>—</td>
</tr>
<tr>
<td>Vss (ml/kg)</td>
<td></td>
<td>573.7</td>
<td>791.8</td>
<td>330.8</td>
<td>56.3</td>
<td>604.6</td>
<td>—</td>
</tr>
</tbody>
</table>

$C_{pt} = A \times e^{-(k_{1}t)} + B \times e^{-(k_{2}t)} + C \times e^{-(k_{3}t)}$, where $C_{pt}$ is the CGP 69846A concentration in plasma at time $t$. ND, not detectable; bql, below quantifiable limit of detection.

**Fig. 2.** Distribution of intact CGP 69846A in selected organs/tissues represented as a per cent of dose and in µg/g concentrations (mean ± SD). Tissues were collected 90 min after a single iv dose.

The decrease in per cent dose associated with liver and spleen as dose was increased are statistically significant. *, significant difference from low dose (0.8 mg/kg); **, $p < 0.05$; ***, $p < 0.001$.

**Fig. 3.** Plasma disposition dependence on dose is represented by (a) 2-min plasma concentrations of CGP 69846A after single and multiple dose administration and (b) AUC plotted as a function of dose after single and repeated administrations.

A large increase in plasma AUC was observed following repeated administration at the 20 mg/kg dose.

100 mg/kg, the per cent of total oligonucleotide increased to 6.5% and 15%, respectively. Indeed, the amount of full-length oligonucleotide and total measurable oligonucleotide in urine increased greater than would be predicted by dose alone. For example, excretion of total measurable oligonucleotide increased from approximately 1.8 µg to 419 µg, over a 25-fold increase in dose (4 mg/kg to 100 mg/kg). Also, the fraction of total excreted oligonucleotide that was accounted as
full-length CGP 69846A increased from approximately 0.09 (ratio of parent:total) at a dose of 0.8 mg/kg to approximately 0.42 (ratio of parent:total) at a dose of 100 mg/kg.

Furthermore, the pattern of metabolites observed in urine collected over a period of 24 hr after CGP 69846A administration was markedly changed over the dose range tested (fig. 9). Chain-shortened metabolites (14-mer and less) were the predominant species apparent in urine samples taken from animals receiving doses of 0.8 and 4 mg/kg. However, at a dose of 20 mg/kg the pattern shifted to include all metabolites (N-1 through N-11), although the highest concentrations of any single metabolite resided with the N-11 species (9-mer). Finally, at the 100 mg/kg dose the full-length oligonucleotide (CGP 69846A) represented the predominant oligonucleotide species, and, in contrast to the lower doses, the metabolites comprising the N-1 and N-2 species exhibited the highest concentrations of any of the chain-shortened metabolites. Nevertheless, excretion in urine was a minor route of elimination for CGP 69846A at all doses.

**Discussion**

**Plasma Pharmacokinetics and Metabolism.** The plasma kinetic disposition of CGP 69846A was similar to other phosphorothioate oligonucleotides and was characterized by rapid distribution into all tissues analyzed (2, 4, 9). However, the prolonged plasma terminal elimination rates reported for radiolabeled phosphorothioate oligodeoxynucleotides in rodents by many previous investigators (40–66 hr) seem to be associated with very low concentrations of circulating oligonucleotide (less than 0.1% of the initial concentrations of parent and/or chain-shortened metabolites) that are not measurable by capillary gel electrophoresis and are not likely associated with any significant pharmacological or toxicological consequence and, indeed, may not be associated with oligonucleotide.
As the dose was increased, changes in clearance and volume of distribution were seen in the absence of any change in extent or pattern of the appearance of metabolites. These results suggest that metabolism is not involved in the observed nonlinear plasma pharmacokinetics. Because published accounts describing the disposition of phosphorothioate oligonucleotides rely primarily on total radioactivity for describing the pharmacokinetics, little to no detailed information on the rate and identity of metabolism in plasma and tissue has been available. This study clearly shows that the metabolism observed in plasma is dose- and time-independent. Immediately following administration, the metabolism of CGP 69846A in plasma is initially very rapid with about 65% of the circulating oligonucleotide composed of intact CGP 69846A by 10 min after iv injection. Subsequent metabolism of the parent compound in plasma seemed to slow with 30–65% of the circulating total detected oligonucleotide seen at 300 min after administration being composed of parent compound. One explanation for the apparent slowing of exonuclease metabolism in plasma may be that the generation of monomer nucleoside or nucleotide metabolites may inhibit the exonucleases, thus resulting in a metabolite inhibition of metabolism. This argument is refuted by the lack of difference in the rate, extent, and pattern of metabolism over the dose range studied. Indeed, rate and extent of CGP 69846A metabolism were dose-independent and therefore predictable over the studied dose range and after repeated administration to 28 days and suggest that there is no induction or inhibition of metabolism over this dose range and regimen in mice. Another explanation for the apparent slowing of the rate of metabolism may be attributed to the stereoisomeric selectivity of exonucleases (20, 21). Phosphorothioate oligonucleotides consist of racemic mixtures of Rp and Sp phosphorothioate diastereoisomers that exhibit very different exonuclease stability. Simulations were conducted using a pharmacokinetic model that assumes only exonuclease base deletion in sequence and assigned a probability of 0.5 that rapid cleavage (Rp) vs. slow cleavage (Sp) would occur at each linkage. This model predicted a similar metabolic profile and rate change as that seen for CGP 69846A in mice (fig. 10).

Parent compound is cleared very slowly from tissue, and the clearance from tissue seems to be predominantly attributed to metabolism. As in plasma, the reproducibility of the observed metabolic pattern seen over time in tissues suggests that metabolism is neither induced or inhibited by this dose regimen in mice.

**Tissue Distribution.** Consistent with recent reports (11, 22), we see evidence of organ saturability for uptake of CGP 69846A. Curiously, very few investigators have reported this dose-dependent pharmacokinetic behavior for other phosphorothioate oligonucleotides. The lack of information in the literature can be attributed to a preponderance of single dose pharmacokinetic study designs. In this study, as dose was increased, there seemed to be a decrease in plasma clearance. This nonlinear disposition may be caused, at least in part, by saturation of major distribution sites such as the liver and spleen and, to a lesser extent, the kidney (23).

The overall tissue distribution seen for CGP 69846A was consistent with data reported for other phosphorothioate oligonucleotides (7, 9, 11, 24, 25). Furthermore, the distribution observed for CGP 69846A in mice seems to be similar to the distribution reported for rats and monkeys for other phosphorothioate oligonucleotides of different target and sequence, thus suggesting both species- and sequence-independent distribution. Accumulation of full-length oligonucleotide after repeat dose administration is consistent with the elimination of half-lives estimated in various tissues after a single dose and suggests that the liver and kidney have the greatest opportunity for tissue accumulation at this dosage regimen (once every other day) because of the longer half-lives (37–60 hr for intact, full-length oligonucleotide). Previous published accounts in mice have not characterized tissue half-lives for intact oligonucleotide. Cossum et al. reported similarly long tissue half-lives (ranging from 62 to 112 hr for liver and renal cortex, respectively) for 14C radioactivity associated with oligonucleotide (total oligonucleotide including metabolites) in rats (9, 10). The slower elimination of oligonucleotide from kidney and liver may be a function of increased retention in these tissues by an as yet
undefined mechanism. Rappaport et al. reported that phosphorothioate oligonucleotides are preferentially taken up in renal proximal tubular epithelial cells (26) with little to no uptake in glomeruli and medullary cells. This observation is consistent with tissue localization of ISIS 2105 in rat kidney where kidney cortex consistently exhibited concentrations of oligonucleotide that exceeded medulla concentrations 2–3-fold (9). Distribution studies utilizing radiolabel autoradiography, fluorescence tagging, and immunohistocytochemistry confirm this distribution in kidney and further implicate Kupffer cell uptake in the liver (27). The cells that seem to more efficiently uptake and accumulate oligonucleotide may retain them in a different manner (such as lysosomal compartmentalization) than “non-scavenging” epithelial cells and thus endow the tissues in which they reside with preferential uptake and prolonged retention of phosphorothioate oligonucleotides.

Elimination of Urine Excretion. Published studies dealing with the recovery of radiolabeled in urine have produced widely varying accounts of total excreted oligonucleotide to date. Based on the results of this study, it seems that the reported differences may be a function of isotope, isotope placement, and dose rather than a function of any real differences in excretion of phosphorothioate oligonucleotides of differing sequence. For example, the use of a $^{35}$S or $^{32}$P label placed on the 5’-end of a phosphorothioate oligonucleotide (30 mg/kg) (7, 8) consistently resulted in greater recovery of label in urine (~30–50% of dose by 24 hr after dose administration) than the use of internally placed $^3$H or $^{14}$C labels (less than 10% of dose by 24 hr after dose administration (2, 9, 22). Single base deletion from the 5’-end would liberate monomer nucleotide that is likely to be readily excreted. If the label is placed at the first internucleotide bridge, the amount of monomer liberated could be quite high and may skew the resulting urinary recovery to the high side, while leaving in tissue an active oligonucleotide that either contains less label or no longer contains label. In addition to placement, the type of isotope should be considered. For example, it is known that $^{14}$C labeled at the carbon-2 position of the thymine ring will be largely cleared via expired air as labeled CO$_2$ (9, 10). This should not obviate the use of $^{35}$S or $^{32}$P as tracer isotopes for phosphorothioate oligonucleotides but rather implicate the need for rational placement of the isotope so that the recovery in excreta may better reflect the actual oligonucleotide excretion rates. In addition, the dose administered in disposition studies must be considered in light of the dose-dependent excretion observed in this study. Certainly, the results of this study indicate that excretion in urine is a minor elimination route for CGP 69846A in mice and that the excretion in urine is dose-dependent in contrast to the $^{35}$S-labeled oligonucleotide studies, suggesting that much of the $^{35}$S isotope may be excreted as the isotope unassociated with oligonucleotide.

The urinary excretion data are consistent with a saturable excretion pathway. Plasma concentrations achieved at the doses of 20 and 100 mg/kg would be expected to exceed the plasma protein binding capacity in the mouse (ref. 13 and internal report) and may explain the overall increase in excretion of oligonucleotide as dose was increased. Renal proximal tubular cell reuptake of phosphorothioate oligonucleotides has been reported (26, 28). At high doses, it may be possible to exceed the reuptake capacity of the renal proximal tubular cells as an increasing concentration of unbound full-length oligonucleotide is filtered by the glomeruli. Therefore, in addition to exceeding plasma protein binding; saturation of the renal reuptake process may also play a role in the observed excretion pattern changes. In the absence of any evidence of metabolism differences occurring systemically over the dose range studied, the dramatic change in the pattern of metabolite excretion suggests a dose-dependent change in the excretion of CGP 69846A.

Summary

In conclusion, this study provides new and unique data regarding the pharmacokinetics and metabolism of phosphorothioate oligonucleotides. Quantitation of intact oligonucleotide allowed for definition of the intact drug pharmacokinetics. The identification and quantitation of oligonucleotide metabolites in plasma, tissue, and urine have provided definitive data to support a greater understanding of the clearance of phosphorothioate oligonucleotides. The data suggest that long elimination half-lives associated with radiolabeled oligonucleotides occur at pharmacologically insignificant concentrations of intact oligonucleotide in plasma and suggest that these low and sustained levels may

### TABLE 4

**Summary of urine excretion as a function of dose**

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Amount of CGP 69846A Excreted (µg)</th>
<th>Amount of Total Oligonucleotide Excreted (µg)</th>
<th>% of Dose Excreted</th>
<th>% Parent$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.8</td>
<td>0.043 ± 0.020</td>
<td>0.508 ± 0.198</td>
<td>0.19</td>
<td>2.3</td>
</tr>
<tr>
<td>4</td>
<td>0.066 ± 0.028</td>
<td>1.77 ± 1.19</td>
<td>0.06</td>
<td>1.6</td>
</tr>
<tr>
<td>20</td>
<td>4.94 ± 3.74</td>
<td>36.50 ± 15.62</td>
<td>0.88</td>
<td>6.5</td>
</tr>
<tr>
<td>100</td>
<td>175.83 ± 47.89</td>
<td>418.55 ± 62.07</td>
<td>6.3</td>
<td>15.0</td>
</tr>
</tbody>
</table>

$^a$ (Amount of CGP 69846A Excreted/Amount of Total Oligonucleotide excreted) × 100.
Fig. 9. Representative capillary gel electropherograms resulting from analysis of urine collected over 24 hr after iv injection of CGP 69846A.

The electropherograms illustrate the dose-dependent nature of urinary excretion of intact oligonucleotide and its metabolites.
Fig. 10. Simulation of plasma pharmacokinetics for intact and base-deleted metabolites as predicted using a model that assumes sequential exonuclease production of metabolites with a differential rate of nuclease activity dependent upon the stereoselective properties of the phosphorothioate linkage.

References


