In Vivo Distribution and Metabolism of a Phosphorothioate Oligonucleotide within Rat Liver after Intravenous Administration

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ABSTRACT
In the rat, the liver represents a major site of phosphorothioate oligodeoxynucleotide deposition after i.v. administration. For this reason, we examined the intracellular fate of ISIS 1082, a 21-base heterosequence phosphorothioate oligodeoxynucleotide, isolated from parenchymal and nonparenchymal cell types after systemic dosing using established perfusion and separation techniques followed by CGE. Isolated cells were further fractionated into nuclear, cytosolic and membrane constituents to assess the intracellular localization, distribution and metabolic profiles as a function of time and dose. After a 10-mg/kg i.v. bolus, intracellular drug levels where maximal after 8 hr and diminished significantly thereafter, suggesting an active efflux mechanism or metabolism. Nonparenchymal (i.e., Kupffer and endothelial) cells contained approximately 80% of the total organ cellular dose, and this was equivalently distributed between the two cell types, while the remaining 20% was associated with hepatocytes. Nonparenchymal cells contained abundant nuclear, cytosolic and membrane drug levels over a wide dose range. In contrast, at doses of less than 25 mg/kg, hepatocytes contained significantly less drug with no detectable nuclear-association. Doses at or above 25 mg/kg appeared to saturate nonparenchymal cell types, whereas hepatocytes continued to accumulate drug in all cellular compartments, including the nucleus. Our results suggest that although pharmacokinetic parameters vary as a function of hepatic cell type, significant intracellular delivery can be readily achieved in the liver after systemic administration.

Antisense oligonucleotides represent an exciting new class of therapeutic agents that are designed to inhibit the expression of viral and mammalian genes in a selective and sequence specific manner. Because unmodified phosphodiester linkages proved quite labile to serum and cellular nucleases (Sands et al., 1994), replacement of each nonbridging oxygen in the backbone with a sulfur atom, thereby forming a phosphorothioate linkage, provided a dramatic improvement in the drug half-life in vitro and in vivo (Crooke et al., 1995). Phosphorothioate oligodeoxynucleotides have demonstrated broad distribution and acceptable toxicity profiles after systemic delivery in a variety of species (Crooke et al., 1996; Agrawal et al., 1995; Iverson et al., 1995 and Rifai et al., 1996). These studies revealed that the major sites of distribution of phosphorothioate oligonucleotides after i.v. administration included the liver, kidney, spleen and bone marrow. Although distribution data are important, these experiments did not provide information concerning localization of phosphorothioate oligonucleotides within the cells of these organs.

More recent in vivo experiments have begun to address suborgan distribution and intracellular localization of phosphorothioate oligodeoxynucleotides in a variety of organs and tumor isolates after systemic administration (Butler et al., 1997; Plenat et al., 1995; Rifai et al., 1996; Nicklin et al., 1998, in press). Endpoints for these analyses included direct detection of rhodamine and digoxigenin-conjugated phosphodiester and phosphorothioate oligomers, autoradiography using 125I, 3H, 14C and 35S labeled compounds, as well as an indirect immunostaining method using a monoclonal antibody recognizing a 20-mer phosphorothioate oligodeoxynucleotide heterosequence (ISIS 2105). Results of these studies suggested that, within 24 hr, phosphorothioate oligodeoxynucleotides accumulated in renal proximal convoluted tubular cells, skin fibroblasts, dendritic cells and within the hepatocytes, Kupffer and endothelial cells of the liver. In hepatocytes, lower concentrations of phosphorothioate oligonucleotides were observed relative to other cell types.

ABBREVIATIONS: CGE, capillary gel electrophoresis; HPLC, high-performance liquid chromatography; DME, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; PECAM-1, platelet endothelial cell adhesion molecule; HRP, horseradish peroxidase; DAB, diamino benzidine; DTT, dithiothreitol; SAX, strong anion exchange; SPE, solid phase extraction.

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Although the aforementioned experiments demonstrated that phosphorothioate oligodeoxynucleotides are grossly localized within cells of the rodent liver, none of these studies provided quantitative pharmacokinetic data. Recently, an attempt to quantify distribution of phosphorothioate oligonucleotides within hepatic cell types using $^{35}$S-labeled oligomer has been reported (Bijsterbosch et al., 1997). Results from these experiments indicated that uptake of radiolabeled oligonucleotides is predominantly found in endothelial and parenchymal cell types, with minimal uptake observed in Kupffer cells. In addition, whole liver subcellular fractionation indicated that a majority of the internalized radiolabeled oligonucleotides were sequestered within lysosomal compartments with minimal nuclear accumulation. It should be noted that Bijsterbosch et al. (1997) made these observations without demonstrating the integrity of the oligonucleotide isolated from the liver after systemic administration. These results differed from most reports using autoradiography and antibody staining which indicated that Kupffer cells internalize substantial levels of phosphorothioate oligonucleotides with nuclear-association evident (Butler et al., 1996).

To provide even greater detail concerning the cellular and subcellular distribution and metabolic fate of a 21-mer phosphorothioate oligonucleotide in rat liver over 24 hr using a variety of dose levels, without the caveats associated with radiolabeled or conjugated phosphorothioate oligonucleotides, we used cellular isolation, fractionation and analytical techniques (Deschenes et al., 1980; Crooke et al., 1995, 1996; Leedes et al., 1996) which were previously standardized in our laboratories. Our data suggest that, although pharmacokinetic parameters vary as a function of liver cell type, there is significant intracellular delivery within parenchymal and nonparenchymal cells after systemic administration.

**Materials and Methods**

**Oligonucleotide Synthesis**

ISIS 1082 (GCCGAGGTCCATGTCGTACGC), a 21-base phosphorothioate oligodeoxynucleotide, was synthesized at Isis Pharmaceuticals on a Milligen 8800 DNA synthesizer by the phosphoramidite method. The thiation reagent was synthesized as described previously (Iyer et al., 1990). The oligomer was purified using reverse phase HPLC and determined to be greater than 85% full length by CGE.

**Preparation of $^{35}$S -1082**

The $^{35}$S radiolabel was incorporated at a single position at the most 5’ linkage of the ISIS 1082 sequence by the after method. P(III) solid phase oligonucleotide synthesis was performed utilizing commercial phosphoramidites and the unlabeled sites oxidized to P(V) phosphorothioates via Beaucage reagent as described previously (Iyer et al., 1990). During the synthetic process, site-specific $^{35}$S was incorporated by initially oxidizing P(III) triesters to P(V) $^{35}$S - phosphorothiotriesters with $^{35}$S$_{0}$. Complete oxidation of the site was insured by after the $^{35}$S oxidation with standard Beaucage reagent oxidation. After complete synthesis of the oligonucleotide, full length product was purified from shorter synthetic failure sequences, as well as any remaining trapped $^{35}$S$_{0}$, by trityl-on reverse phase HPLC. The final product was more than 90% full length radiolabeled compound with a specific activity of $1.9 \times 10^{9}$ dpm/umole.

**Formulation of Radiolabeled ISIS 1082**

The $^{35}$S labeled ISIS 1082 was formulated in 1X PBS (pH 7.4, without calcium or magnesium). Unlabeled and radiolabeled oligonucleotides were combined to produce a 600 $\mu$M dosing solution and were sterilized using a 0.2 $\mu$m Centrixfuge centrifugal filter (Schleicher & Schuell, Keene, NH) before administration. The specific activity for the radiolabeled compound was $2.48 \times 10^{9}$ dpm/umole.

**Animals**

Male Sprague-Dawley rats (130–200 g) were obtained from Harlan Sprague-Dawley (Madison, WI). Results from multiple experiments performed within this age and weight range revealed no significant hepatic oligonucleotide uptake or distribution differences. The animals were housed in polycarbonate cages and fed ad libitum. Doses of 5, 10, 25 or 50 mg/kg were administered by i.v. injection (tail vein) and animals killed after 24 hr. Time course experiments were conducted using a 10-mg/kg dose.

For radioactive studies, two animals were injected with approximately 25 $\mu$Ci of $^{35}$S, corresponding to a 10-mg/kg dose. This dose was chosen because it was within a relevant pharmacological and toxicological range (Crooke et al., 1995). After 4 hr, animals were sacrificed and various isolation procedures were performed. For whole organ determinations, the liver was perfused with 200 ml of wash buffer (10 mM HEPES, 142 mM NaCl and 6.7 mM KCl pH 7.4) to remove the blood, excised and weighed, followed by direct homogenization of the entire organ in Proteinase K digestion buffer (0.5% NP-40, 25 mM Tris pH 8.0, 25 mM EDTA and 100 mM NaCl). Quadruplicate (100 mg) aliquots were combined with 10 ml Beckman Ready Safe (Fullerton, CA) scintillation cocktail and counted using a Beckman LS 6000IC Liquid Scintillation Counter using dual source counting to correct for quench differences. $^{35}$S counting efficiency of physiological isolates under these conditions was approximately 93%.

**Liver Perfusion**

The liver was perfused, with minor modification, as described previously (Berry and Friend, 1972; Deschenes et al., 1980; Seglen, 1976). Briefly, rats were anesthetized with an i.p. injection of sodium pentobarbital. The inferior vena cava was catheterized and perfused in a retrograde flow with the inferior vena cava clamped proximal to the liver with the mesenteric vessels cut for drainage. Perfusion was performed using 250 ml of a wash buffer (described previously), followed by and additional 200 ml of wash buffer supplemented with 1.26 mM CaCl$_2$ and 0.5 mg/ml collagenase (Sigma Chemical Co., St. Louis, MO) at a flow rate of 30 ml/min. Buffers maintained an organ temperature of approximately 37°C. After collagenase treatment, the liver was removed and placed in 100 ml of ice cold 1X PBS. After gentle mincing, the suspension was poured through sterile 150-µm nylon mesh (Tetko, Buffalo, NY).

**Purification of Parenchymal and Nonparenchymal Cells**

Hepatocytes, Kupffer and endothelial cell types were isolated from whole liver as described previously (Seljelid and Smedsrod, 1981; Berry and Friend, 1972; Deschenes et al., 1980; Seglen, 1976). Hepatocytes were isolated from the liver perfusate described above by centrifugation at 50 x g for 5 min. in a Beckman table top centrifuge (Fullerton, CA). The supernatant containing nonparenchymal cells was removed and retained on ice. Centrifugation was repeated three additional times to remove contaminating cells. After the final pelleting, hepatocytes were resuspended in 1X PBS, counted and viability determined using trypan blue. Viability of hepatocytes was generally more than 90% after this procedure.

The initial supernatant, containing nonparenchymal cells, was further separated into endothelial and Kupffer cell populations by brief adherence to plastic at 37°C followed by trypsinization and cell scraping to take advantage of the differential adherence characteristics of the two cell types. It has been well documented that Kupffer
cells interact, in a trypsin insensitive manner, with plastic or glass substrates (Seljelid and Smedsrod, 1981; Garvey and Caperna, 1981). By isolating endothelial cells with trypsin, washing and then removing Kupffer cells by scraping, a rapid and effective nonparenchymal cell type enrichment was accomplished.

More specifically, the washes from the hepatocyte preparations were pooled and centrifuged at 500 × g for 10 min to recover the mixed nonparenchymal cell types. The resulting cell pellet was resuspended in 40 ml of DME (Gibco/BRL, Grand Island, NY) containing 15% heat inactivated FCS (HyClone, Logan, UT), transferred to a T-175 flask (Falcon, Becton/Dickinson; Franklin Lakes, NJ) and allowed to adhere for 1 hr at 37°C in a humidified incubator with 5% CO₂. After adherence, endothelial cells were removed using 7 ml of trypsin, followed by suspension in 7 ml of 15% DME. The flask was then washed with an additional 25 ml of 15% DME before isolation of Kupffer cells. To recover the Kupffer cells, 15 ml of the media containing serum were added to the flask and cells removed using a cell scraper. Subsequently, both cell isolates were recovered by centrifugation at 500 × g for 10 min. Typically, 20 to 30 × 10⁶ endothelial cells and 1 to 3 × 10⁶ Kupffer cells were obtained, with viabilities more than 85% using trypan blue dye exclusion. For distribution and metabolism studies, 4 to 10⁶ hepatocytes and endothelial cells and the total yield of Kupffer cells were used for each sample.

Validation of Parenchymal and Nonparenchymal Cells

Flow cytometry. Because hepatocytes are considerably larger than nonparenchymal cell types, the enrichment of parenchymal cells was confirmed by observing the forward and side scatter display using a flow cytometer (Becton Dickinson FacsScan, San Jose, CA). Comparison of pre- vs. postseparated cells revealed that hepatocytes were 95% free of other contaminating cell types, although the nonparenchymal cell isolates were 92% free of parenchymal cells (data not shown).

Immunohistochemistry

Cytocentrifuge preparations. The isolated liver cell populations were washed in PBS and suspended at a concentration of approximately 5 × 10⁶/ml. A 150-μl cell suspension was added to cytocentrifuge funnels clipped to glass slides. The slides were spun at 520 × g for 5 min and air dried overnight at room temperature before fixation and immunohistochemical staining.

Double label immunohistochemistry. Endothelial cell preparations were stained with monoclonal antibodies to rat PECAM-1 (Seroxtech MCA1334, Oxford, England) and detected using HRP-donkey anti-mouse IgG F(ab')₂ (Jackson Immunoresearch laboratories, West Grove, PA). Hepatocyte preparations were stained with rabbit antibodies to mouse albumin (ICN, Costa Mesa, CA) and detected using alkaline phosphatase-donkey anti-rabbit IgG F(ab')₂.

Kupffer cells were stained with mouse anti-rat mononuclear phagocyte antibodies (Pharmingen clone IC7, San Diego, CA) and were detected with either HRP or alkaline phosphatase-conjugated donkey anti-mouse IgG F(ab')₂. All slides were stained sequentially on a DakoAutostainer (Dako Corporation, Carpenteria, CA). The substrate used for HRP conjugated detection was DAB and new fuchsin for the alkaline phosphatase-conjugated antibodies (Dako Corp.). All slides were counterstained with hematoxylin and mounted with Gel/ Mount (Biomedica Corp., Foster City, CA).

Double label antibody staining revealed that, in nonparenchymal preparations, approximately 70% of cells were labeled with the appropriate antibody marker. Hepatocyte preparations were greater than 90% free of nonparenchymal cell types under the same conditions. Immunohistochemical results from a typical cell separation are shown (fig. 1).

Subcellular fractionation. The fractionation procedure used to isolate nuclear, membrane and cytosolic constituents was performed as described previously, with minor modification (Crooke et al., 1995). Cell lysis was accomplished using an isotonic buffer consisting of 0.5% NP-40, 10 mM Tris-Cl (pH 7.4), 140 mM KCl, 5 mM MgCl₂ and 1 mM DTT (Berger and Chirgwin, 1989). This buffer preserved the morphology of the isolated nuclei as assessed by phase-contrast microscopy (data not shown). After lysis, nuclei were maintained at 4°C and isolated by centrifugation for 5 min at 1310 × g. The supernatant, which was retained, contained the cytosolic/membrane fraction. The pellet was washed and centrifuged once again under the same conditions, with the final pellet representing the purified nuclear fraction and was confirmed by phase contrast microscopy. Membrane constituents were separated from the cytosol by transfer to thick-walled polycarbonate tubes and centrifugation (200,000 × g) for 30 min at 4°C in a Beckman TLA 100.2 fixed angle rotor using a
Beckman TL-100 benchtop ultracentrifuge (Fullerton, CA). After centrifugation, the supernatant represented purified cytosol although the pellet contained plasma membrane and various subcellular organelles including endosomes and lysosomes. Previous control experiments using this technique revealed minimal cross-contamination between the various isolated fractions (Crooke et al., 1995).

Cellular digestion and organic extraction for CGE analysis. Whole cells and cellular fractions were digested using proteinase K extraction solution, as described previously. Proteinase K enzyme (Boehringer Mannheim, GmbH, Germany) was added to each sample to a final concentration of approximately 100 μg/mL. In addition, 30 pmol of an internal standard (polyT 27-mer phosphorothioate oligodeoxyxynucleotide) was added before enzymatic digestion to permit quantitation of ISIS 1082. Samples were incubated for 2 hr at 55°C to digest proteins. After digestion, 200 μL of 30% ammonium hydroxide was added to each sample before organic extraction using 1 ml of phenol/isooamyl alcohol/chloroform (24:1:24), as described previously (Crooke et al., 1996).

Solid phase extraction. To purify samples sufficiently for CGE, two solid phase extraction columns were required (Crooke et al., 1996). Briefly, removal of residual contaminants was accomplished using a SAX SPE column (J & W Scientific, Folsom, CA) followed by desalting using a reverse phase SPE column (Isolute C18(EC), Mid Glamorgan, U.K.). A final desalting step was employed prior to CGE analysis to further reduce the amount of competitive anions that would be loaded during electrokinetic injections. Samples were placed on 0.025-μm dialysis membranes (Millipore, Bedford, MA) and floated over 60-mm culture dishes containing 10 mL of 18.3 MΩ-cm dH2O for 30 min before analysis.

CGE analysis. Samples were placed into microvials and analyzed using a Beckman PA/CE System Gold 5010 capillary electrophoresis system (Fullerton, CA) with a UV- detection at 260 nm. Samples were resolved using a 100 μM ID capillary column (Polymer micro Technologies Inc, Phoenix, AZ) filled with 11% polymerized acrylamide (Fluka, Neu-Ulm, Switzerland). The electrophoretic buffer used in the capillary and running buffer contained 200 mM biss[2-Hydroxyethyl] iminotris [hydroxymethyl]-methane (Sigma Chemical Co., St Louis, MO), 200 mM boric acid (Fluka) and 8.3 M urea (Boehringer Mannheim, GmbH, Germany). Samples were electrokinetically applied using 5 to 10 kV for 5 to 10 sec, although separations were achieved operating at 20 kV constant voltage in approximately 5 min at 50°C. Samples were injected and quantified within the linear range of the detector which spanned approximately 0.01 to 0.001 measured absorbance units.

Comparison of the absorbance and migration time of the internal standard (T27) with the absorbance and migration time of oligonucleotide allowed for sample quantitation. Because the absolute volume of each subcellular compartment was difficult to assess, the amount of oligonucleotide detected in each fraction was expressed as the number of molecules detected per cell.

Results

Total Organ Distribution

To delineate the ratio of the systemic dose distributed between whole liver, parenchymal and nonparenchymal cells and the extracellular matrix, in vivo uptake and fractionation experiments were performed using whole livers isolated 4 hr after i.v. bolus administration of a 10-mg/kg dose of 35S labeled ISIS 1082. Results, which were derived from quadruplicate 100-mg samples of whole liver homogenates, indicated that liver-associated radioactivity represented 12.3% (±0.047) of the total dose, corresponding to an organ oligomer concentration of 2.88 μM. Results from CGE analysis performed under the same conditions revealed a concentration of intact drug and metabolites of 3.83 μM, which correlated well with the radioactivity derived determination.

After collagenase treatment, radioactivity in the total unpurified cell isolates was measured and this value compared to the level determined in whole liver. Results from this experiment indicated that 53.6% (±1.32) of the total liver dose was cell-associated, although the remaining 46.4% of the drug was found in the perfusate. The material within the perfusate could arise from drug associated with connective tissue, extracellular spaces and cells damaged during the perfusion.

Kinetics of Whole Liver Association

To assess the pharmacokinetic behavior of ISIS 1082, rats were dosed by i.v. bolus injection and killed at 1, 4, 8 and 24 hr after a 10-mg/kg drug dose. The concentration of intact drug and metabolites as a function of time was determined by CGE analysis using 100 mg of total liver homogenate (fig. 2). After 1 hr, total drug level reached 4.21 μM and remained relatively constant for up to 8 hr. Between 8 and 24 hr, oligomer levels decreased by approximately 50% to 2.72 μM.

Effect of Dose

The effect of dose on the concentration of intact drug and metabolites detected within whole liver after 24 hr was measured after i.v. administration of 10, 25 and 50 mg/kg of ISIS 1082 (fig. 3). As might be expected, levels of liver-associated oligomer increased as dose was escalated. At a dose of 10 mg/kg, measured drug levels were approximately 3 μM, although at 50 mg/kg, the total liver concentration achieved was approximately 12.3 μM.

Suborgan Localization

Liver cell distribution. The changes in oligonucleotide abundance in the total liver cell population as a function of

![Fig. 2. Effect of time on the level of ISIS 1082 and metabolites within 100 mg of homogenized whole rat liver after 10 mg/kg i.v. bolus. Whole liver is expressed in μM amount detected and each point represents the mean and S.E.M. of quadruplicate samples.]
time were determined (fig. 4). One hour after i.v. bolus dosing, the amount of ISIS 1082 and metabolites was $2 \times 10^8$ molecules/cell. No increase was detected by 4 hr, however, by 8 hr, levels had increased to approximately $5.5 \times 10^8$ molecules/cell. At 24 hr levels decreased to roughly $3.5 \times 10^8$ molecules/cell. The kinetics of cellular accumulation differed slightly from those observed for whole liver (fig. 2). The higher clearance rate observed after 24 hr in whole liver is probably the result of hepatic interstitial efflux.

The effects of increasing dose on cellular accumulation of oligonucleotide were measured 24 hr after i.v. bolus (fig. 5). Hepatocellular drug levels increased as the dose was escalated from 5 to 50 mg/kg, ranging from approximately $1.7 \times 10^8$ molecules/cell at the 5 mg/kg dose to roughly $4.2 \times 10^8$ molecules/cell at the highest dose, 50 mg/kg. The increase in cellular drug was approximately 3-fold, which was similar to that observed for whole liver (fig. 3).

**Accumulation of ISIS 1082 and Metabolites into Different Liver Cell Types**

**Kinetics.** The effect of time on the proportion of ISIS 1082 and metabolites found within hepatocytes, Kupffer and endothelial cells over a 24-hr period after a 10 mg/kg i.v. bolus dose was evaluated (fig. 6). One hour after dosing, substantial differences in the uptake of drug by hepatocytes and nonparenchymal cell types were evident. In hepatocytes, the proportion of ISIS 1082 and metabolites was approximately 20% of the total liver cell dose for the first 8 hr, decreasing to approximately 7% after 24 hr. In contrast, both Kupffer and endothelial cells retained roughly 80% of the total cell-associated dose. After 1 hr, endothelial cells contained a greater proportion of ISIS 1082 relative to the Kupffer cells, but thereafter, maintained equivalent proportions of ISIS 1082 and metabolites for the remaining 24 hr.

**Dose response.** When cellular distribution was evaluated after 24 hr, as a function of ISIS 1082 dose, some interesting trends emerged (fig. 7). At the 5- and 10-mg/kg doses, nonparenchymal cells contained the majority of the total liver cell-associated drug, results that were consistent with data...
presented previously in figure 6. However, as the dose was increased to 25 mg/kg, substantial shifts in relative accumulation amongst the cell types became evident. The percentage of cell-associated ISIS 1082 and metabolites in hepatocytes and endothelial cells increased, but Kupffer levels declined. This suggests that, at doses more than 10 mg/kg, Kupffer cells were saturated. At 50 mg/kg, the percentage of the dose found in the endothelial cells also declined, with saturation of those cell types occurring at approximately 25 mg/kg. In contrast, hepatocytes continued to internalize drug, with no evidence of saturation even at the highest dose evaluated.

Subcellular Localization

Kinetics. To fully characterize the intracellular localization of ISIS 1082 and metabolites within liver cell types after systemic administration of the compound, fractionation of cell isolates was performed. The intracellular distribution patterns in hepatocytes, Kupffer cells and endothelial cells were evaluated over a 24-hr period (fig. 8). In hepatocytes, at 10 mg/kg, no nuclear-associated drug was detected at any time. However, both cytosolic and membrane drug levels appeared to increase from 1 to 8 hr followed by a significant decrease by 24 hr. In these cells, as well as nonparenchymal cell types, the amount of membrane-associated ISIS 1082 and metabolites was lower than that seen in the cytosolic fractions.

In contrast to the distribution pattern seen in hepatocytes, Kupffer and endothelial cells contained readily detectable nuclear-associated ISIS 1082 and metabolites throughout the entire time course. Consistent with results from whole cell

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**Fig. 6.** The effect of time on the proportion of ISIS 1082 and metabolites in hepatocytes (n = 9), Kupffer cells (n = 3) and endothelial cells (n = 6) 1, 4, 8 and 24 hr after 10 mg/kg i.v. bolus. The mean and S.E.M. for each cell type are plotted. Symbols are as follows: [squlo], hepatocytes; [trio], Kupffer cells; [circo], endothelial cells.

**Fig. 7.** The effect of dose on the proportion of ISIS 1082 and metabolites in hepatocytes (n = 9), Kupffer cells (n = 3) and endothelial cells (n = 6) 24 hr after i.v. bolus dosing at 5, 10, 25 and 50 mg/kg. The mean and S.E.M. for each cell type are plotted. Symbols are as follows: [squlo], hepatocytes; [trio], Kupffer cells; [circo], endothelial cells.

**Fig. 8.** The subcellular localization of ISIS 1082 and metabolites over a 24-hr period in rat hepatocytes (A), Kupffer cells (B) and endothelial cells (C), respectively. Values for each fraction represent the mean and S.E.M. of intact drug and metabolites expressed in molecules/cell. Replicates are as follows: Hepatocytes (n = 9), Kupffer values (n = 3) and endothelial cells (n = 9). Bars are as follows: [squlo], nuclear;[rhbox], cytosolic and [squlp], membrane.
experiments, these levels were at least 2-fold higher in all fractions relative to hepatocytes under the same conditions.

**Dose response.** To determine the effect of dose on the subcellular localization of ISIS 1082 and metabolites in subcellular isolates, the same dose regimen as described previously for whole liver and liver cells was performed. The effect of dose on subcellular distribution was most pronounced in hepatocytes (fig. 9A). After 24 hr, at the dose of 5 mg/kg, ISIS 1082 and metabolites were detected only in cytosolic fractions. However, when the dose was increased to 10 mg/kg, membrane-associated drug was detected. Finally, as the dosage was escalated from 25 to 50 mg/kg, nuclear-associated ISIS 1082 and metabolites were evident. Thus, to achieve nuclear drug levels in rat hepatocytes after i.v. administration, a minimal dose of 25 mg/kg was required. At 50 mg/kg, ISIS 1082 and metabolites levels were still increasing with no suggestion of cell saturation.

In contrast to the dramatic dose-dependent changes seen in the subcellular distribution in hepatocytes, only a modest change in distribution patterns in Kupffer cells was observed (fig. 9B). Although cytosolic drug levels increased from 5 to 10 mg/kg, nuclear and membrane levels remained constant over the entire range.

Finally, when endothelial cells were analyzed (fig. 9C), there appeared to be a dose-dependent increase in ISIS 1082 and metabolites in all fractions up to a dose of 25 mg/kg. At the 50-mg/kg dose no further increases were observed, again suggestive of a saturation process. Distribution to all three subcellular fractions increased as a function of dose, but the cytosolic fraction accumulated the greatest proportion of the drug.

**Metabolism**

The metabolic profiles of ISIS 1082 isolated from whole organ, cellular and subcellular fractions were evaluated. Typical electropherograms generated from hepatocyte, Kupffer and endothelial cellular compartments 24 hr after a 10-mg/kg i.v. bolus are shown (fig. 10).

Results from whole liver drug stability studies evaluating the integrity of ISIS 1082 as a function of time are shown in figure 11, with A representing the percent of intact ISIS 1082 and B representing full length drug, as well as the first two nucleotide elimination products (n-1, n-2). Because it has been demonstrated that, in general, loss of the two residues results in metabolic products with some, albeit reduced, pharmacological antisense activity (Crooke, 1995), we believed it worthwhile to include these data. One striking feature of these data is that it appears that a majority of the observed drug metabolism occurred within the first hour after administration to animals. In general, the amount of intraorgan metabolism appears to be rather modest over the rest of the 24-hr period, with percent full length being reduced from approximately 38% after 1 hr to 28% after 24 hr. This would suggest that a majority of the metabolism observed occurred extracellularly. After 24 hr, when full length and the first two metabolic products are included, more than 50% of the total oligomer represented pharmacologically active drug and metabolites. The effects of dose on stability were also evaluated (fig. 12). Once again, after trends observed in the 10-mg/kg time course experiments, only a moderate increase intact ISIS 1082 was observed over the entire dose range.

**Subcellular Metabolism**

The extent of metabolism as a function of liver cell type and cell compartment was also compared (fig. 13). In general, the fractions prepared from all cell types displayed minimal time-dependent metabolism. As was seen in whole liver, the greatest degradation was apparent as early as 1 hr after 10 mg/kg i.v. bolus. Of the cell types evaluated, hepatocytes appeared to metabolize the drug to the greatest extent, with percent full length averaging 40% over the entire time course. Kupffer and endothelial cells displayed somewhat less drug metabolism, approximately 50 to 60% over the same period.

The effect of dose on the stability of ISIS 1082 isolated from liver cell types was also assessed (fig. 14). In hepatocytes, the stability of ISIS 1082 increased as the dose was increased to 10 mg/kg, although at higher doses, the metabolic rate appeared constant. In Kupffer cells, 24 hr after 5 mg/kg dosing,
there was difference in compartmental nuclease activity observed, with the nuclear-associated oligonucleotide roughly 50% full length, with cytosolic and membrane-associated oligonucleotide 30 and 35% intact, respectively. At higher doses this difference was not as dramatic, but in general, oligonucleotides isolated from the Kupffer cell nuclei were less metabolized than in either cytosolic or membrane isolates. In contrast to the other cell types evaluated, endothelial cell

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**Fig. 10.** Electropherograms generated from hepatocyte (A), Kupffer cell (B) and endothelial cell (C) subcellular fractions 24 hr after 10 mg/kg i.v. bolus. Nuclear, cytosolic and membrane isolates are labeled and the positions of full length ISIS 1082 and internal standard T27 are indicated by arrowheads. The migration time (in min) is indicated.
fractions revealed no dose-dependent trends in stability. Additionally, the subcellular compartments behaved similarly.

Discussion

Previous studies have reported the in vivo distribution and metabolism of phosphorothioate oligodeoxynucleotides after i.v. administration (Nicklin et al., 1998, in press; Iverson et al., 1995; Rifai et al., 1996; Geary et al., 1997b). Although these studies provided pharmacokinetic information, they did not address intraorgan distribution. In contrast, more recent experiments have determined the intracellular localization of oligonucleotides within a variety of organ systems using autoradiography, immunostaining and rhodamine-conjugated phosphorothioate oligodeoxynucleotides (Butler et al., 1997; Rappaport et al., 1995; Rifai et al., 1996; Carome et al., 1997). Although these studies identified cell types within organs that internalized the oligonucleotides, they did not provide quantitative pharmacokinetic information. The most recent hepatic cell fractionation experiments used radiolabeled materials as a means of quantitation (Bijsterbosch et al., 1997) but could not evaluate oligonucleotide metabolism, which is a critical component of pharmacokinetic analysis.

By combining established separation, fractionation and analytical techniques described previously (Seglen, 1976; Crooke et al., 1995; Crooke et al., 1996), the first complete analysis of phosphorothioate oligodeoxynucleotide disposi-
been localized to connective tissue and can bind to various proteins within matrices such as laminin and fibronectin (Butler et al., 1997; Plenat et al., 1995; Benimetskaya et al., 1995).

After a 10-mg/kg i.v. bolus dose, peak organ concentration was achieved after 8 hr, with slow efflux evident for 24 hr. Over a range of 5 to 50 mg/kg, organ concentrations rose from 2.7 to 12.3 μM, respectively, after 24 hr. These data suggest that a nonlinear relationship exists between dose and hepatic drug levels that was consistent with previous observations from our own and other laboratories (Geary et al., 1997b; Rafai et al., 1996; Nicklin et al., 1998, in press).

Within 1 hr after a 10-mg/kg i.v. bolus, a majority of the oligonucleotide degradation detected within the liver had already occurred. At this timepoint, the compound was approximately 38% full length, and yet after 24 hr, the drug was still approximately 28% full length. These data suggest one or more of the following are occurring: 1) a majority of ISIS 1082 metabolism occurred from serum nuclease activity, although the drug was being cleared from the blood compartment to the liver, 2) endogenous hepatic nucleases are actually inhibited by the phosphorothioates and/or their metabolite breakdown products or not localized in the same cellular compartments, 3) there is a differential rate of metabolism for the Rp and Sp diastereoisomers present at each phosphorothioate linkage or 4) there is preferential efflux of degradation products although intact drug continues to be distributed from other sites in the body to the liver. It is likely, in fact, that all four processes occur in vivo, as suggested previously by others (Nicklin et al., 1998, in press; Glover et al., 1997). These studies demonstrated that within 1 hr after i.v. administration, metabolism was evident in both rodent and human plasma extracts, using a variety of different phosphorothioate oligonucleotide heterosequences. This degradation process appeared to be very rapid, with a majority occurring within 10 min and minimal metabolism observed thereafter, which would also agree with the diastereoisomeric nuclease selectivity seen by others (Spitzer and Eckstein, 1988). Various experiments, both in vitro and in vivo, have demonstrated the principal serum enzymatic activity results from 3’ exonucleolytic cleavage (Crooke, 1993, 1995) resulting in progressive nucleotide deletions and formation of n-1, n-2, etc. degradation products. It has also been suggested that metabolism is the principal means of drug clearance in vivo (Nicklin et al., 1997).

Phosphorothioates have also been reported to inhibit a variety of enzymes, including RNase H, topoisomerases, DNA polymerases and nucleases (Crooke, 1995). Our data are consistent with those previous observations and in vitro experiments from our laboratories (Crooke, 1995). This trend was also noted in whole rat liver, where metabolism of ISIS 1082 appeared to be moderately attenuated as delivery concentration escalated. Specifically, 24 hr after dosing at 5 mg/kg approximately 18.2% of ISIS 1082 was still intact, whereas, after a 50-mg/kg dose, 34.9% represented full length material.

Suborgan localization studies revealed dramatic differences among liver cell types. On a per cell basis, nonparenchymal cells contained approximately 80% of the total liver cell-associated drug, being equally distributed between Kupffer and endothelial cells. Additionally, the kinetics of drug accumulation and elimination in these cellular popula-

Fig. 14. Effect of dose on the integrity of ISIS 1082 in hepatocyte (A), Kupffer cell (B) and endothelial cell (C) subcellular fractions 24 hr after a single 5, 10, 25 or 50 mg/kg i.v. bolus. Note that in hepatocytes, above 10 mg/kg dosing, oligonucleotide was present within the nuclei. Bars are as follows: [squlo], nuclear; [rhbox], cytosolic and [squlf], membrane.
tions were comparable. In contrast, hepatocytes accumulated the remaining 20% of the liver dose, with maximal levels being observed 4 to 8 hr after i.v. administration. Our data also suggest that ISIS 1082 was not extensively transferred from one cell type to another within the liver, as the pattern of hepatic cell distribution observed after 1 hr remained relatively constant.

Although these results differ from those described in a recent report (Bijsterbosch et al., 1997), suborgan distribution studies performed in our laboratories as well as others, using both autoradiography and immunohistochemical detection methods, have demonstrated substantial phosphorothioate oligodeoxynucleotide accumulation within Kupffer cells after i.v. administration (Butler et al., 1997; Plenat et al., 1995; Riffai et al., 1996; Inagaki et al., 1992). It should also be noted that the oligonucleotide doses given by Bijsterbosch et al. (1997) were much lower than ours. This may have influenced the results. Finally, Bijsterbosch et al. (1997) relied on radioactivity measurements and did not determine the integrity of the material in the liver or cellular compartments. In contrast, we have used CGE to extensively characterize the oligonucleotide metabolites in various cellular fractions. This difference in approach would also account for some of the discrepancies.

After 24 hr, at doses above 10 mg, Kupffer cell drug levels appeared to saturate, although in endothelial cells, maximal drug levels were observed at the 25-mg/kg dose. In contrast, above 25-mg/kg doses, cellular levels continued to increase in hepatocytes, with no evidence of saturation. This enhancement in uptake capacity observed in hepatocytes might not be surprising in light of their substantially greater cell volume relative to the nonparenchymal cell types. These results also suggest that, to deliver phosphorothioate oligodeoxynucleotides to hepatocytes at levels necessary for antisense inhibition, higher doses may be required relative to nonparenchymal cell types. This is consistent with studies in which effects on target mRNA levels in whole liver have been measured after systemic administration (Dean and McKay, 1994; Brown-Driver V, unpublished data). Specifically, when a 20-mer rodent specific C-raf kinase antisense deoxypo-

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References


