Hepatic Distribution and Clearance of Antisense Oligonucleotides in the Isolated Perfused Rat Liver

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Received October 21, 1996; accepted January 28, 1997

Purpose. This study was conducted to investigate the impact of backbone modifications on the hepatobiliary disposition of oligonucleotides.
Methods. The disposition of backbone-modified antisense oligonucleotides [phosphorothioate (PS) and methylphosphonate (MP)] of the same base-length and sequence (5'-TAC-GCC-AAC-AGC-TCC-3'), complementary to the codon 12 activating mutation of Ki-ras, was investigated in the isolated perfused rat liver. Livers were perfused for 2 hr; perfusate and bile concentrations were analyzed by HPLC. Hepatocellular distribution was examined by measuring the amount of radiolabeled PS oligonucleotide associated with hepatocytes and Kupffer cells. Protein binding of the PS and MP oligonucleotides was determined in rat serum by ultrafiltration.
Results. MP oligonucleotide perfusate concentrations remained constant during the 2-hour perfusion. In contrast, PS oligonucleotide was eliminated slowly by the isolated perfused liver [Cl = 1.05 ± 0.21 mL/min; extraction ratio = 0.06 ± 0.01]. Uptake of PS oligonucleotide by Kupffer cells appeared to exceed uptake by hepatocytes, based on standard cell separation techniques as well as confocal microscopy. The degree of protein binding in rat serum was greater for the PS oligonucleotide (79.9 ± 2.2%) than for the MP oligonucleotide (53.0 ± 4.7%).
Conclusions. Backbone modifications significantly influence the hepatic clearance of oligonucleotides. Uncharged MP oligonucleotides are not extracted by the isolated perfused rat liver, whereas the charged PS oligonucleotide is processed more readily.

KEY WORDS: isolated perfused rat liver; oligonucleotides; hepatocellular distribution.

INTRODUCTION

Antisense oligonucleotides are designed to block the expression of specific proteins that are associated with disease states such as viral infections or cancer (1–3). Due to the inherent instability of the unmodified phosphodiesters in serum, various backbone modifications, such as phosphorothioate (PS), phosphorodithioate (PS_2) or methylphosphonate (MP), were introduced. The impact of these backbone modifications on the pharmacokinetics and tissue distribution of oligonucleotides are poorly understood. Sufficient understanding of the influence of backbone modifications on *in vivo* disposition of oligonucleotides, however, is prerequisite to successful application of these analogs as therapeutic agents.

The majority of pharmacokinetic studies to date have focused on PS oligonucleotides (4–6). A wide range of distribution [10 min (5) to 1 hr (7)] and elimination [1.9 hr (4) to 42–56 hr (7)] half-lives have been reported for these compounds. A systematic approach to comparing various backbone modifications has been undertaken in only a few studies. One investigation (8) concluded that oligonucleotides, irrespective of backbone modification (PS, PS_2 or MP), were cleared rapidly in nude, tumor-bearing mice ($t_{1/2\beta}$ ranging from 30 to 60 min) after intravenous administration. Organs with the highest oligonucleotide uptake included the kidney and liver. Taken together, these data suggest that antisense oligonucleotides may need to be administered chronically, on a multiple-dose-per-day basis, to achieve and maintain the desired therapeutic effects. Because the oral route of administration is most suitable for chronic dosing, and because previous studies indicated that a moderate amount of oligonucleotide may be absorbed from the GI tract (15% in rat everted gut [9]; 26% in rats after oral administration [10]), the extent of hepatic extraction of oligonucleotides is of importance.

The aim of the present study was to characterize the hepatic clearance of backbone-modified oligonucleotides in the isolated perfused rat liver, a system that avoids confounding factors such as extrahepatic distribution and elimination. It was of particular interest to investigate whether the charge of the backbone modification (negatively-charged PS backbone versus uncharged MP backbone) would result in differences in hepatic handling of these compounds.

Materials and Methods

Substances

The oligonucleotides used in these studies were complementary to the codon 12 activating mutation of the Ki-Ras (11). The sequence for all investigated oligonucleotides was 5'-TAC-GCC-AAC-AGC-TCC-3'. MP oligonucleotide was synthesized according to the method described by Agrawal and Goodchild (12). The PS oligonucleotide was obtained from Oligos Etc. Inc. (Wilsonville, OR). ^{14}C-labeled oligonucleotides were prepared by the method of Hughes *et al.* (9). In brief, oligonucleotides were reacted with ^{14}C-formaldehyde, reduced with sodium cyanoborohydride and precipitated in isopropyl (PS) or butyl (MP) alcohol. Purification of the labeled oligonucleotides was accomplished by preparative thin layer chromatography. The method of Sambrook *et al.* (13) was employed to prepare and to purify ^{32}P-labeled oligonucleotides. Cyanine-labeled PS olgonucleotides were obtained from the Lineberger Cancer Center of the University of North Carolina.

Animals

Male Sprague-Dawley rats (Hilltop Laboratory Animals, Scottdale, PA; 200–250 g) were used as liver donors in this study. Animals were anesthetized with ethyl carbamate (1g/kg i.p.). After opening the abdominal cavity the bile duct was

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0724-8741/97/0400-0516$12.50/0

cannulated with polyethylene tubing (I.D.: 0.011", OD.: 0.024", Becton Dickinson, Sparks, MD). Upon cannulation of the portal vein (14), the liver was perfused immediately with oxygenated saline, removed from the carcass and transferred to a temperature-controlled humidified perfusion chamber. Once in the chamber, the liver was maintained at 37°C and perfused in a recirculating manner via the portal vein with heparinized male rat blood (20%, v/v) in Krebs-Ringer bicarbonate buffer, containing 1% (w/v) glucose. Perfusate flow was maintained at a constant rate for each liver with a peristaltic pump (Buchler Instruments Inc., Fort Lee, NJ). The total volume of recirculating perfusion medium was 80 mL. Perfusate was equilibrated with 95% O_2 and 5% CO_2 using an oxygenation system described by Hamilton *et al.* (15). The perfusate was maintained between pH 7.3 and 7.4 by adding sodium bicarbonate solution to the reservoir as needed. Bile flow, oxygen consumption, inflow perfusion pressure and liver enzyme levels (ALT) were monitored as measures of liver viability. ALT levels were measured with an UV alanine aminotransferase test kit (Sigma Diagnostics, St. Louis, MO).

Experimental Design

The liver and perfusion fluid were allowed to equilibrate for 15 min before the addition of oligonucleotide (0.2 μmol bolus) to the reservoir. Bile was collected at 15-min intervals throughout the experiment, and the volume of bile was determined gravimetrically. Perfusate samples (approximately 300 μL) were taken from the reservoir at 10, 20, 30, 45, 60, 75, 90, 105 and 120 min after addition of oligonucleotide to the reservoir. The liver was removed from the apparatus at the end of the experiment, blotted and weighed.

Quantification of Oligonucleotides in Perfusate and Bile

Oligonucleotide concentrations in the perfusate were determined by reverse-phase high-performance liquid chromatography (HPLC). Oligonucleotides were extracted from the perfusate according to the method of DeLong *et al.* (8). In brief, samples were centrifuged at 10,000 rpm for 5 min; supernatant (200 μL) was precipitated with an equal volume of acetonitrile and mixed by vortex. After further centrifugation (10,000 rpm for 5 min), the supernatant was evaporated under vacuum. The residue was reconstituted in 200 μL of 20% (v/v) acetonitrile in water (for PS) or 30% (v/v) acetonitrile in water (for MP) and injected into the HPLC. Chromatographic separation was achieved with a gradient system [buffer A: 2% (v/v) acetonitrile in water, buffer B: 50% acetonitrile (v/v) in water; for PS, buffers A and B contained 5 mM of reagent PIC-A (tetrabutylammonium phosphate, Waters Corporation, Milford, MA) and for MP, buffers A and B contained 2 mM of triethylamine acetate]. The gradient was increased from 0 to 100% buffer B over 15 min (for MP) or 20 min (for PS) at a flow rate of 1 mL/min. Furosemide and antipyrine (Sigma, Chemical Company, St. Louis, MO) were employed as the internal standards for the PS and MP oligonucleotides, respectively. Detection of the compounds was achieved at 267 nm on an LKB 2151 variable wavelength UV detector (Pharmacia Biotech, Piscataway, NJ). The recovery was 85–90% for all compounds. Oligonucleotide concentrations were determined based on the peak area or peak height ratio of oligonucleotide to internal standard. Standard curves were linear in the concentration range from 0.5 to 4 μM. The coefficient of variation ranged from 3.9 (3.5 μM) to 16.0 (0.5 μM). The limit of quantification was 0.3 μM.

Pharmacokinetic Analysis

Perfusate concentration-time data were fit with both mono- and biexponential equations. The most appropriate function for each data set was identified with standard statistical criteria. Model-independent pharmacokinetic parameters (clearance [Cl], steady-state volume of distribution [V_{ss}], terminal half-life [$t_{1/2\beta}$]) were obtained by integrating the optimal exponential function (16). The extraction ratio was calculated as the ratio of Cl to perfusate plasma flow (i.e., 18 mL/min).

Protein Binding Experiments

Protein binding studies were performed in 500 μL of rat serum at 37°C by ultrafiltration. Oligonucleotides, dissolved in PBS buffer, were added to 500 μL of rat serum (final concentration 2 μM) and samples were centrifuged (IEC, Centra-8R Centrifuge, Needham HTS, MA) for 30 minutes at 3,000 rpm in low-binding regenerated cellulose filter units (Millipore, Bedford, MA) with a molecular weight cut-off of 10,000. Concentrations of compound in the filtrate and retentate were determined by HPLC. The percentage unbound was calculated as the concentration ratio of filtrate to retentate. For each compound, non-specific binding to filter material was determined by measuring filtrate and retentate concentrations in buffer solutions. The non-specific binding was less than 10% in all cases. All binding data were corrected for non-specific binding.

Liver Cell Fractionation

Perfused liver experiments were conducted with ^{32}P- and ^{14}C-labeled PS oligonucleotide to investigate the hepatic distribution of oligonucleotides. The stability of the ^{14}C-labeled compound in the perfusion fluid was monitored by HPLC on a radiomatic detector (Hewlett Packard Instrument Company, Downers Grove, IL). At the end of the 2-hr perfusion, hepatocytes and Kupffer cells were isolated according to the method described by Pertoft and Smedsrod (17). Livers were first perfused with Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (HBSS) for 10 min at a flow rate of 15 mL/min (NaCl 8g/L, KCl 0.4 g/L, $Na_2HPO_4 \cdot 7H_2O$ 0.09 g/L, KH_2PO_4 0.06 g/L, glucose 1 g/L, $NaHCO_3$ 0.35 g/L). Thereafter, livers were perfused with HBSS, containing $MgSO_4 \cdot 7H_2O$ 0.2 g/L and $CaCl_2 \cdot 2H_2O$ 0.185 g/L and 0.032% (w/v) collagenase Type II (Worthington Biochemical Corporation, Freehold, NJ) for 10 min at a flow rate of 15 mL/min. At the end of the perfusion, livers were dispersed gently with tweezers. After filtration and centrifugation at 500 rpm for 3 min, the pellet was washed three times with 10 mL HBSS buffer. The purified pellet was resuspended in 5 mL HBSS buffer for determination of hepatocyte counts and associated total radioactivity. The combined supernatants were centrifuged at 1500 rpm for 7 min. The pellet was resuspended in 10 mL HBSS buffer and placed on a double layer (50% and 25%) of Percoll (Pharmacia Bitoech, Piscataway, NJ) and centrifuged at 2500 rpm for 15 min. The layer (approximately 10 mL) in the lower part of the 25% Percoll solution contained

the Kupffer cells. This layer was centrifuged at 1500 rpm for 7 min. The pellet was re-suspended in HBSS buffer and used for determination of Kupffer cell count and associated radioactivity. The hepatocytes and Kupffer cells could be discriminated clearly, based on their morphology. The number of cells in suspension was determined with a hemacytometer (Fisher Scientific, Pittsburgh, PA). The percentage of viable cells was determined by staining with 0.4% (w/v) Trypan Blue.

Confocal Microscopy

To investigate further the hepatocellular distribution of oligonucleotides, livers were perfused for 2 hours with fluorescently labeled PS oligonucleotide, followed by a 5-min perfusion with Krebs-Ringer buffer to rinse residual blood; tissue was fixed by perfusion with 4% paraformaldehyde solution. Small slices were taken from each lobe, embedded and sectioned for confocal microscopy. Images were obtained on a Leica TCS 4D microscope (Leica, Heidelberg, Germany) with a 1.4 NA oil objective (magnification 40×) and were processed using Adobe Photoshop.

Since the cyanine fluorophore had excitation and emission wavelengths of 643 and 667 nm, respectively, there was no background fluorescence of the liver tissue, which only occurs at shorter wavelengths. Autofluorescence, at an excitation wavelength of 494 nm and an emission wavelength of 525 nm, was used to visualize the architecture of the hepatic tissue.

RESULTS

Representative HPLC chromatograms for the PS and MP oligonucleotides are shown in Figure 1. Baseline separation of the PS and MP oligonucleotide from the respective internal standards, furosemide and antipyrine, and other peaks was achieved. Retention times for the PS and MP oligonucleotides were 19.5 and 10.4 min, respectively. The retention times for the corresponding internal standards, furosemide and antipyrine, were 16.8 and 12.1 min, respectively.

The results of the isolated perfused liver experiments are displayed in Figure 2. In panel A the data are normalized to the first measured concentration; a representative plot for the PS oligonucleotide is shown in panel B. The charged PS oligonucleotide was eliminated slowly by the liver (Cl = 1.05 ± 0.21 mL/min; V_{ss} = 109 ± 19.2 mL; extraction ratio = 0.06 ± 0.01). There was no loss of compound in the system due to chemical instability or binding to the tubing (data not shown). In contrast, the concentrations of the uncharged MP oligonucleotide remained constant during the 2-hr perfusion. Due to the apparent lack of hepatic uptake and/or metabolism of the MP oligonucleotide, further analyses were not performed. Biliary concentrations of the PS and MP oligonucleotides were below the limit of detection.

Concentrations of the liver-specific enzyme ALT were measured in the perfusate during each experiment to monitor potential liver toxicity. Average ALT concentrations, ranging from 35 to 115 units/L, were not significantly different in the absence or presence of oligonucleotides, suggesting a lack of oligonucleotide toxicity during the procedure.

Modest differences were observed in the serum protein binding behavior of the two oligonucleotides. The PS oligonucleotide was bound (mean ± SD) more extensively (79.9 ± 2.2 %; n = 4) than the MP oligonucleotide (53.0 ± 4.7 %; n = 3).

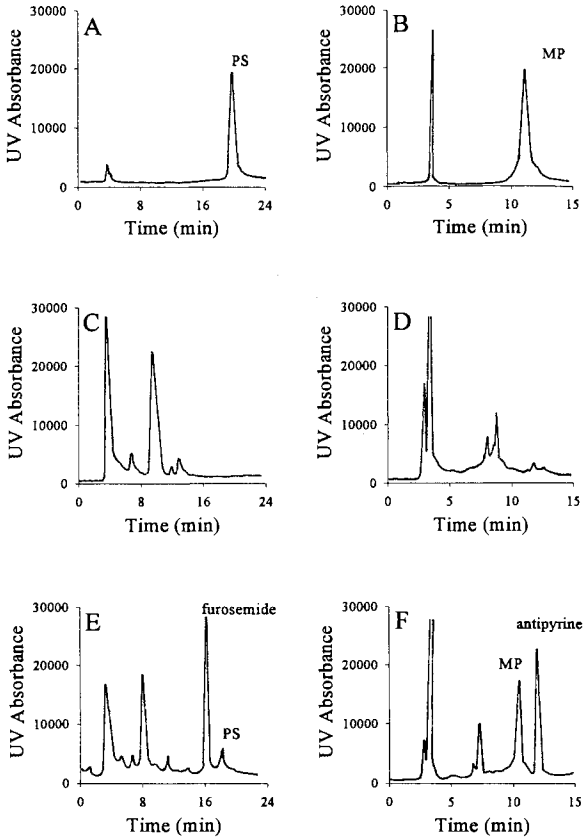

Fig. 1. Representative chromatograms for (A) PS oligonucleotide (2.5 μM) injected in 20% (v/v) acetonitrile, (B) MP oligonucleotide (2 μM) injected in 35% (v/v) acetonitrile, (C) blank perfusion fluid for PS, (D) blank perfusion fluid for MP, (E) PS oligonucleotide and internal standard (furosemide) extracted from perfusion fluid, and (F) MP oligonucleotide and internal standard (antipyrine), extracted from perfusion fluid.

The ratios of radioactivity in the Kupffer cell fraction relative to the hepatocyte fraction, normalized for the number of viable cells, was determined with ^{32}P- (2.0 ± 0.4; n = 4) and ^{14}C-labeled (1.6; n = 1) PS oligonucleotide (Figure 3). The percentage of viable cells was greater than 70% in all cases. Analysis of perfusion fluid by HPLC with radiomatic detection indicated the presence of intact compound at the end of the 2-hr experiment and the appearance of fast-eluting breakdown products. The concentrations of intact compound in the perfusion fluid were in good agreement with expected concentrations based on the half-life of unlabeled PS oligonucleotide.

The distribution of PS oligonucleotide was investigated further by confocal microscopy (Figure 4). Hepatocytes could be visualized clearly using their autofluorescence in the FITC channel. It was difficult, however, to identify Kupffer cells in this channel, even at higher magnifications (100×). Fluorescence derived from the cyanine labeled PS oligonucleotide was highest in the sinusoids and the walls of blood vessels, regions in which Kupffer cells reside predominantly. Fluorescence asso-

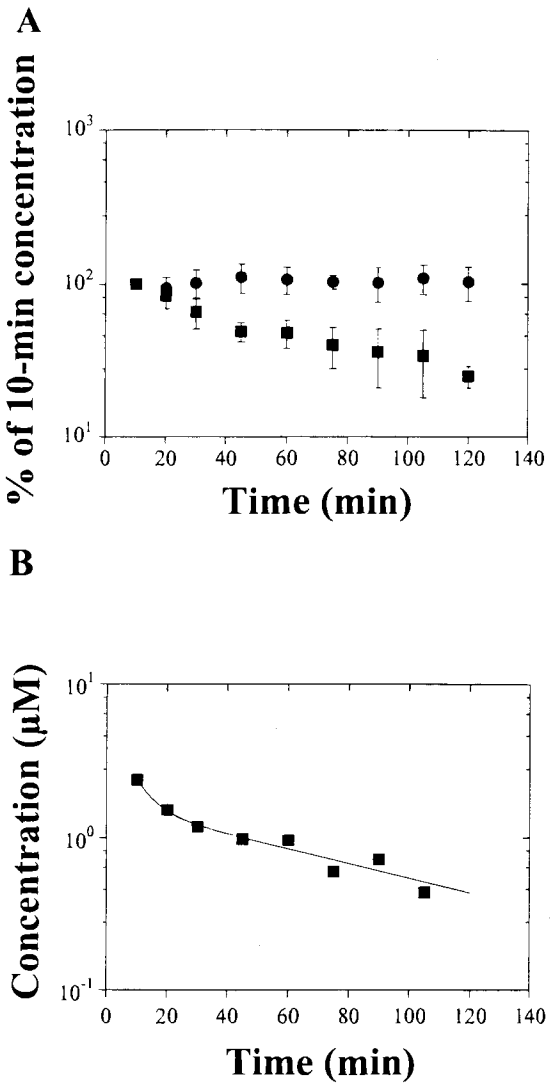

Fig. 2. Time course of oligonucleotides during liver perfusion. Data are presented as (**A**) percent of the corresponding 10-min concentration (mean ± SD) for PS (■; n = 4) and MP (●; n = 3); (**B**) representative concentration-time profile for PS. The line in Panel B represents the fit of a biexponential function to the concentration-time data.

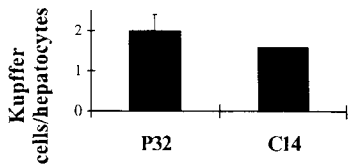

Fig. 3. Hepatocellular distribution (mean ± SD) of ^{32}P-labeled (2.0 ± 0.4; n = 4) and ^{14}C-labeled (1.6; n = 1) PS oligonucleotide. Data are expressed as radioactivity associated with Kupffer cells relative to radioactivity associated with hepatocytes, normalized for number of viable cells.

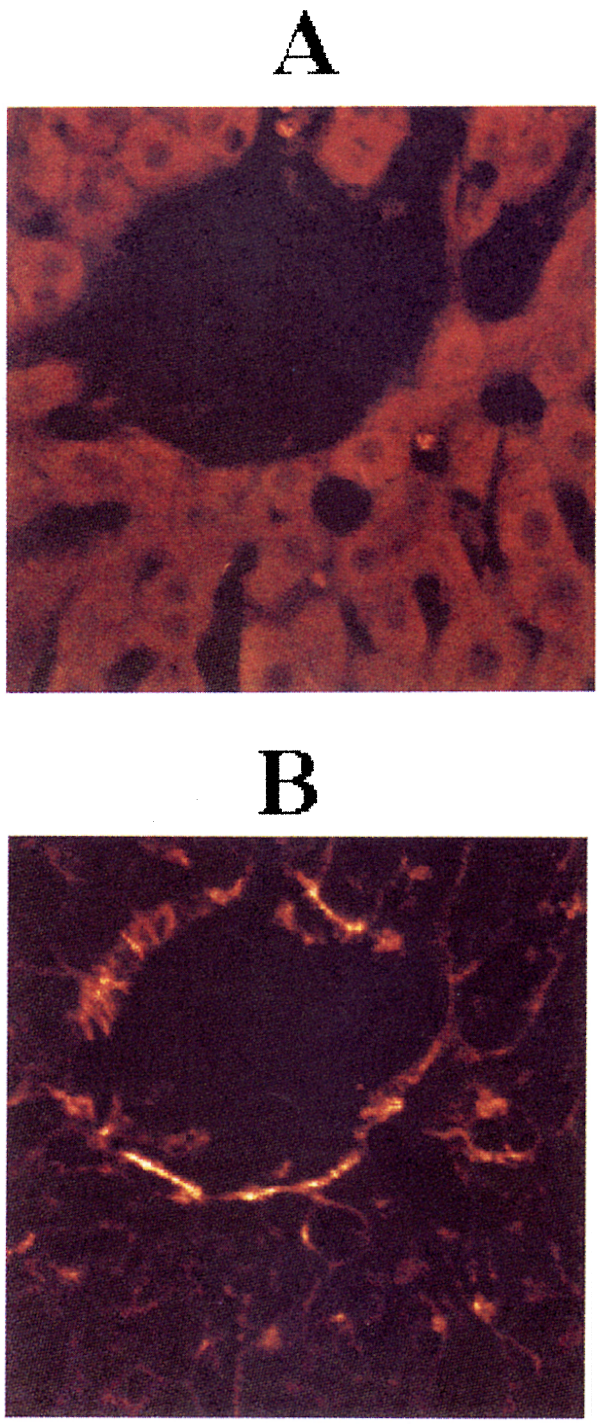

Fig. 4. Hepatocellular distribution of cyanine labeled PS oligonucleotide. Image A shows autofluorescence in a liver slice as viewed in the FITC channel (excitation: 494 nm; emission: 525 nm). Image B displays the same slice as viewed by the cyanine channel (excitation: 643 nm; emission: 667 nm). Magnification for both cases is 40×.

ciated with hepatocytes was observed, but only to a limited extent.

DISCUSSION

In the present study, the hepatic clearance of two backbone-modified oligonucleotides with the same base sequence was

characterized in isolated perfused rat livers. The doses of oligonucleotides used in this study resulted in initial perfusate concentrations (2.5–3 μM) which are considered pharmacologically relevant (0.1–10 μM) for this class of compound. This model, in contrast to whole animal experiments, allows the specific determination of hepatic uptake and/or metabolism without the interfering effects of other tissues or organs. Numerous *in vivo* studies (18,19) have shown that accumulation of PS oligonucleotides is most pronounced in the kidney and liver. These findings are consistent with the results of the present study; hepatic clearance was low for the PS oligonucleotide. Despite the low extaction ratio (the efficiency of the liver to remove compound from the blood in a single pass) it is still possible for a significant amount of PS oligonucleotide to accumulate in the hepatic compartment after a single dose. For example, at an initial concentration of 2.5 μM (similar to the initial concentrations observed in this study) a hepatic clearance of 1 mL/min would yield accumulation of approximately 1% of the oligonucleotide dose in the liver during the first minute. Thus, over prolonged periods of time, substantial amounts of oligonucleotides can accumulate in the liver.

Another important finding of this study is that the charge or chemistry of the backbone modification may influence the extent of hepatic uptake of oligonucleotides. The negatively-charged PS oligonucleotide was cleared to a limited extent by the liver, whereas the uncharged MP oligonucleotide was not extracted by the liver. Further studies involving charged and uncharged backbone modifications of oligonucleotides with various base lengths and sequences will be necessary to confirm the hypothesis that the charge of the backbone modification influences the extent of hepatic uptake of oligonucleotides. In an attempt to elucidate further the hepatocellular distribution of the PS oligonucleotide, the accumulation of radiolabeled compound in parenchymal cells (hepatocytes) and non-parenchymal cells (Kupffer cells) was investigated. Kupffer cells accumulated the PS oligonucleotide to a greater degree than hepatocytes suggesting that metabolism of oligonucleotides by liver parenchymal cells plays a lesser role in the elimination of these compounds from the perfusion medium. The hepatic distribution of oligonucleotide was evaluated further by confocal microscopy. The confocal images suggest that the PS oligonucleotide is distributed mainly into sinusoids and the walls of blood vessels, regions in which Kupffer cells are located. Uptake into hepatocytes appears to occur to a lesser extent. These observations are consistent with the cellular distribution studies using radiolabeled oligonucleotides.

Both the MP and, in particular, the PS oligonucleotide, posed challenges regarding the analytical methodologies. Initial experiments with PS, using triethylamine acetate instead of PIC-A reagent in the mobile phase, resulted in insufficient separation (broad oligonucleotide peaks) and sensitivity. The addition of PIC-A reagent to buffers A and B improved the quality of the chromatograms considerably. It should be noted that the use of reverse-phase HPLC affords greater specificity than monitoring total radioactivity derived from labeled oligonucleotides, a method commonly employed in other studies. Future studies will explore the use of capillary electrophoresis, a technique which exhibits high sensitivity and specificity for a number of compounds, to characterize the metabolic fate of oligonucleotides in perfusion fluid and bile.

To date there are only a few reports regarding the protein binding of oligonucleotides. Employing ultrafiltration, Sands *et al.* (20) found that 65% of a 20-mer PS oligonucleotide was bound. The backbone modification of oligonucleotides influences the serum protein binding of these compounds; the more lipophilic MP oligonucleotide was bound less extensively to rat serum proteins. However, there was no obvious correlation between protein binding characteristics and hepatic clearance of the two types of oligonucleotides.

In summary, hepatic clearance of oligonucleotides is influenced by the charge on the backbone modification. Modest hepatic extraction was observed for the PS oligonucleotide, whereas the MP oligonucleotide was not extracted by the isolated perfused rat liver. Whether chemical modifications other than those on the backbone result in more extensive hepatic metabolism and/or biliary excretion remains to be investigated. The low extraction ratio of oligonucleotides may have great impact on the delivery of these compounds. Because there is some evidence for at least moderate uptake of the PS oligonucleotide from the GI tract (9,10), oral delivery of these compounds may be possible.

ACKNOWLEDGMENTS

This work was supported in part by a grant from the North Carolina Biotechnology Center and NIH grants CA 47044 and CA 60139.

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