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In vivo fate of phosphorothioate antisense oligodeoxynucleotides: predominant uptake by scavenger receptors on endothelial liver cells

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ABSTRACT

Systemically administered phosphorothioate antisense oligodeoxynucleotides can specifically affect the expression of their target genes, which affords an exciting new strategy for therapeutic intervention. Earlier studies point to a major role of the liver in the disposition of these oligonucleotides. The aim of the present study was to identify the cell type(s) responsible for the liver uptake of phosphorothioate oligodeoxynucleotides and to examine the mechanisms involved. In our study we used ISIS-3082, a phosphorothioate antisense oligodeoxynucleotide specific for murine ICAM-1. Intravenously injected [^{3}H]ISIS-3082 (dose: 1 mg/kg) was cleared from the circulation of rats with a half-life of 23.3 ± 3.8 min. At 90 min after injection (>90% of [^{3}H]ISIS-3082 cleared), the liver contained the most radioactivity, whereas the second-highest amount was recovered in the kidneys (40.5 ± 1.4% and 17.9 ± 1.3% of the dose, respectively). Of the remaining tissues, only spleen and bone marrow actively accumulated [^{3}H]ISIS-3082. By injecting different doses of [^{3}H]ISIS-3082, it was found that uptake by liver, spleen, bone marrow, and kidneys is saturable, which points to a receptor-mediated process. Subcellular fractionation of the liver indicates that ISIS-3082 is internalized and delivered to the lysosomes. Liver uptake occurs mainly (for 56.1 ± 3.0%) by endothelial cells, whereas parenchymal and Kupffer cells account for 39.6 ± 4.5 and 4.3 ± 1.7% of the total liver uptake, respectively. Preinjection of polyinosinic acid substantially reduced uptake by liver and bone marrow, whereas polyadenylic acid was ineffective, which indicates that in these tissues scavenger receptors are involved in uptake. Polyadenylic acid, but not polyinosinic acid, reduced uptake by kidneys, which suggests renal uptake by scavenger receptors different from those in the liver. We conclude that scavenger receptors on rat liver endothelial cells play a predominant role in the plasma clearance of ISIS-3082. As scavenger receptors are also expressed on human endothelial liver cells, our findings are probably highly relevant for the therapeutic application of phosphorothioate oligodeoxynucleotides in humans. If the target gene is not localized in endothelial liver cells, the therapeutic effectiveness might be improved by developing delivery strategies that redirect the oligonucleotides to the actual target cells.

INTRODUCTION

The technology of cloning and sequencing of pathogenic genes enables specific therapeutic approaches at the level of nucleic acids. Oligonucleotides complementary to sequences in pathogenic genes (antisense oligonucleotides) can hybridize sequence-specifically with these genes or their messenger RNA, and can consequently highly specifically affect the expression of their target genes (1–4). Such a specific inhibition of the expression of pathogenic genes affords an exciting new strategy for therapeutic intervention. An initial drawback for the therapeutic application of the antisense technology was the low stability of unmodified oligonucleotides in a biological environment, due to hydrolysis of their phosphodiester bonds by exo- and endonucleases. To overcome this problem, a wide variety of oligonucleotide analogues with modified internucleoside linkages or modified ribose moieties have been synthesized (2,3,5). These analogues are more resistant to nuclease activity than the unmodified phosphodiester oligonucleotides. Phosphorothioate oligodeoxynucleotides, in which a non-bridging oxygen atom is replaced by a sulfur atom, are the most extensively studied analogues. These compounds are much more stable in a biological environment than phosphodiester oligodeoxynucleotides (1,4). Numerous studies have demonstrated that phosphorothioate oligodeoxynucleotides can be effective inhibitors of the expression of a broad range of genes, including pathogenic genes such as oncogenes and viral genes (4). Further, it was found in animal studies that phosphorothioate oligodeoxynucleotides are well tolerated, and can be therapeutically effective *in vivo* (7–11). Several phosphorothioate oligodeoxynucleotides have now entered clinical trials (3,6).

To fully understand the possibilities and limitations of the therapeutic application of phosphorothioate antisense oligodeoxynucleotides, it is necessary to evaluate their *in vivo* fate. The

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disposition of phosphorothioate oligodeoxynucleotides has been studied in several species, and the general conclusion from these studies was that the liver is the major site of uptake (reviewed in refs 7,8). However, the liver contains three major cell types: parenchymal cells, Kupffer cells, and endothelial cells, each equipped with specific receptor systems that are capable of internalizing extracellular ligands (12,13). The role of these different liver cell types in the clearance of phosphorothioate oligodeoxynucleotides, and the receptor systems involved, has thus far not been studied. The aim of the present study is, therefore, to address these unresolved issues, which are especially relevant for antisense phosphorothioate oligodeoxynucleotides aimed to affect gene expression in the liver. We investigated in rats the disposition of ISIS-3082, a 20mer phosphorothioate oligodeoxynucleotide specific for the murine adhesion molecule ICAM-I (9). The results indicate that endothelial cells are mainly responsible for the hepatic uptake of the oligonucleotide, and that uptake is mediated by 'scavenger-type' receptors

MATERIALS AND METHODS

Reagents

Polyinosinic acid (5′) and polyadenylic acid (5′) were from Sigma (St. Louis, MO, USA). Emulsifier Safe™ or Hionic Fluor™ scintillation cocktails were from Packard (Downers Grove, IL, USA). All other reagents were of analytical grade.

Oligonucleotide synthesis

ISIS-3082 phosphorothioate oligodeoxynucleotide, sequence 5′-TGCATCCCCCAGGCCACCAT-3′, was synthesized as described before (9). The oligonucleotide was radiolabeled with ^{3}H by heat-catalyzed exchange at C8 positions of the purine nucleotides as described earlier (14). The specific radioactivity of the radiolabeled oligonucleotide was 48.5×10^6 d.p.m./mg. The radiochemical purity, determined by HPLC (see below), was ~90%.

HPLC analysis

The integrity of [^{3}H]ISIS-3082 in stock solutions, plasma samples, and urine was assessed by ion-exchange HPLC, using a Partisil SAX-10 column (0.46 × 25.0 cm; Alltech, Deerfield, IL, USA). The column was eluted at a flow rate of 1 ml/min, using the following mobile phases: buffer A, 0.1 M Tris–HCl (pH 7.4) + 25% (v/v) acetonitrile; buffer B, 0.1 M Tris–HCl (pH 7.4) + 25% (v/v) acetonitrile + 2.5 M KCl. After injection of the samples (0.5 ml), the column was washed for 5 min with buffer A, followed by gradient of 0–100% buffer B (20 min). Subsequently, the column was washed for 50 min with buffer B. The retention time of ISIS-3082 under these conditions was ~57 min. A series of 3′-truncated ISIS-3082 oligodeoxynucelotides were used as standards. It was found that 12-, 14-, 16-, and 18mer oligonucleotides eluted at ~27, 30, 34, and 42 min, respectively. Plasma or urine samples were processed before separation as follows. Aliquots of 0.2 ml were diluted with 0.4 ml H_2O, and spiked with 40 µg of authentic ISIS-3082, which served both as carrier to minimize non-specific adsorption and as internal standard. To eliminate non-specific interactions with serum proteins, plasma samples were heated for 2 min at 95°C and then rapidly chilled on ice, immediately before separation (15).

Determination of plasma clearance and tissue distribution

Male Wistar rats weighing between 200 and 350 g were used. The animals were anaesthesized by intraperitoneal injection of sodium pentobarbital (60 mg/kg body weight), and the abdomen was opened. Radiolabeled oligonucleotide, dissolved in phosphate-buffered saline (10 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl), was injected via the vena penis (2 ml/kg body weight). At the indicated times, blood samples of 0.2–0.3 ml were taken from the inferior vena cava and collected in heparinized tubes. The samples were centrifuged for 2 min at 16 000 *g*, and the plasma assayed for radioactivity. The total amount of radioactivity in plasma was calculated using the equation: plasma volume (ml) = [0.0219 × body weight (g)] + 2.66 (16). At the indicated times, liver lobules were tied off and excised, and at the end of the experiment the remainder of the liver was removed. The amount of liver tissue tied off successively did not exceed 15% of the total liver mass. The amount of radioactivity in the liver at each time point was calculated from the radioactivities and weights of the liver samples. Uptake by extrahepatic tissues was determined by removing the tissues at the end of the experiment, and counting of radioactivity. Uptake by bone marrow was determined by sampling from femurs and tibiae, and assuming that bone marrow constitutes 1.32 ± 0.14% of the body weight (calculated from data in refs 17,18). Radioactivity in the tissues was corrected for radioactivity in plasma present in the tissue at the time of sampling (19).

Determination of the distribution over liver cell types

Rats were anaesthesized and injected with radiolabeled ISIS-3082 as described above. The liver was perfused at 60 min after injection, and parenchymal, Kupffer and endothelial cells were isolated from the liver as described in detail earlier (20). The cell fractions were assayed for radioactivity and protein. Shortly before separation of the cells, a liver lobule was tied off and excised to determine the total liver uptake. The contributions of the various cell types to the total liver uptake was calculated as described previously (20). As found with other ligands (16,20), no significant amounts of radioactivity were lost from the cells during the isolation procedure. This was checked in each experiment by comparing the calculated liver uptake (i.e. the summation of the contributions of the various cell types) with the value actually measured in the liver lobule.

Subcellular fractionation

Rats were anaesthesized and injected with radiolabeled ISIS-3082 as described above. After 60 min, the liver was perfused with ice-cold 0.25 M sucrose + 10 mM Tris–HCl (pH 7.4), and subsequently divided into subcellular fractions as described previously (21). In brief, the liver was homogenized in sucrose/Tris–HCl (see above) using a homogenizer of the Potter–Elvehjem type. Fractions enriched in nuclei, mitochondria, lysosomes and microsomes were obtained by collecting pellets obtained after consecutive centrifugation steps of 5 min at 1200 *g*, 5 min at 9000 *g*, 15 min at 22 000 *g* and 30 min at 210 000 *g*, respectively (*g* forces in middle of tubes). The final supernatant was the cytosol fraction. The fractions were assayed for the activity of marker enzymes as described earlier (21). Radioactivity and protein were determined as described below.

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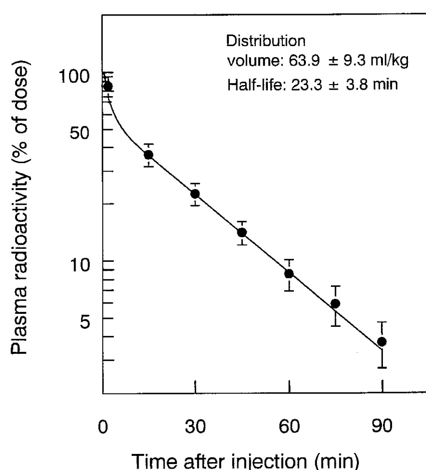

Figure 1. Plasma clearance of intravenously injected [^{3}H]ISIS-3082. Rats were intravenously injected with [^{3}H]ISIS-3082 at a dose of 1 mg/kg body weight, and the clearance of radioactivity from the blood plasma was monitored. Values are means ± S.E.M. of four rats.

Determination of proteins

Protein concentrations in cell suspensions and subcellular fractions were determined by the method of Lowry *et al.* (22), with a bovine serum albumin standard.

Determination of radioactivity

Liquid samples were counted without further processing by liquid scintillation spectroscopy, using Emulsifier Safe™ or Hionic Fluor™ scintillation cocktails. Tissue samples were processed using a Packard 306 Sample Oxidizer. Some tissues (e.g. bone) were dissolved in 10 M NaOH at 95°C before counting. All radioactivity measurements were performed in a Packard Tri-Carb 1500 liquid scintillation counter.

RESULTS

Plasma clearance and tissue uptake of ISIS-3082

The disposition of ISIS-3082 was studied after a bolus injection of the radiolabeled oligonucleotide into rats. The administered amount, 1 mg/kg body weight, was in the range of phosphorothioate oligodeoxynucleotide doses that have been found to be effective *in vivo* (9–11). Figure 1 shows that after an initial rapid distribution phase (distribution volume 63.9 ± 9.3 ml/kg body weight), radioactivity was cleared from the circulation with a half-life of 23.3 ± 3.8 min (means ± S.E.M. of four rats). HPLC-analysis of plasma samples indicated that at 60 and 90 min after injection >85% and >75% of the radioactivity, respectively, represented oligonucleotide that was at least 70% full length. At 90 min after injection, when >90% of the injected dose had been cleared from the circulation, the distribution of the radioactivity over the body was determined (Table 1). A small amount of the administered radioactivity (2.5 ± 0.4% of the dose) was recovered in the urine. HPLC analysis indicated that the radioactivity in urine represented degradation products. The liver, which contained 40.5 ± 1.4% of the dose, was found to be mainly responsible for the clearance of the oligonucleotide. Spleen and bone marrow, which constitute with the liver the reticuloendothelial system (RES), contained smaller amounts of radioactivity (0.7 ± 0.1 and 6.7 ± 0.7% of the dose, respectively). The kidneys contained the second highest amount of radioactivity (17.9 ± 1.3% of the dose), and the remainder of the radioactivity was distributed over a large number of tissues. Table 1 only lists tissues containing >1% of the dose. Bulky tissues, like muscle and skin contained 5.5 ± 0.8 and 8.4 ± 1.7% of the dose, respectively. The carcass contained a substantial amount of radioactivity (10.4 ± 0.5% of the dose), which is mainly present in bone marrow (see above). The specific uptake, expressed as relative specific radioactivity, was highest in the kidneys and in the RES organs liver, spleen, and bone marrow.

Table 1. Tissue distribution of intravenously injected [^{3}H]ISIS-3082

Tissue	Radioactivity (% of recovered)	Relative specific radioactivity
Blood plasma	2.1 ± 0.3	
Urine	2.5 ± 0.4	
Liver	40.5 ± 1.4	8.1 ± 0.2
Spleen	0.7 ± 0.1	3.3 ± 0.4
Bone marrow	6.7 ± 0.7	4.7 ± 0.5
Kidneys	17.9 ± 1.3	22.4 ± 2.0
Intestines	6.4 ± 0.6	1.0 ± 0.1
Pancreas	2.2 ± 4.4	2.2 ± 0.3
Muscles	5.5 ± 0.8	0.2 ± 0.0
Skin	8.4 ± 1.7	0.5 ± 0.1
Carcass (including marrow)	10.4 ± 0.5	0.5 ± 0.0
All other tissues	3.3 ± 0.1	0.3 ± 0.0

Rats were intravenously injected with [^{3}H]ISIS-3082 at a dose of 1 mg/kg body weight. At 90 min after injection, the distribution of radioactivity over all tissues was determined. The results are expressed as percent of the recovered amount of radioactivity and as relative specific activity (percent recovered radioactivity divided by percent recovered weight). Recoveries of radioactivity and tissues were 84.6 ± 4.2% and 94.1 ± 0.7%, respectively. Only tissues containing >1.0% of the recovered dose and/or a relative specific radioactivity >2.0 are listed. Values are means ± S.E.M. of three rats.

Dose-dependence of plasma clearance and tissue uptake of ISIS-3082

The high specific uptake of ISIS-3082 by the RES organs and kidneys points to a selective uptake mechanism, possibly receptor-mediated endocytosis. As receptor-mediated clearance mechanisms are saturable, we investigated the effect of the injection of different doses of ISIS-3082. Figure 2 compares the plasma clearance of radioactivity after injection of [^{3}H]ISIS-3082 at a dose of 0.05 mg/kg or 1.0 mg/kg. After injection of 0.05 mg of ISIS-3082/kg body weight, radioactivity was cleared from the circulation with a half-life of 13.1 ± 2.0 min, which is significantly (P <0.05) faster than observed after injection of the oligonucleotide at a dose of 1.0 mg/kg (half-life 23.3 ± 3.8 min). The distribution volume was not significantly different (83.2 ± 13.0 ml/kg versus 63.9 ± 9.3 ml/kg). The rate of uptake by the liver, being the most important organ for disposition, was also investigated. Figure 3 shows that the hepatic uptake of [^{3}H]ISIS-3082 proceeded more

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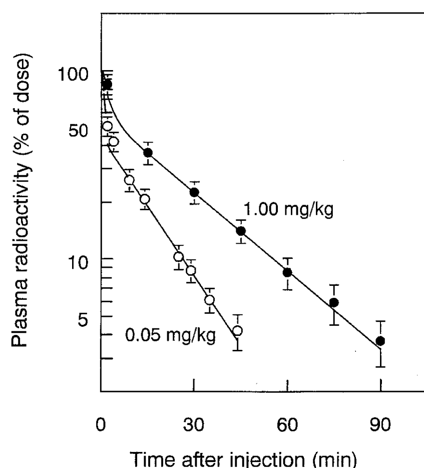

Figure 2. Effect of dose on the plasma clearance of [^{3}H]ISIS-3082. Rats were intravenously injected with [^{3}H]ISIS-3082 at a dose of 0.05 mg/kg body weight (○) or 1.00 mg/kg body weight (●), and the clearance of radioactivity from the blood plasma was monitored. Values are means ± S.E.M. of four rats.

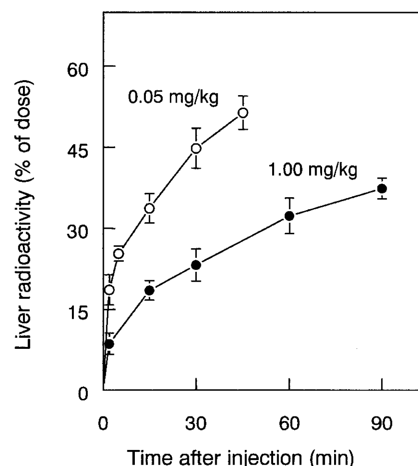

Figure 3. Effect of dose on the liver uptake of [^{3}H]ISIS-3082. Rats were intravenously injected with [^{3}H]ISIS-3082 at a dose of 0.05 mg/kg body weight (○) or 1.00 mg/kg body weight (●). At the indicated times, the association of radioactivity with the liver was determined. Values are means ± S.E.M. of 3–4 rats.

rapidly at a dose of 0.05 mg/kg than at 1.0 mg/kg. The dose-dependence of extrahepatic uptake of [^{3}H]ISIS-3082 was also investigated (Fig. 4). As the sampling times and rates of plasma clearance are different for the two different doses, the uptake of the oligonucleotide is expressed as uptake index, which allows the most accurate comparison. The uptake indexes of liver, spleen, bone marrow, and kidneys were significantly lower at a dose of 1.0 mg/kg than at 0.05 mg/kg, which indicates that the uptake systems in these organs are saturable. The uptake indexes of muscle, skin and intestine were dose-independent and thus uptake in these organs is, at least in the range of 0.05–1.0 mg/kg, not saturable.

Cellular and subcellular distribution of ISIS-3082 in the liver

The liver contains several actively endocytosing cell types (12,13). To identify the cell type(s) responsible for the hepatic uptake of ISIS-3082, rats were injected with the radiolabeled oligonucleotide (1 mg/kg). After 60 min, parenchymal, endothelial, and Kupffer cells were isolated from the liver and assayed for radioactivity. The cell isolation procedure was performed at a low temperature (8°C) to prevent processing of internalized oligonucleotide. The results are shown in Table 2. Endothelial cells were found to be at least ten times more active in uptake than Kupffer and parenchymal cells. From the uptake/mg of cell protein and the contribution of each cell type to the total liver protein, it can be calculated that the endothelial cells accounted for 56.1 ± 3.0% of the total liver uptake. Parenchymal cells (which show a low specific uptake, but constitute >90% of the liver mass) were responsible for 39.6 ± 4.5% of the hepatic uptake, whereas Kupffer cells accounted for only 4.3 ± 1.7%.

To investigate the intracellular routing of ISIS-3082, the liver was subjected to a subcellular fractionation at 60 min after injection of [^{3}H]ISIS-3082 (Fig. 5). The relative specific radioactivity was, like the relative specific activity of the lysosomal marker acid phosphatase, the highest in the lysosomal fraction. The subcellular fractions of interest for intracellular delivery of antisense oligonucleotides (i.e. the nuclear- and cytosol fractions) also contained some radioactivity. However, these fractions contained similar amounts of the lysosomal marker acid phosphatase, indicating that the radioactivity recovered in the nuclear- and lysosomal fractions can probably be ascribed to the presence of lysosomes. The microsomal marker glucose-6-phosphatase and cytoplasmic marker lactate dehydrogenase showed clearly different distributions. These findings indicate that ISIS-3082 is internalized and transported to the lysosomal compartment.

Table 2. Uptake of intravenously injected ISIS-3082 by liver cell types

Cell type	Uptake of ISIS-3082	
	ng/mg of cell protein	Percent total liver uptake
Parenchymal cells	23.0 ± 3.8	39.6 ± 4.5
Kupffer cells	90.0 ± 33.8	4.3 ± 1.7
Endothelial cells	901.0 ± 7.5	56.1 ± 3.0

Rats were injected with [^{3}H]ISIS-3082 at a dose of 1 mg/kg body weight. After 60 min, parenchymal, endothelial and Kupffer cells were isolated, and the association of radioactivity to each cell type was determined. Uptake by each cell type is expressed as ng of ISIS-3082 per mg of cell protein and as percent of the total liver uptake. These latter values were calculated from the uptake per mg of cell protein and the contribution of each cell type to the total liver protein (20). Values are means ± S.E.M. of three rats.

Effects of polyanions on tissue uptake of ISIS-3082

The distribution of ISIS-3082 over liver cell types strongly resembles that of acetylated low density lipoprotein, the classical ligand of the scavenger receptor type AI/AII. These receptors are highly expressed on liver endothelial cells, and they bind and internalize a wide variety of polyanionic ligands (20,23). Uptake by type AI/AII scavenger receptors can effectively be inhibited by the polyribonucleotide polyinosinic acid (poly-I), whereas poly-adenylic acid (poly-A), which has a different tertiary structure, is not effective (24). To study the possible role of type AI/AII scavenger receptors in the tissue uptake of ISIS-3082, rats were

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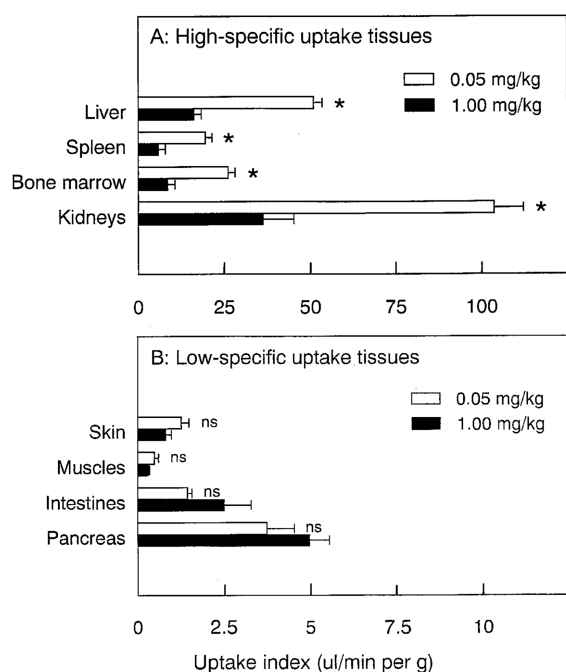

Figure 4. Effect of dose on the tissue uptake of [^{3}H]ISIS-3082. Rats were intravenously injected with [^{3}H]ISIS-3082 at a dose of 0.05 mg/kg body weight (□) or 1.00 mg/kg body weight (■). The association of radioactivity with the indicated tissues was determined at 45 min after injection (0.05 mg/kg dose) or 90 min after injection (1.0 mg/kg dose). Uptake indices (expressed as rate of the plasma clearance of ISIS-3082/g of tissue) were calculated as described earlier (18). (**A**) Rates of uptake by high-specific uptake tissues; (**B**) rates of uptake by low-specific uptake tissues. Note the difference in scales. Values are means ± S.E.M. of 3–4 rats. Differences were tested for significance by Wilcoxon's two-sample test (39). $^*P$ <0.05; ns, not significant.

preinjected with poly-I or poly-A shortly before injection of [^{3}H]ISIS-3082. Poly-I strongly reduced the rate of plasma clearance, whereas poly-A had no significant effect (Table 3). Poly-I and poly-A did not significantly affect the distribution volume of ISIS-3082 (101.9 ± 14.9 ml/kg and 59.7 ± 4.7 ml/kg, respectively, versus 70.0 ± 3.1 ml/kg for the solvent controls). Uptake by liver and bone marrow was significantly reduced by poly-I, but not by poly-A (Fig. 6). This finding suggests that uptake of ISIS-3082 by liver and bone marrow occurs largely by scavenger receptors. Interestingly, uptake by the kidneys was significantly reduced by poly-A, but not by poly-I, suggesting uptake by a scavenger receptor other than the liver receptor. Uptake by spleen, muscles, and skin was not significantly affected by either polyribonucleotide.

DISCUSSION

The plasma clearance of ISIS-3082 and the characteristics of its disposition (i.e. high uptake by liver and kidney) are in good agreement with earlier reported data obtained with other phosphorothioate oligodeoxynucleotides (7,8). We further obtained evidence that uptake of the oligonucleotide by liver, bone marrow, spleen, and kidneys is saturable, and might thus be receptor-mediated. However, the most important novel findings in our study are that endothelial cells are mainly responsible for the liver uptake of ISIS-3082, and that receptors of the 'scavenger type' are likely to be involved in the uptake.

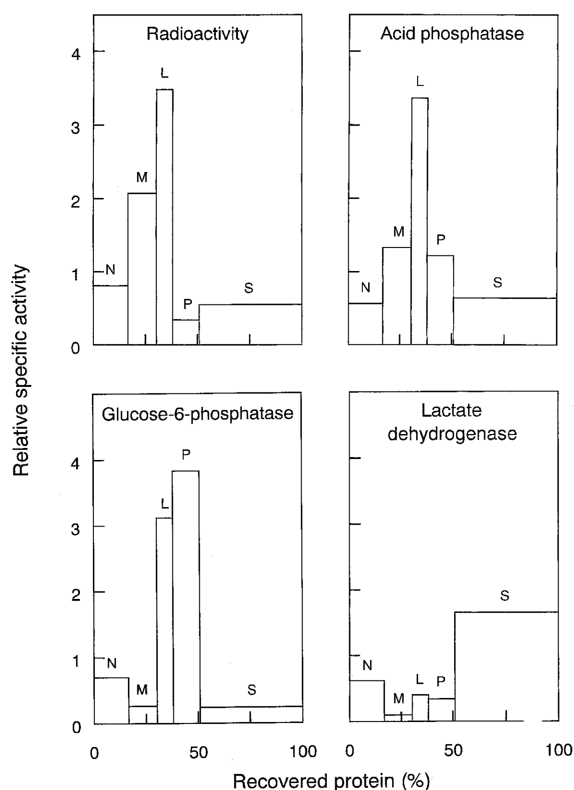

Figure 5. Distribution patterns of radioactivity and marker enzymes over subcellular fractions of the liver after injection of [^{3}H]ISIS-3082. Rats were intravenously injected with [^{3}H]ISIS-3082 at a dose of 1.0 mg/kg body weight. After 60 min, the liver was perfused with ice-cold 0.25 M sucrose + 10 mM Tris–HCl (pH 7.4) and divided into subcellular fractions by differential centrifugation. The fractions were assayed for radioactivity, protein and the activities of marker enzymes; recoveries were 70–92%. Blocks from left to right represent: nuclear (N), mitochondrial (M), lysosomal (L), microsomal (P) and supernatant (cytosol: S) fractions. The relative protein concentration is given on the abscissa. The ordinate represents the relative specific activity (percent of total recovered activity divided by percent of total recovered protein).

Table 3. Plasma clearance of intravenously injected [^{3}H]ISIS-3082; effects of polyanions

Preinjection	Half-life (min)
Saline	22.3 ± 2.5
poly-I	54.1 ± 14.5[a]
poly-A	25.2 ± 1.3[b]

Rats were intravenously injected with [^{3}H]ISIS-3082 at a dose of 1 mg/kg body weight. Shortly (1 min) prior to the injection of the labeled ligand, the animals received poly-I (10 mg/kg), poly-A (10 mg/kg), or an equal volume of solvent (phosphate-buffered saline; 2 ml/kg). The plasma clearance was followed for 90 min. Values are means ± S.E.M. of 3–4 rats. Differences with respect to the saline controls were tested for significance by Wilcoxon's two-sample test (39).
[a]P <0.05; [b]not significant.

The intrahepatic distribution of phosphorothioate oligodeoxynucleotides was recently also examined by Rifai *et al.,* who performed radioautography on liver sections obtained from mice injected with a 15mer radioiodinated phosphorothioate oligodeoxynucleotide (25). Light microscopy autoradiographs demonstrated

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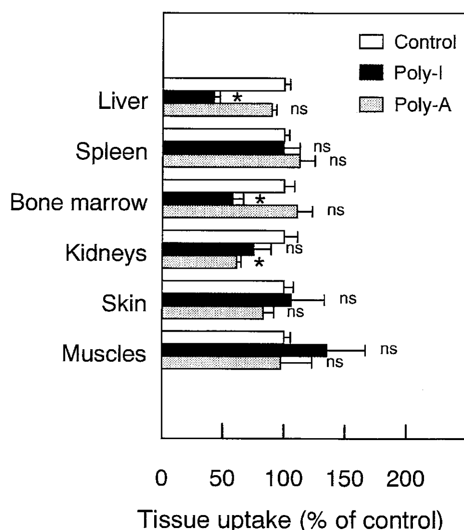

Figure 6. Tissue uptake of intravenously injected (^{3}H)ISIS-3082; effects of competing polyanions. Rats were intravenously injected with [^{3}H]ISIS-3082 at a dose of 1.0 mg/kg body weight. Shortly (1 min) prior to the injection of the labeled ligand, the animals were preinjected with poly-I (10 mg/kg) or poly-A (10 mg/kg). Control animals received an equal volume of solvent (phosphate-buffered saline; 2 ml/kg). The distribution of radioactivity over tissues was determined at 90 min after injection. The results are expressed as percent of uptake in control (phosphate-buffered saline-injected) animals. Control values for liver, bone marrow, spleen, kidneys, muscles and skin were 41.8 ± 1.9, 4.8 ± 0.4, 0.8 ± 0.0, 13.1 ± 1.4, 3.9 ± 0.2, and 9.4 ± 0.7% of the dose, respectively. Values are means ± S.E.M. of 3–4 rats. Differences with respect to the saline controls were tested for significance by Wilcoxon's two-sample test (39). $^*P < 0.05$; ns, not significant.

that the majority of the grains were associated with the sinusoidal lining. Based on electron microscope autoradiography, the authors suggested major uptake by the Kupffer cells. However, no data were given on endothelial cells, which in our present quantitiative study were found to be the major site of uptake.

Three main classes of scavenger receptors have been described: the mammalian class A and B receptors, and a class C receptor isolated from *Drosophila* (23,26–29). Class A receptors are the best characterized. Two isoforms, type AI and type AII, which display very similar binding characteristics, have been described (23). Unlike most cell-surface receptors, AI/AII scavenger receptors exhibit a broad ligand specificity. They bind a wide variety of polyanionic ligands including modified (lipo)proteins, polynucleotides, and polysaccharides. However, not all polyanionic ligands are recognized. Some polyribonucleotides, like poly-I, that form stable four-stranded helixes (quadruplexes), bind tightly to the receptors. Others, like poly-A, that do not form such structures, are poorly bound (24). The difference in affinities of poly-I and poly-A for AI/AII scavenger receptors was utilized in our study to examine the role of these receptors in the clearance of ISIS-3082. The liver uptake of ISIS-3082 was inhibited by poly-I, whereas poly-A was ineffective. This finding points to a prominent role of AI/AII scavenger receptors in the hepatic uptake of ISIS-3082. A further indication is that the distribution of ISIS-3082 over liver cell types strongly resembles that of acetylated low density lipoprotein, the classical ligand of AI/AII scavenger receptors (20,23). Subcellular fractionation of the liver indicates that ISIS-3082 does not merely associate to cells, but is internalized and delivered to the lysosomal compartment. This finding is also compatible with a role of type AI/AII receptors, as it has been shown that ligands of these receptors are internalized and transported to lysosomes (30). We conclude therefore that the dispostion of ISIS-3082 in the liver is largely due to internalization by AI/AII scavenger receptors on endothelial cells. Uptake of ISIS-3082 in the bone marrow follows a similar pattern as in liver, and occurs possibly also via AI/AII scavenger receptors. As uptake by the spleen is saturable, it is presumably also receptor-mediated. Splenic uptake was, however, not significantly affected by poly-I or poly-A, and is thus probably not mediated by scavenger receptors.

The kidneys also play an important role in the clearance of ISIS-3082. When the absolute organ uptake is compared, uptake by the kidneys is the second highest. The relative specific uptake by the kidneys (i.e. uptake per mass unit) was even the highest. Uptake of circulating ligands by the kidneys can occur in two possible ways. The ligand can be ultrafiltrated by the glomeruli and subsequently reabsorbed by the tubules, or it can be taken up directly at the capillary side. ISIS-3082 (MW 6300) is sufficiently small for filtration by the glomeruli. However, phosphorothioates are tightly bound to serum proteins, which will affect glomerular filtration (4,15,31). If ISIS-3082 is taken up by tubular reapsorbtion after glomerular filtration, inhibtion of renal uptake by poly-A would result in an increase in urinary excretion (the polyanions would only block reabsorbtion, but not filtration). As only small amounts of radioactivity (<1% of the dose) were recovered in the urine of animals treated with poly-A, uptake probably occurs predominantly at the capillary side. Sawai *et al.* recently reached a similar conclusion, based on kinetic studies with a 20mer phosphorothioate oligodeoxynucleotide in an isolated rat kidney perfusion system (32). The saturation of renal uptake of ISIS-3082 points to a receptor-mediated process, and the inhibition by poly-A suggest that scavenger receptors are implicated. However, because poly-I had no effect, the renal scavenger receptors are probably different from the type AI/AII receptors. Sawai *et al.* also performed competition experiments with polyanions, and suggested a role for scavenger receptors in the renal uptake of oligonucleotides (32). However, they used for these latter experiments oligonucleotides that contained predominantly (85%) phosphodiester linkages. Their findings are therefore not entirely comparable with the present data as it has been established that kidney uptake of phosphodiester oligonucleotides is very different from the uptake of the phosphororothioate analogues (32,33). The cellular localization of phosphorothioate oligodeoxynucleotides in kidneys was recently investigated by Oberbauer *et al.* (34). Their histological studies provided evidence for a major role of proximal tubular cells in uptake of phosphorothioate oligonucleotides. In addition, glomerular mesangial cells are possible candidates. It has been shown that scavenger receptors are expressed on cultured rat mesengial cells (35,36), and the glomerular mesangium is only separated from the bloodstream by a fenestrated and leaky endothelium (32).

We recovered in our study no significant amounts of oligonucleotide in urine. In other studies, excretion of intact phoshorothioate oligodeoxynucleotides into the urine was reported (7,37). However, the animals in these earlier studies received much higher (up to 50 mg/kg) amounts of the oligonucleotides. Due to saturation of protein-binding, a larger proportion of the phosphorothioate oligodeoxynucleotides is in free form under these conditions, which may lead to glomerular filtration and excretion into the urine.

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In conclusion, the present study indicates that scavenger receptors on rat liver endothelial cells play a predominant role in the plasma clearance of the phosphorothioate oligodeoxynucleotide ISIS-3082. Endothelial liver cells in other species, including man, also express functional scavenger receptors (23,38). The present findings are therefore probably highly relevant for phosphorothioate-based therapeutic strategies in humans. Uptake of antisense oligonucleotides by endothelial liver cells is only beneficial when the target gene is localized in this cell type. If a phosphorothioate oligodeoxynucleotide is designed to exert its action elsewhere, it is advantageous to develop delivery strategies that avoid uptake by endothelial cells and promote uptake by the actual target cell. Such strategies could be based on conjugating the oligonucleotides to carriers that are specifically taken up by the target cells.

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