Pharmacokinetics and Tissue Disposition in Monkeys of an Antisense Oligonucleotide Inhibitor of Ha-*Ras* Encapsulated in Stealth Liposomes

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Purpose. This study examined the pharmacokinetics and tissue distribution of an antisense oligonucleotide ISIS 2503, formulated in stealth (pegylated) liposomes (encapsulated) or in phosphate-buffered saline (unencapsulated).

Methods. Encapsulated or unencapsulated ISIS 2503 was administered to rhesus monkeys by intravenous infusion. The concentrations of ISIS 2503 and metabolites in blood, plasma, and tissue samples were determined by capillary gel electrophoresis.

Results. Plasma concentrations of encapsulated ISIS 2503 decreased mono-exponentially after infusion with a mean half-life of 57.8 hours. In contrast, the concentration of unencapsulated ISIS 2503 in plasma decreased rapidly with a mean half-life of 1.07 hours. Both encapsulated and unencapsulated ISIS 2503 distributed widely into tissues. Encapsulated ISIS 2503 distributed primarily to the reticulo-endothelial system and there were few metabolites observed. In contrast, unencapsulated ISIS 2503 distributed rapidly to tissue with highest concentration seen in kidney and liver. Nuclease-mediated metabolism was extensive for unencapsulated oligonucleotide in plasma and tissues.

Conclusions. The data suggest that stealth liposomes protect ISIS 2503 from nucleases in blood and tissues, slow tissue uptake, and slow the rate of clearance from the systemic circulation. These attributes may make these formulations attractive for delivering oligonucleotides to sites with increased vasculature permeability such as tumors or sites of inflammation.

KEY WORDS: antisense phosphorothioate oligonucleotide; stealth liposome; pharmacokinetics; monkey; capillary gel electrophoresis.

INTRODUCTION

ISIS 2503 is a phosphorothioate oligonucleotide consisting of 20 bases, complementary to a region near the Ha-*ras* AUG codon (1–2). This oligonucleotide specifically inhibits Ha-*ras* gene expression by an antisense mechanism, and shows sequence-specific anti-tumor activity (2–3). ISIS 2503 is in clinical trials for the treatment of various types of cancers. The pharmacokinetics and tissue distribution of phosphorothioate oligonucleotides have been characterized in rodents (4–6), primates (7), and humans (8–10). The pharmacokinetics are sequence-independent and they exhibit rapid plasma distribution half-life (36–83 min), and distribute broadly to tissues with the kidney and the liver showing the highest concentrations (5,11–13).

By formulating oligonucleotides in cationic liposomes, immunoliposomes, or pH-sensitive liposomes oligonucleotide delivery to some target organs can be increased (14–16). However, the pharmacokinetics and tissue disposition of phosphorothioate oligonucleotide formulated in stealth liposomes has not been reported in the literature. Compared with conventional liposomes, stealth liposomes are long-circulating, sterically stabilized liposomes, which are formulated with surface bound segments of methoxypolyethylene glycol (MPEG) (17). Stealth liposomes exhibit a relatively lower macrophage uptake that results in a longer circulating half-life. Because of their small size (approximately 100 nm diameter), stealth liposomes are able to extravasate from the abnormal leaky vasculature of tumors, sites of infection and inflammation, thus enhancing distribution to potential target tissues (17–21). The objective of this study was to compare the kinetics of encapsulated ISIS 2503 and a nonencapsulated formulation.

MATERIALS AND METHODS

Materials

Synthesis and Purity of ISIS 2503

The sequence of ISIS 2503 is TCC GTC ATC GCT CCT CAG GG. ISIS 2503 was synthesized via conventional phosphoramidite coupling chemistry (22) and purified by HPLC. The compound used in this study was found to be 96.12% full length. The remainder consisted of elongation failures typically one nucleotide shorter than ISIS 2503.

Encapsulated ISIS 2503 was formulated by Sequus Pharmaceuticals, Inc. (Menlo Park, CA) as 7 mg/ml ISIS 2503 with a nominal lipid concentration of 100 mg/ml. ISIS 2503 formulated in phosphate-buffered saline (2.0 mg/ml) was supplied by ISIS Pharmaceuticals, Inc. (Carlsbad, CA).

Liposome Encapsulation

ISIS 2503 was encapsulated using the thin-film hydration method. The liposomes contained three lipids: the hydrogenated soy phosphatidylcholine (HSPC), N- (carbamoyl-methoxypolyethylene glycol 2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine sodium salt (MPEG-DSPE), and cholesterol in a target molar ratio of 56.4:5.3:38.3. ISIS 2503 was dissolved in Tris EDTA/sodium chloride (TE/NaCl) buffer pH 7.4 at 10 mg ISIS 2503/ml buffer. Particle size of 100–110 nm was achieved by extrusion through polycarbonate membranes. Sephadex G200-120 resin with TE/NaCl buffer as eluent was used to remove the unencapsulated oligonucleotide. Entrapment efficiency of 25–30% was achieved, and ISIS 2503 concentration in the liposomes was determined by solvent extraction method using phenol:choloroform: isoamyl alcohol (25:24:1, V:V:V).

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Animal Studies

Liposome Formulation

Twelve rhesus monkeys (macaca mulatta) (six males and six females) were used in the liposome formulation group. These animals were in the age range of 3–7 years, with body weights ranging from 3–4 kg. Each animal received one intravenous infusion of ISIS 2503 encapsulated in stealth liposomes (10 mg/kg) over approximately 30 minutes. Blood samples for pharmacokinetic analysis were collected prior to dosing and at 0, 1, 2, 6, 12, 24, 40, 60, 96, 120, 144, 168, 192, 240, 384, 576 hours after dosing. Two animals (1 male and 1 female) were euthanized 24, 60, 120, 168, 384, or 576 hours after the end of infusion and brain, heart, pancreas, prostate, ovaries, spleen, intestine, kidney cortex, kidney medulla, liver, mesenteric and mandibular (combined, M & M) lymph nodes, axillary and inguinal (combined, A & I) lymph nodes, lung, back skin, and hand skin were collected from each animal.

Saline Formulation

In a separate study, six rhesus monkeys (three males and three females) received a 2-hour iv infusion of 10 mg/kg ISIS 2503 formulated in saline solution every-other-day for 28 days. A two-hour infusion was employed in this portion of the study to avoid high peak plasma concentrations and resultant complement activation and other toxicological sequelae associated with high peak plasma concentrations of phosphorothioate oligonucleotide (23). Blood samples were collected in EDTA tubes pre-dose, at 1 and 2 hrs during infusion, and at 1, 2, and 22 hrs after the end of the infusion of the first dose. Monkeys were sacrificed 48 hours after the last dose. The following tissues were collected from each animal: heart, pancreas, spleen, kidney cortex, kidney medulla, liver, axillary and inguinal lymph nodes, lung, colon, bone marrow, prostate, ovaries, and uterus.

Sample Extraction and Analysis

Plasma samples were extracted directly using a solid phase extraction method as described previously (24) with the following exceptions: the KCl concentration was decreased from 0.5 M to 0.25 M in the strong anion exchange running buffer, the reverse-phase solid phase extraction column used was the Glen Research Poly-Pack™ column (Glen Research, Sterling, VA) in place of the Isolute reverse-phase column, and purified oligonucleotide was eluted with 3 ml of freshly prepared 50% acetonitrile rather than 20% acetonitrile. Extraction of oligonucleotide from whole blood (100 μl) and tissue (approximately 100 mg) samples combined the proteinase K digestion method (12) with solid phase extraction method (24). For tissues the method was similar except that monkey tissues were weighed, homogenized in a Bio Savant (Bio 101, Inc., Vista, CA) and then the material was extracted as described (24). Extracted samples were analyzed by capillary gel electrophoresis (CGE) using a Beckman P/ACE Model 5010 capillary electrophoresis instrument (Beckman Instruments, Irvine, CA) with UV detection at 260 nm (24).

Quantitation of intact ISIS 2503 and metabolites for whole blood samples was based on the calibration curve with T_{27} (a 27-mer phosphorothioate oligodeoxythymidine) as the internal standard. The limit of quantitation for this assay has been estimated to be 0.10 μg/ml ISIS 2503 in blood. In contrast, the concentrations of ISIS 2503 and metabolites in the plasma and tissue samples were calculated from the ratio of the absorbances, based on the starting concentration of internal standard (T_{27}) as previously described (24). The limit of quantitation for this assay has been estimated to be 0.07 μg/ml in plasma and 0.35 μg/g in tissue. Both parent compound (ISIS 2503) and total oligonucleotide (sum of ISIS 2503 and all detected metabolites) concentrations are presented in the results section because many of the chain-shortened metabolites retain similar physical-chemical properties and hence toxicologic activities. The method for quantitating ISIS 2503 concentrations in blood or tissues does not distinguish between free and liposome encapsulated oligonucleotides.

Plasma Protein Binding

The protein binding of ISIS 2503 in monkey plasma was determined using ultrafiltration. (^{32}P) labeled ISIS 2503 was added to 2 ml of monkey plasma at concentrations of 1, 2, 4, 6, 8, and 10 μM. After incubation for 30 minutes at 37°C, a 300-μl aliquot of the plasma drug mixture was placed into a disposable Ultrafree-MC filter (Millipore, Bedford, MA) and was centrifuged at 2000 g for 20 minutes. Radioactivity in aliquots of both ultrafiltrate and the pre-filtered plasma were determined by liquid scintillation counting (Beckman LS 6500 Fullerton, CA). The bound fraction (F_b) was calculated according to the following formula:

$$F_b = 100 - (F/T)*100$$

where, F and T are the concentration of radioactivity in the ultrafiltrate and in the plasma, respectively.

Distribution of ISIS 2503 (Encapsulated and Unencapsulated) in Whole Blood

Encapsulated or unencapsulated ISIS 2503 (1 μM) were added to triplicate aliquots of 1 ml of monkey blood (1ml). After centrifugation at 1500 rpm (2000 g) for 10 minutes, the upper plasma layer was separated from red blood cell (RBC) layer. From each layer 100 μl was extracted and analyzed using the CGE methods described above. T_{27} (5 μM) was added to each sample prior to sample extraction as internal standard.

Pharmacokinetic Analysis

Liposome Formulation

There was no binding or distribution of ISIS 2503 (either encapsulated and unencapsulated) on or in the RBCs. Therefore, concentrations of ISIS 2503 in plasma were calculated from concentrations in blood by correcting for hematocrit values (assumed to be 0.41 ref. 25).

Inspection of the semi-logarithmic plots of intact ISIS 2503 (full-length) plasma concentration-versus time curves indicated that they could be described by a monoexponential equation. First order elimination was assumed. Initial estimates of parameters were obtained by linear regression of the terminal concentration time points. Nonlinear regression was accomplished using a one compartment model for each individual animal

(WinNonlin 1.0). C_{max} was determined by visual inspection and the data summarized using descriptive statistics.

Four animals were excluded from complete individual pharmacokinetic analysis of plasma concentrations because they were sacrificed before a complete plasma profile could be collected (2 at 24 hr and 2 at 60 hr).

Tissue disposition kinetics was analyzed by noncompartmental methods using WinNonlin 1.0. Tissue half-lives were estimated by linear regression analysis of the log-linear terminal phase of the tissue concentration-time curve. The area under the tissue concentration-time curve ($AUC_{0\to\infty}$) and the area under the first moment of the concentration-time curve ($AUMC_{0\to\infty}$) were calculated using the linear trapezoidal rule, up to the last measured time point, plus the extrapolated area. The mean residence time (MRT) was calculated as the ratio of the $AUMC_{(0\to\infty)}$ to the $AUC_{(0\to\infty)}$. C_{max} and T_{max} were determined by visual inspection and the data summarized using descriptive statistics.

Saline (Unencapsulated) Formulation

The plasma pharmacokinetics of ISIS 2503 formulated in the saline solution after single dose was analyzed by noncompartmental methods using WinNonlin 1.0. AUC and AUMC were calculated using the linear trapezoidal rule with extrapolation to time-infinity. Plasma half-life for ISIS 2503 was calculated from the slope of the log-linear terminal phase of the plasma concentration-time curve. AUC and AUMC were used to estimate plasma clearance (CL = dose/AUC), and volume of distribution at steady state (V_{ss} = dose*AUMC/AUC^2). C_{max} was determined by visual inspection and the data summarized using descriptive statistics.

Statistics

Statistical analysis for gender difference of kinetic parameters was performed by F-test (Excel 6.0) for the analysis of variance, and t-test (Excel 6.0) for the analysis of mean at the $p = 0.05$ level.

RESULTS

Plasma Protein Binding

ISIS 2503 is highly bound to monkey plasma proteins (Table 1). Percentage bound of (^{32}P)-ISIS 2503 ranged 96.26% to 95.90% over a concentration range of 1 to 10 μM. Because protein binding was almost constant at the studied concentration range, %bound was averaged and was found to be 96.14 ± 0.13%.

Table 1. Plasma Protein Binding in Monkey Plasma

ISIS 2503 concentrations (μM)	F_b
1	96.13
2	96.22
4	96.26
6	96.20
8	96.15
10	95.90
Mean ± SD	96.14 ± 0.13

Distribution of ISIS 2503 (Encapsulated and Unencapsulated) in Whole Blood

There were no detectable concentrations of ISIS 2503 in the RBC layer. ISIS 2503 was recovered 100% in the plasma portion for both encapsulated and unencapsulated formulation.

Plasma Pharmacokinetics and Metabolism

The clearance of ISIS 2503 and oligonucleotide metabolites from plasma is greatly prolonged for the liposome formulation compared with the saline formulation (Table 2, Fig. 1). C_{max} of intact ISIS 2503 in the liposome formulation group was approximately 172 μg/ml and was generally observed at the end of the 30-minute infusion. Concentrations of encapsulated ISIS 2503 in plasma decreased slowly to approximately 18 μg/ml at 144 hours after infusion. In contrast, after administration of unencapsulated ISIS 2503, C_{max} of intact ISIS 2503 was approximately 65 μg/ml and was observed at the end of the 2-hr infusion.

Unencapsulated ISIS 2503 concentration in plasma declined four-fold 2 hrs after infusion, and was below the limit of detection at 22 hrs after infusion (<0.07 μg/ml).

No statistical significance was seen between genders. The mean half-life in plasma for the encapsulated ISIS 2503 was 57.8 ± 13.1 hours, whereas the mean half-life in plasma for saline formulated ISIS 2503 was only 1.07 ± 0.19 hours (Table 3).

Table 2. Concentrations (μg/ml) of ISIS 2503 in Plasma After the End of 0.5 hr (for the Liposome Formulation) and 2 hr (for the Saline Formulation) Intravenous Infusion of 10 mg/kg ISIS 2503 to Rhesus Monkeys

	Liposome formulation		Saline formulation	
Time (hr)	ISIS 2503 (μg/ml) (mean ± SD)	Full length % intact[a]	ISIS 2503 (μg/ml) (mean ± SD)	Full length % intact
0	151 ± 41.8	95.6	65.0 ± 12.5	61.0
1	153 ± 32.0	96.1	30.2 ± 5.7	55.3
2	139 ± 37.3	96.1	17.4 ± 4.3	55.0
6	133 ± 34.6	96.0	NA	NA
12	109 ± 31.5	97.8	NA	NA
22	NA[c]	NA	nd[b]	nd
24	107 ± 30.8	96.7	NA	NA
40	84.8 ± 25.2	96.8	NA	NA
60	85.8 ± 15.9	92.2	NA	NA
96	38.3 ± 21.8	91.9	NA	NA
120	26.6 ± 17.3	89.2	NA	NA
144	17.9 ± 10.7	94.2	NA	NA
168	10.5 ± 6.55	95.1	NA	NA
192	6.14	84.5	NA	NA
240	3.61	64.2	NA	NA
384	nd	nd	NA	NA
576	nd	nd	NA	NA

[a] % Intact = percent of total detectable oligonucleotide represented by intact ISIS 2503.
[b] nd = not detected.
[c] NA = not applicable (samples were not collected).

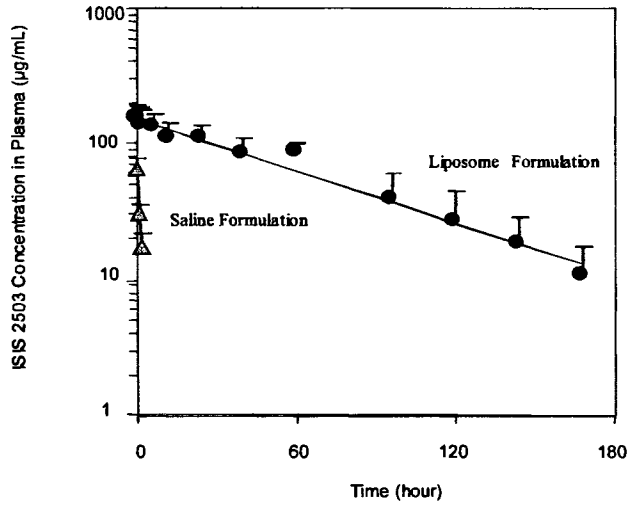

Fig. 1. Concentration time profile of intact ISIS 2503 in plasma after Intravenous Infusion of 10 mg/kg ISIS 2503 to rhesus monkeys. Time point 0 hr refers to the end of infusion. Closed circles represent observed plasma concentrations from the liposome formulation group. Open triangles represent plasma concentrations from the saline formulation group. Plasma samples were collected at various time points after the infusion and assayed to determine the concentration of ISIS 2503. Each point is represented as the mean (symbol) ± standard deviation (n = 4–8). Nonlinear regression was performed (solid line).

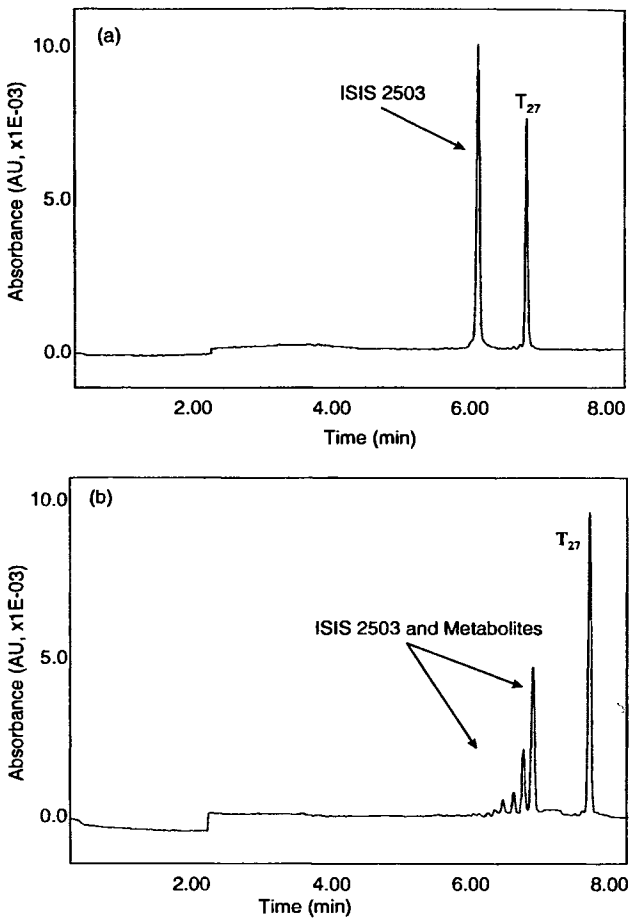

Fig. 2. Electropherograms of representative extracts. (a) A whole blood sample from a monkey at 60 hrs after 0.5 hr iv infusion of 10 mg/kg ISIS 2503 formulated in stealth liposomes, and (b) a plasma sample from a monkey at 1 hr after initiation of a 2 hr infusion of 10 mg/kg of ISIS 2503 formulated in saline solution.

The average plasma clearance and Vd_{ss} for the liposome formulation were 0.892 ± 0.161 ml/hr/kg and 73.0 ± 15.3 ml/kg, respectively. The average plasma clearance and Vd_{ss} for the saline formulated ISIS 2503 were 54.0 ± 12.0 ml/hr/kg and 85.0 ± 17.0 ml/kg, respectively. Plasma clearance after administration of the saline formulated ISIS 2503 was over 50 times faster than after administration of the liposome formulation, indicating that ISIS 2503 in the liposome formulation was distributed and metabolized at a much slower rate compared with the saline formulation.

For the liposome formulation group, only a few species of chain-shortened metabolites were found at any time point (Table 2, Fig. 2a). In contrast, metabolites were more abundant in the saline formulation group, where the chain-shortened metabolites represented 35 to 45% of total oligonucleotides in plasma (Table 2) and there were more species of metabolites (compare Fig. 2a with 2b). These metabolites which resulted from progressive shortening of ISIS 2503 by nucleases were observed within 1 hr.

Table 3. Summary of Estimated Pharmacokinetic Parameters in Plasma for ISIS 2503 (10 mg/kg) Encapsulated in Stealth Liposome Administered to Rhesus Monkeys by 0.5 hr Infusion (n = 8), and in Saline Formulation Administered to Rhesus Monkeys by 2 hr Infusion (n = 6)

Parameter	Liposome formulation (mean ± SD)	Saline formulation (mean ± SD)
AUC (µg*hr/ml)	$1.15 \times 10^4 \pm 2.07 \times 10^3$	156 ± 30
$t_{1/2}$ (hr)	57.8 ± 13.1	1.07 ± 0.19
C_{max} (µg/ml)	172[a] ± 33	65.0 ± 12.5
Cl (ml/hr/kg)	0.892 ± 0.161	54.0 ± 12.0
Vd_{ss} (ml/kg)	73.0 ± 15.3	85.0 ± 17.0

[a] Data obtained from 12 animals.

Tissue Distribution, Elimination Kinetics, and Metabolism

ISIS 2503 was distributed widely into tissues after administration of the liposome formulation. The highest tissue concentrations of total oligonucleotide were measured in liver, with slightly lower concentrations detected in spleen, followed by the lymph nodes, lung, kidney cortex and medulla, pancreas, heart, back skin, and colon (Fig. 3). It appears that the reticuloendothelial system played an important role in the distribution of encapsulated ISIS 2503. Tissue distribution of encapsulated ISIS 2503 differed from unencapsulated ISIS 2503 in the saline formulations (Fig. 3) and other unencapsulated oligonucleotides studied previously (5,11–13), where the highest concentration of oligonucleotide is consistently observed in kidney cortex. Moreover, oligonucleotide concentration in kidney cortex was significantly higher than the concentration in kidney medulla for unencapsulated ISIS 2503, and this concentration difference between cortex and medulla was not observed for encapsulated ISIS 2503. Although the tissue distribution for unencapsulated

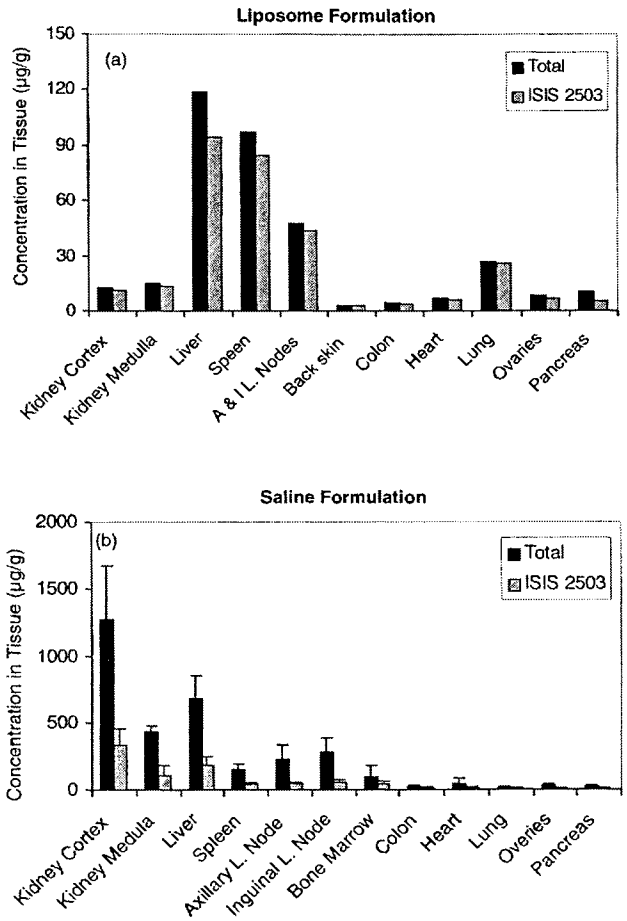

Fig. 3. Tissue distribution of ISIS 2503 and total oligonucleotide in monkey tissues. (a) At 60 hrs following single intravenous infusion of 10 mg/kg ISIS 2503 formulated in stealth liposomes, and (b) at 48 hrs from the last dose following multiple intravenous infusion of 10 mg/kg ISIS 2503 formulated in saline solution administered every-other-day. A & I L. Nodes, Axillary and inguinal lymph nodes.

ISIS 2503 was obtained from monkeys that received multiple doses, the overall pattern of oligonucleotide distribution to tissues should not be altered (7).

Relatively long half-lives of ISIS 2503 were observed in all tissues studied (Table 4) following administration of ISIS 2503 encapsulated in stealth liposomes. The mean residence time (15 days) of ISIS 2503 in the kidney cortex was the longest among all the tissues examined. Uptake was slow in all tissues with time to peak concentration from 1–7 days (Table 4). The concentration of ISIS 2503 in brain, prostate, and ovaries was still increasing up to seven days post-dosing. However, the concentration of ISIS 2503 in these tissues was below the limit of quantitation for the CGE analysis by 384 hours (the next data point after the 7-day time point).

For the liposome formulation group, the percentage of metabolites remained low even 576 hours after infusion (data not shown). Only 10 to 20% of the total oligonucleotide was in the form of metabolites in most organs. In liver, kidney cortex, and pancreas, 30 to 60% of the total detected oligonucleotide were metabolites. Significant amounts of oligonucleotide metabolites were observed as early as 24 hours after infusion in the pancreas. At later time points (≥120 hr), increasing

Table 4. Estimated Tissue Pharmacokinetic Parameters for ISIS 2503 (10 mg/kg) Encapsulated in Stealth Liposomes Administered to Rhesus Monkeys by 0.5 hr Intravenous Infusion

Tissue	$t_{1/2}$ (day)	MRT (day)	T_{max} (day)	C_{max} (μg/g)
Kidney cortex	11	15	1	13.4
Kidney medulla	5.6	8.2	1	14.3
Liver	4.2	8.1	7	106
A & I lymph node[b]	NA	18	5	68.2
M & M lymph node[c]	7.7	13	5	43.8
Spleen	9.7	14	5	94.0
Back skin	3.1	7.0	7	11.1
Hand skin	4.2	10	2	25.7
Lung	2.0	3.4	2	26.1
Heart	3.0	5.7	1	10.3
Pancreas	3.2	6.4	2	5.53
Brain	NA	NA	1	2.27
Colon	NA	NA	7[a]	6.88
Ovary	NA	NA	2	6.93
Prostate	NA	NA	7[a]	6.89

Note: NA = not available.
[a] Concentration was still increasing at the last analyzed time point.
[b] Axillary and inguinal lymph node.
[c] Mesenteric and mandibular lymph node.

concentrations of chain-shortened oligonucleotide metabolites were seen in liver and kidney. In comparison, a much higher percentage of metabolites (~62–84%) was observed in the tissues of the saline formulation group at 48 hrs from the last dose, as illustrated in Fig. 4a and 4b.

In addition to chain-shortened metabolites, there was a peak (n + 1) that migrated more slowly than parent oligonucleotide in the extracts of tissues primarily from the saline formulation group (Fig. 4b). Slower migrating oligonucleotide peaks have been identified for other phosphorothioate oligonucleotides in tissue. Slower migration suggests that the mass to charge ratio was increased either from the addition of a substituent or loss of charge. For another phosphorothioate oligonucleotide, these metabolites were identified by mass spectral analysis to be intact oligonucleotide plus an additional nucleotide or two (25).

DISCUSSION AND CONCLUSIONS

In vitro studies with ISIS 2503 demonstrated that this compound is highly bound to plasma proteins and not cellular elements of blood. This observation was in agreement with data reported for other phosphorothioate oligonucleotides of different targets and sequences (4,5,26–27).

The half-life of encapsulated ISIS 2503 (57.8 hours) is significantly greater than that observed for unencapsulated oligonucleotides (1.07 hours) suggesting that ISIS 2503 in liposomes is cleared more slowly from circulation than unencapsulated ISIS 2503. The apparent volume of distribution at steady state (Vd_{ss}) for both formulations was significantly higher than the plasma volume in monkeys, 45 ml/kg (28), suggesting extravascular distribution. The apparent distribution volume at steady state for encapsulated and unencapsulated based on unbound ISIS 2503 (Vd_{ss}/fu) was 1.9 L/kg and 2.2 L/kg respectively, indicating extensive tissue distribution for

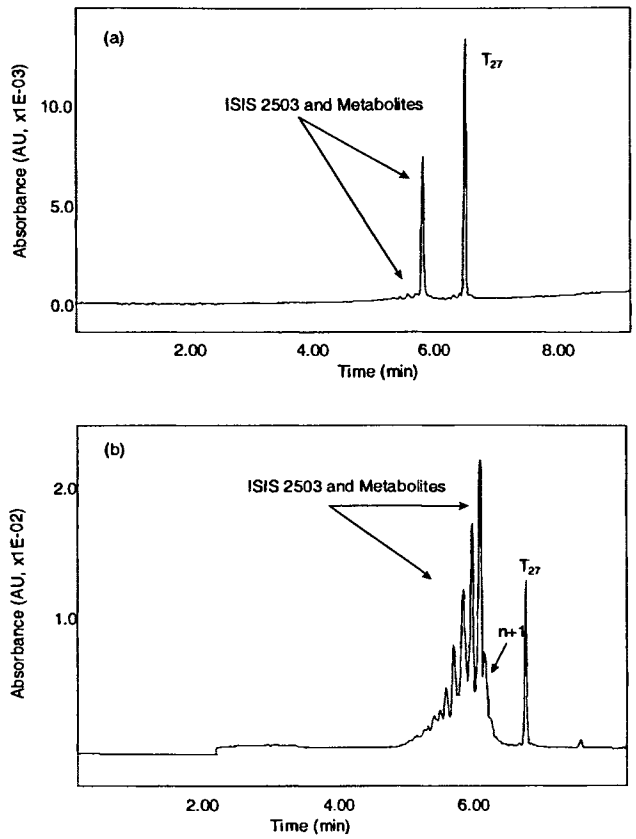

Fig. 4. Electropherograms of representative extracts. (a) A kidney cortex sample from a monkey at 60 hrs after 0.5 hr iv infusion of 10 mg/kg ISIS 2503 formulated in stealth liposomes, and (b) a kidney cortex sample from a monkey at 48 hrs after the last 2-hr infusion of 10 mg/kg of ISIS 2503 formulated in saline solution (total doses received, 14 doses administered every-other-day).

the unbound ISIS 2503. Because the major clearance routes for oligodeoxynucleotides from the general circulation are distribution to tissues, and to a lesser extent, metabolism, these data suggest that encapsulation reduces metabolism but more importantly, slows the tissue uptake dramatically.

Much less metabolism was observed in the circulation and tissues in the liposome formulation group compared with the saline formulation group. Phosphorothioate oligonucleotides are metabolized by exonuclease cleavage of terminal nucleotides resulting in a shortened oligonucleotide and the release of a mononucleotide. Nucleotides can be cleaved from either the 3′ or the 5′ ends of the molecule in tissues, although 3′ is more common (29). Capillary gel electrophoresis separates oligonucleotides according to length but does not provide unique identification. (The metabolite formed from a single nucleotide deletion from the 5′ end of ISIS 2503 will migrate the same as the metabolite formed from a single nucleotide deletion from the 3′ end.) The low concentrations of metabolites in blood and tissues from the liposome formulation group suggests that liposomal encapsulation protects the oligonucleotide from blood (and tissue) nucleases. This finding supports the concept that there was very little leakage of ISIS 2503 from the stealth liposomes.

In the liposome formulation group, the greater abundance of metabolites observed in kidney, liver, and pancreas compared to other organs could be explained by digestion of the liposomes in these tissues. For the pancreas, it is tempting to speculate that this phenomenon could be related to the activity of lipases in this organ allowing more ISIS 2503 to escape from liposomes and be metabolized. This phenomenon may also occur in liver and kidney. Alternatively, kidney and/or liver may be primary sites where oligonucleotide released from liposomes elsewhere in the body is accumulated and metabolized. This behavior would be consistent with the distribution of unencapsulated ISIS 2503.

Although ISIS 2503 is highly bound to plasma proteins, ISIS 2503 is extensively distributed to tissues after administration of both formulations. This observation indicated that the binding affinity of ISIS 2503 to tissues is much higher than the binding to plasma proteins. Encapsulated ISIS 2503 distributed primarily into organs of the reticulo-endothelial system (RES), and oligonucleotide distribution to the kidneys is markedly reduced compared to unencapsulated oligonucleotide. In the saline formulation group, ISIS 2503 was preferably taken up by the kidney cortex, and to a lesser extent, by the liver. Moreover, ISIS 2503 concentrations in the kidney cortex were much higher than the concentrations in kidney medulla. This observation is consistent with reported results, which show that phosphorothioate oligonucleotides are preferentially taken up in renal proximal tubule epithelial cells, with little to no uptake in glomeruli and medullary cells (30). However, this concentration difference in kidney cortex and medulla was not observed in the liposome formulation group of this study. The size difference between encapsulated versus unencapsulated formulation may account for the pattern of ISIS 2503 tissue distribution.

Encapsulated ISIS 2503 has a much longer tissue half-life, approximately 2–11 days (Table 4). On the other hand, unencapsulated oligonucleotide had an average reported tissue half-life of 21–44 hours in mice (4). Intact ISIS 2503 is the predominant oligonucleotide species measured in the liposome formulation group, indicating slow metabolism in tissues and supporting the concept that liposomes remain intact in tissues which is consistent with earlier studies. Clearance of oligonucleotide from organs is likely dominated by metabolism. The persistence and abundance of intact ISIS 2503 in tissues is best explained by the protection from nucleases afforded by liposomal encapsulation.

This study shows stealth liposome encapsulation changes the pharmacokinetics and tissue distribution of ISIS 2503 by prolonging circulation time reducing metabolism, shifting primary distribution to RES, prolonging tissue residence and inhibiting metabolism. As a consequence, these data will enable us to design alternative dosage forms to prolong the circulation time of oligonucleotide. Owing to long circulation time and small size, stealth liposome encapsulated doxorubicin has been shown to extravasate through the leaky vasculature of tumors, and enhance the drug level in tumors compared with unencapsulated doxorubicin. ISIS 2503 is intended for use in oncology and stealth liposome encapsulation may allow for more effective delivery of oligonucleotide to tumor sites with less frequent dosing. The enhancement of the circulation time, and delivery to tumor vasculature with stealth liposome formulation is a promising approach for delivery of oligonucleotides.

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REFERENCES

1. B. P. Monia, J. F. Johnston, D. J. Ecker, M. Zounes, W. F. Lima, and S. M. Freier. Selective inhibition of mutant Ha-ras mRNA expression by antisense oligonucleotides. *J. Biol. Chem.* 19954–19962 (1992).
2. C. F. Bennett, N. Dean, D. J. Ecker, and B. P. Monia. Pharmacology of antisense therapeutic agents. In S. Agrawal (ed.), *Methods in Molecular Medicine: Antisense Therapeutics*, Humana Press Inc., Totowa, NJ, 1996, pp. 13–46.
3. L. M. Cowsert. In vitro and in vivo activity of antisense inhibitors of ras: potential for clinical development. *Anti-Cancer Drug Des.* **12**:359–371 (1997).
4. P. A. Cossum, L. Troung, S. R. Owens, P. M. Markham, J. P. Shea, and S. T. Crooke. Pharmacokinetics of a 14C-labeled phosphorothioate oligonucleotide, ISIS 2105, after intradermal administration to rats. *J. Pharmacol. Exp. Ther.* **1**:89–94 (1994).
5. P. A. Cossum, H. Sasmor, D. Dellinger, L. Troung, L. Cummins, S. R. Owens, P. M. Markham, J. P. Shea, and S. Crooke. Disposition of the 14C-labeled phosphorothioate oligonucleotide ISIS 2105 after intravenous administration to rats. *J. Pharmacol. Exp. Ther.* **3**:1181–1190 (1993).
6. R. S. Geary, J. M. Leeds, J. Fitchett, T. Burckin, L. Troung, C. Spainhour, M. Creek, and A. A. Levin. Pharmacokinetics and metabolism in mice of a phosphorothioate oligonucleotide antisense inhibitor of C-raf-1 kinase expression. *Drug Metab. Dispos.* **25**:1272–1281 (1997).
7. S. Agrawal, J. Temsamani, W. Galbraith and J. Tang. Pharmacokinetics of antisense oligonucleotides. *Clin. Pharmacokinet.* **28**:7–16 (1995).
8. R. Zhang, J. Yan, H. Shahinian, G. Amin, Z. Lu, T. Liu, M. S. Saag, Z. Jiang, J. Temsamani, R. Martin, P. J. Schechter, S. Agrawal, and R. B. Diasio. Pharmacokinetics of an anti-human immunodeficiency virus antisense oligodeoxynucleotide phosphorothioate (GEM 91) in HIV-infected subjects. *Clin. Pharmacol. Ther.* **58**:45–53 (1995).
9. E. Bayever, P. L. Iversen, M. R. Bishop, J. G. Sharp, H. K. Tewary, M. A. Arneson, S. J. Pirruccello, R. W. Ruddon, A. Kessinger, G. Zon, and J. O. Armitage. Systemic administration of a phosphorothioate oligonucleotide with a sequence complementary to p53 for Acute Myelogenous Leukemia and Myelodysplastic syndrome: initial results of a phase 1 trial. *Antisense Res. Dev.* **3**:383–390 (1993).
10. J. M. Glover, J. M. Leeds, T. G. Mant, D. Amin, D. L. Kisner, J. E. Zuckerman, R. S. Geary, A. A. Levin, and W. R. Shanahan. Phase 1 safety and pharmacokinetic profile of an ICAM-1 antisense oligodeoxynucleotide (ISIS 2302). *J. Pharmacol. Exp. Ther.* **282**:1173–1180 (1997).
11. R. S. Geary, J. M. Leeds, S. P. Henry, D. K. Monteith, and A. A. Levin. Antisense oligonucleotide inhibitors for treatment of cancer: 1. Pharmacokinetic properties of phosphorothioate oligodeoxynucleotides. *Anti-Cancer Drug Des.* **12**:383–393 (1997).
12. S. Agrawal, J. Temsamani, and J. Y. Tang. Pharmacokinetics, biodistribution, and stability of oligodeoxynucleotide phosphorothioates in mice. *Proc. Natl. Acad. Sci. U. S. A.* **88**:7595–7599 (1991).
13. L. Iversen, J. Mata, W. G. Tracewell, and G. Zon. Pharmacokinetics of an antisense phosphorothioate oligodeoxynucleotide against rev from human immunodeficiency virus type 1 in the adult male rat following single injections and continuous infusion. *Antisense Res. Dev.* **4**:43–52 (1994).
14. D. C. Litzinger, J. M. Brown, I. Wala, S. A. Kaufman, G. Y. Van, C. L. Farrell, and D. Collins. Fate of cationic liposomes and their complex with oligonucleotide in vivo. *Biochim. Biophys. Acta.* **1281**:139–149 (1996).
15. D. D. Ma and A. Q. Wei. Enhanced delivery of synthetic oligonucleotides to human leukaemic cells by liposomes and immunoliposomes. *Leuk. Res.* **20**:925–930 (1996).
16. O. Zelphati, J. L. Imbach, N. Signoret, G. Zon, B. Rayner, and L. Leserman. Antisense oligonucleotides in solution or encapsulated in immunoliposomes inhibit replication of HIV-1 by several different mechanisms. *Nucleic Acids Res.* **22**:4307–4314 (1994).
17. F. Yuan, M. Leunig, S. K. Huang, D. A. Berk, D. Papahadjopoulos, and R. K. Jain. Microvascular permeability and interstitial penetration of sterically stabilized (stealth) liposomes in a human tumor xenograft. *Cancer Res.* **54**:3352–6 (1994).
18. P. K. Working and A. D. Dayan. CAELYX: Pharmacological-toxicological expert report. *Hum. Exp. Toxicol.* **15**:751–85 (1996).
19. A. Gabizon and F. Martin. Polyethylene glycol-coated (pegylated) liposomal doxorubicin. Rationale for use in solid tumors. *Drugs* **54** Suppl. 4:15–21 (1997).
20. M. Grunaug, J. R. Bogner, O. Loch, and F. D. Goebel. Liposomal doxorubicin in pulmonary Kaposi's sarcoma: improved survival as compared to patients without liposomal doxorubicin. *Eur. J. Med. Res.* **3**:13–9 (1998).
21. M. S. Newman, G. T. Colbern, P. K. Working, C. Engbers, and M. A. Amantea. Comparative pharmacokinetics, tissue distribution, and therapeutic effectiveness of cisplatin encapsulated in long-circulating, pegylated liposomes (SPI-077) in tumor bearing mice. *Cancer Chemther. Pharmacol.* **43**:1–7 (1999).
22. L. Beaucage and R. P. Iyer. Advances in the synthesis of oligonucleotides by the phosphoramidite approach. *Tetrahedron.* **48**:2223–2311 (1992).
23. S. P. Henry, P. C. Giclas, J. M. Leeds, M. Pangburn, C. Auletta, A. A. Levin, and D. J. Kornbrust. Activation of the alternative pathway of complement by a phosphorothioate oligonucleotide: potential mechanism of action. *J. Pharmacol. Exp. Ther.* **281**:810–816 (1997).
24. J. M. Leeds, M. J. Graham, L. Truong and L. L. Cummins. Quantitation of phosphorothioate oligonucleotides in human plasma. *Anal. Biochem.* **235**:36–43 (1996).
25. R. H. Griffey, M. J. Greig, H. J. Gaus, K. Liu, D. K. Monteith, M. Winniman, and L. L. Cummins. Characterization of oligonucleotide metabolism in vivo via liquid chromatography/electrospray tandem mass spectrometry with a quadrupole ion trap mass spectrometer. *J. Mass. Spectrom.* **32**:305–313 (1997).
26. J. C. Bigelow, L. R. Chirin, L. A. Mathews, and J. J. McCormack. High-performance liquid chromatographic analysis of phosphorothioate analogues of oligodeoxynucleotides in biological fluids. *J. Chromatogr. Biomed. Appl.* 133–140 (1990).
27. S. Agrawal, X. Zhang, Q. Cai, E. R. Kandimalla, A. Manning, Z. Jiang, T. Marcel, and R. Zhang. Effect of asprin on protein binding and tissue disposition of oligonucleotide phosphorothioate in rats. *J. Drug Target.* **5**:303–312 (1998).
28. B. Davies and T. Morris. Physiological Parameters in laboratory animals and humans. *Pharm. Res.* **10**:1093–1095 (1993).
29. H. J. Gaus, S. R. Owens, M. Winniman, S. Cooper, and L. L. Cummins. On-line HPLC electrospray mass spectrometry of phosphorothioate oligonucleotide metabolites. *Anal. Chem.* **69**:313–319 (1997).
30. J. Rappaport, B. Hanss, J. B. Kopp, T. D. Copeland, L. A. Bruggeman, T M. Coffman, and P. E. Klotman. Transport of phosphorothioate oligonucleotide in kidney: Implications for molecular therapy. *Kidney Int.* **47**:1462–1469 (1995).