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IN VIVO STABILITY, DISPOSITION AND METABOLISM OF A "HYBRID" OLIGONUCLEOTIDE PHOSPHOROTHIOATE IN RATS

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Abstract—Oligodeoxynucleotide phosphorothioates containing segments of 2′-*O*-methyloligoribonucleotide phosphorothioates at both 3′- and 5′-ends (hybrid oligonucleotide) have been shown to be potent antisense agents. In the present study, *in vivo* biostability, disposition, and excretion of a 25-mer hybrid oligonucleotide were determined in rats after i.v. bolus administration of the ^{35}S-labeled oligonucleotide at a dose of 30 mg/kg. The plasma disappearance curve for the hybrid oligonucleotide could be described by a two-compartmental model, with half-lives of 0.34 and 52.02 hr, respectively. The majority of the radioactivity in plasma was associated with the intact hybrid oligonucleotide. Urinary excretion represented the major pathway of elimination, with 21.98 ± 3.21% (mean ± SD) of the administered dose excreted within 24 hr and 38.13 ± 2.99% over 240 hr post-dosing. The majority of the radioactivity in urine was associated with the degradative products with lower molecular weights, but the intact form was also detected by HPLC analysis. Fecal excretion was a minor pathway of elimination with 2.34 ± 0.13% of the administered dose excreted over 24 hr and 6.74 ± 0.40% over 240 hr post-dosing. A wide tissue distribution of hybrid oligonucleotide was observed based on radioactivity levels, and analysis by HPLC showed that the majority of the radioactivity in tissues was associated with the intact hybrid oligonucleotide. Further analyses of the experimental data provided a comprehensive pharmacokinetic analysis of hybrid oligonucleotide in each tissue. Compared with a previously examined oligodeoxynucleotide phosphorothioate (GEM 91) that has a similar nucleotide sequence, the hybrid oligonucleotide had a shorter distribution half-life and a longer elimination half-life, based on the quantitation of radioactivity in plasma. Although it had a similar tissue distribution pattern compared with other oligonucleotide phosphorothioates such as GEM 91, the hybrid oligonucleotide was more stable *in vivo*, which may be important in the development of antisense oligonucleotides as therapeutic agents.

Key words: antisense oligodeoxynucleotide phosphorothioate; biostability; HIV; pharmacokinetics; metabolism

Development of antisense oligonucleotides as therapeutic agents for the treatment of viral infections and cancer has been shown to be a promising approach [1]. Unmodified and various modified antisense oligodeoxynucleotides have been demonstrated to have antisense activity both *in vitro* and *in vivo* [1–4]. PS-oligonucleotides§ have been studied extensively for their antisense properties [5–11]. Several human clinical trials are ongoing for viral infections and cancer using PS-oligonucleotides. Pharmacokinetic studies in mice and rats have demonstrated that PS-oligonucleotides have a short distribution and a longer elimination half-life in plasma and are distributed widely into all major tissues [12–17]. The highest concentration is present in kidney, liver and bone marrow [12–17]. Analysis of the extracted radioactivity showed that PS-oligonucleotides are metabolized in various tissues primarily by 3′-exonucleases [12, 17]. Degradation of PS-oligonucleotides *in vivo* reduces their effectiveness for longer duration. Therefore, stabilization of PS-oligonucleotides to avoid degradation *in vivo* may improve their effectiveness by increasing the duration of antisense activity of PS-oligonucleotides.

In the present report, we describe the *in vivo* stability, disposition, and excretion of an oligodeoxynucleotide phosphorothioate containing segments of four 2′-*O*-methyloligoribonucleotide phosphorothioates at both 3′- and 5′-ends. It has been observed that 2′-*O*-methyloligoribonucleotide phosphorothioates are more resistant to nucleases than the PS-oligonucleotide *in vitro*.‖ By incorporating segments of 2′-*O*-methyloligoribonucleotide phosphorothioate at the ends of the PS-

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§ Abbreviations: PS-oligonucleotide, oligodeoxynucleotide phosphorothioate; HIV, human immunodeficiency virus; GEM 91, Gene Expression Modulator 91; and hybrid oligonucleotide, hybrid oligonucleotide phosphorothioate.

‖ Metelev V and Agrawal S, Hybrid oligonucleotide phosphorothioates: Synthesis, properties and anti-HIV-activity. *Proceedings of International Conferences on Nucleic Acid Medical Applications*, Cancun, January 1993, Abstract 1-1.

C*U*C*U*CGCACCCATCTCTCTCC*U*U*C*U

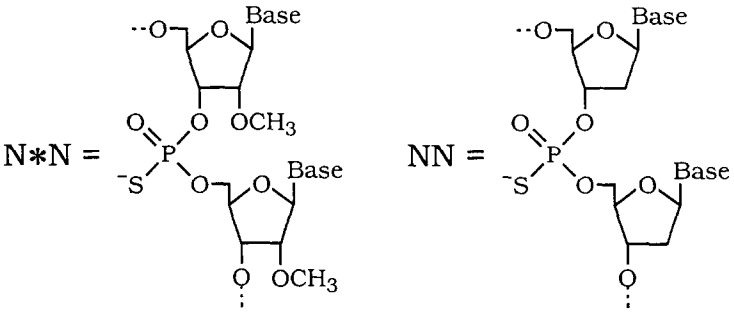

Fig. 1. Structure of hybrid oligonucleotide phosphorothioate. The hybrid oligodeoxynucleotide phosphorothioate containing segments of four 2′-*O*-methyloligoribonucleotide phosphorothioates at both the 3′- and 5′-ends.

oligonucleotide, a significant increase in protection against exonuclease has been observed *in vitro*.‖ In various assay models, these hybrid oligonucleotides have been shown to have improved antisense activity over PS-oligonucleotides [18, 19].

MATERIALS AND METHODS

Preparation of oligonucleotides

Synthesis of hybrid oligonucleotide. Synthesis of the 25-mer hybrid oligonucleotide (Fig. 1) was carried out using deoxynucleoside phosphoramidites (Milligen, Milford, MA) and 2′-*O*-methylribonucleoside phosphoramidites (Glen Research, Sterling, VA) on automated synthesizer (Biosearch 8800). The synthesis was carried out on a 1 mM scale by using the protocols reported earlier [18]. The first three couplings were carried out using 2′-*O*-methylribonucleoside β-cyanoethylphosphoramidite onto 2′-*O*-methylribonucleoside derivatized controlled pore glass (CPG) support. After the first three couplings, the next 17 couplings were carried out using deoxynucleoside β-cyanoethylphosphoramidite. The last four couplings of the sequence were carried out using 2′-*O*-methylribonucleoside β-cyanoethylphosphoramidite. After each coupling, the linkage was oxidized with 3*H*-1,2-benzodithiol-3-one-1,1-dioxide to generate phosphorothioate linkage [18]. Deprotection and purification of hybrid oligonucleotide were carried out by the same procedures as reported earlier [18]. The purified hybrid oligonucleotide was analyzed by ^{31}P NMR, ion exchange HPLC, polyacrylamide gel electrophoresis and melting temperature (both UV and circular dichroism). The percentage purity of the 25-mer in the lyophilized sample was 88% by capillary gel electrophoresis, with the remainder being n-1 and n-2 products.

Synthesis of ^{35}S-labeled hybrid oligonucleotide. To prepare the ^{35}S-labeled hybrid oligonucleotide, synthesis was carried out in a way similar to that as described above except that the last four couplings were carried out using 2′-*O*-methylribonucleoside *H*-phosphonate. 2′-*O*-Methylribonucleoside *H*-phosphonates, U and C, were synthesized by following the PCl_3/triazole method [20], starting from the appropriate 2′-*O*-methylribonucleoside and isolated as triethylammonium salts. The isolated 2′-*O*-methylribonucleoside *H*-phosphonates, U and C, were analyzed by both ^{31}P and ^{1}H NMR spectroscopies. Prior to their use in the oligonucleotide synthesis, the nucleoside *H*-phosphonates were evaporated to dryness twice with anhydrous pyridine and dissolved into anhydrous pyridine/CH_3CN (1/1) to a concentration of 40 mM. After the assembly, the CPG-bound oligonucleotide containing four *H*-phosphonate linkages was oxidized with ^{35}S elemental sulfur (Amersham, 0.5 to 2.5 Ci/milliatom) and deprotected by the same procedure as reported earlier [18]. Purification of the ^{35}S-labeled hybrid oligonucleotide was carried out on 20% PAGE (containing 7 M urea). The bands under UV light were excised, extracted in 100 mM ammonium acetate and desalted using a Sep-Pak C18 Column (Waters, Milford, MA). The specific activity of the hybrid oligonucleotide obtained was 0.25 μCi/μg.

Experimental design

Animals. The protocol of animal use and care was approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham. Male Sprague–Dawley rats (110 ± 10 g, Harlan Laboratories, Indianapolis, IN) were utilized in the study. The animals were fed with commercial diet and water *ad lib.* for 1 week prior to the study.

Kinetic and tissue distribution study. Thirty animals were used in the present study for 10 time points (3 animals for each time point), i.e. 5, 30, and 60 min and 3, 6, 12, 24, 48, 72, and 240 hr. Unlabeled and ^{35}S-labeled hybrid oligonucleotide were mixed in physiological saline (0.9% NaCl) at a concentration of 10 mg/mL (20 μCi/mL). Animals were dosed via a single i.v. bolus injection into a tail vein at a dose of 30 mg/kg body weight. Doses were based on the

pretreatment body weight and rounded to the nearest 0.01 mL. After i.v. injection, each animal was placed in a metabolism cage and fed with commercial diet and water *ad lib*. Total voided urine was collected, and each metabolism cage was then washed following the collection intervals. Total excreted feces was collected from each animal at various time points, and feces samples were homogenized in a 9-fold vol. of 0.9% NaCl prior to quantitation of radioactivity. Blood samples were collected in heparinized tubes from animals at the various time points. Plasma was separated by centrifugation.

Animals were euthanized by exsanguination under sodium pentobarbital anesthesia. Following euthanasia, the tissues were collected from each animal. Each tissue/organ was blotted immediately on Whatman No. 1 filter paper, trimmed of extraneous fat or connective tissue, emptied and cleaned of all contents, and then weighed. Prior to being homogenized in 0.9% NaCl (3–5 mL/g wet weight), each tissue/organ was washed using 0.9% NaCl. The resultant homogenates were kept at $\leq -70°$ until further analyses.

Analytical methods

Total radioactivity measurements in biological fluids and tissue. The total radioactivities in tissues and body fluids were determined by liquid scintillation spectrometry (LS 6000TA; Beckman, Irvine, CA) using methods described previously [17]. In short, biological fluids (plasma, 50–100 μL; urine, 50–100 μL) were mixed with 6 mL scintillation solvent (Beckman, Irvine, CA) to determine total radioactivity. An aliquot of tissue or feces homogenate (100 μL) was mixed with 200 μL solubilizer (TS-2; RPI, Mt. Prospect, IL) and then with the scintillation solvent (6 mL) to measure total radioactivity.

Gel electrophoresis of hybrid oligonucleotides and metabolites from biological samples. PAGE of the hybrid oligonucleotide and its metabolites was carried out by methods described previously [12, 17]. Plasma and tissue homogenates were incubated with proteinase K (2 mg/mL) in extraction buffer (0.5% SDS/10 mM NaCl/20 mM Tris–HCl, pH 7.6/10 mM EDTA) for 1 hr at 60°. The samples were then extracted twice with phenol:chloroform (1:1, v/v) and once with chloroform. After ethanol precipitation, the extracts were analyzed by electrophoresis in 20% polyacrylamide gels containing 7 M urea. Urine samples were filtered, desalted, and then analyzed by PAGE. The gels were fixed in 10% acetic acid/10% methanol solution and then dried before autoradiography.

HPLC analysis. The radioactivities in plasma and urine samples were analyzed by ion-paired HPLC using a modification of the method described previously [15, 17]. Urine samples were centrifuged and passed through a 0.2 μm Acro filter (Gelman, Ann Arbor, MI) prior to analysis. Hybrid oligonucleotide and its metabolites in plasma samples were extracted by using the above methods in the sample preparation for PAGE. A Microsorb MV-C4 column (Rainin Instruments, Woburn, MA) was employed in HPLC using a Hewlett–Packard 1050

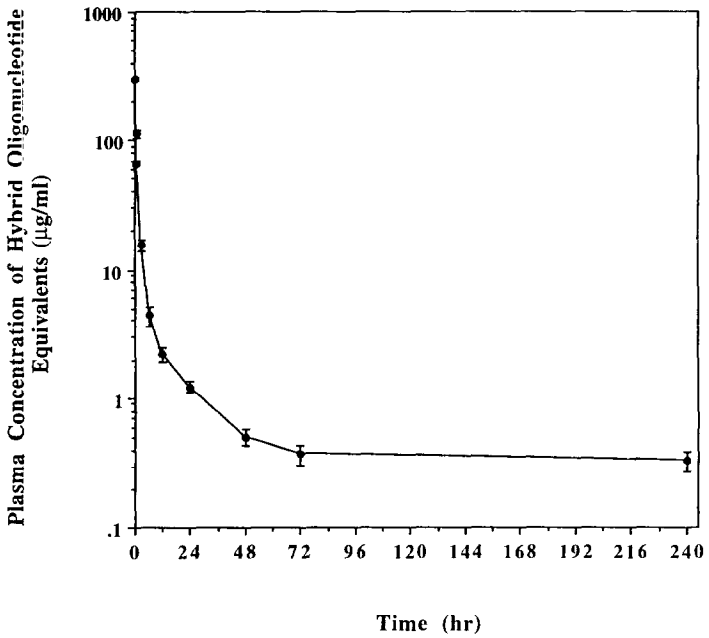

Fig. 2. Plasma concentration–time course. Plasma concentrations were expressed as micrograms of hybrid oligonucleotide equivalents per milliliter (mean ± SD) after i.v. bolus administration of ^{35}S-labeled hybrid oligonucleotide to rats at a dose of 30 mg/kg. Three animals were used for each time point. The plasma concentration was based on the quantitation of radioactivity. Pharmacokinetic analysis revealed that plasma disappearance curves for hybrid oligonucleotide-derived radioactivity could be described by the sum of two exponentials, with half-lives of 0.34 and 52.02 hr (see Table 1).

Table 1. Pharmacokinetic parameters for hybrid oligonucleotide in various tissues (two-compartmental i.v. bolus model)*

Tissue	C_{max} ($\mu g \cdot mL^{-1}$ or $\mu g \cdot mg^{-1}$)	$T_{1/2\beta}$ (hr)	$T_{1/2\beta}$ (hr)	AUC ($\mu g \cdot hr \cdot mL^{-1}$)	MRT (hr)	CL ($mL \cdot kg^{-1} \cdot hr^{-1}$)
Plasma	339.73	0.34	52.02	704.09	57.53	42.61
Adrenal	47.49	0.96	163.32	5745.29	234.36	
Brain	4.82	0.16	172.41	384.69	248.30	
Fat	8.45	7.48	287.43	2440.64	410.06	
Heart	29.29	0.86	252.19	4016.00	361.85	
Large intestine	41.22	5.57	181.49	8154.99	259.29	
Pancreas	28.14	0.53	314.83	4795.25	453.01	
Skeletal muscle	9.84	1.02	255.70	1768.30	367.43	
Skin	44.29	16.76	113.95	4724.98	151.33	
Small intestine	52.20	1.26	157.65	4725.93	224.27	
Spleen	56.29	0.55	265.75	7233.67	381.89	
Stomach	54.03	0.32	115.76	2919.24	166.07	
Testes	8.38	2.45	252.57	2858.54	364.22	
Thyroid	36.61	0.16	242.27	8789.45	349.49	

* Values are means based on the experimental data from 30 rats following administration of ^{35}S-labeled hybrid oligonucleotide. Concentrations in various tissues were based on the quantitation of radioactivity. Abbreviations: C_{max}, maximal concentration; $T_{1/2\alpha}$, distribution half-life; $T_{1/2\beta}$, elimination half-life; AUC, area under the curve; MRT, mean residue time; and CL, clearance.

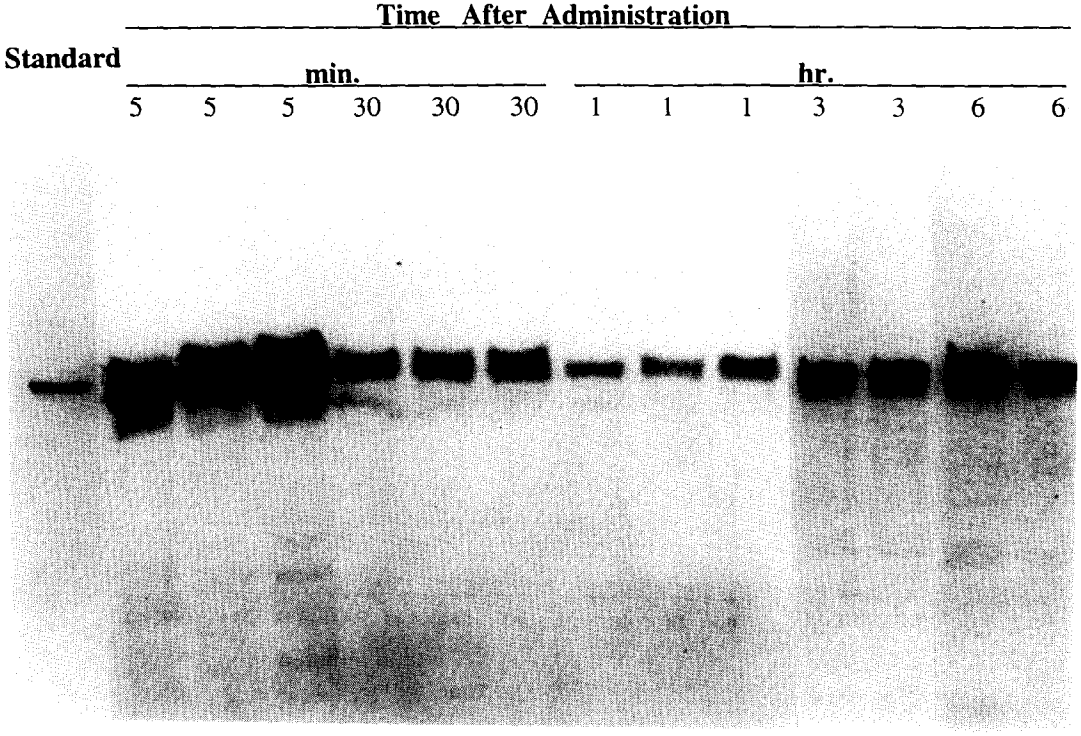

Fig. 3. Gel electrophoresis of plasma radioactivity. Plasma samples were treated with proteinase K (2 mg/mL) in extraction buffer (see text) for 2 hr at 37°. The samples were then extracted twice with phenol : chloroform (1 : 1, v/v) and once with chloroform. After ethanol precipitation, the oligonucleotides were analyzed by PAGE (20%, 7 M urea). Each lane represents one sample from an individual animal collected at various times.

HPLC with a quaternary pump for gradient making. Mobile phase included two buffers; Buffer A was 5 mM PIC-A reagent (Waters Co., Bedford, MA) in water and Buffer B was 4:1 (v/v) acetonitrile (Fisher):water. The column was eluted at a flow rate of 1.5 mL/min, using the following gradient: (1) 0–5 min, 0% Buffer B; (2) 5–15 min, 0–35% Buffer B; and (3) 15–70 min, 35–80% Buffer B. The column was equilibrated with Buffer A for at least 30 min prior to the next run. By using a RediFrac fraction collector (Pharmacia LKB Biotechnology, Piscataway, NJ), 1-min fractions (1.5 mL) were collected into 7-mL scintillation vials and mixed with 5 mL scintillation solvent to determine radioactivity in each fraction.

Data analysis

The concentrations of hybrid oligonucleotide-derived radioactivity were expressed as hybrid-oligonucleotide equivalents in either micrograms per milliliter of biological fluids (e.g. plasma) or micrograms per gram of wet tissue/organ. The results were expressed as mean ± SD.

The pharmacokinetic parameters of hybrid oligonucleotide were estimated by using the NLIN procedure of SAS programs [21, 22]. Functions consisting of the sum of one-, two-, or three-exponential components ($C_t = \sum_{i=1}^{n} A_i e^{-K_i t}$, N = 1, 2, 3) were fitted to data by a least squares method, where C_t is the concentration at time point t; A_i is the concentration coefficient; and K_i is the elimination or absorption rate coefficient. Selections of models were based on comparison of Akaikie's Information Criterion (AIC) and standard errors (SE) of estimated parameters. One-, two-, and three-compartmental models of i.v. bolus injection or first order absorption administration models were tested to fit for each tissue/biological fluid. For example, it was shown that a two-compartmental model of i.v. bolus injection best fit the plasma concentration–time curve. The two-compartmental model of the first order absorption administration model best fit the kidney concentration–time curve. The area under the curve (AUC) was calculated from $\sum_{i=1}^{n} A_i/K_i$. Elimination $T_{1/2}$ values of hybrid oligonucleotide were calculated from $0.693/K_i$. The clearance rate (CL) of hybrid oligonucleotide was calculated by dividing the dose by the AUC.

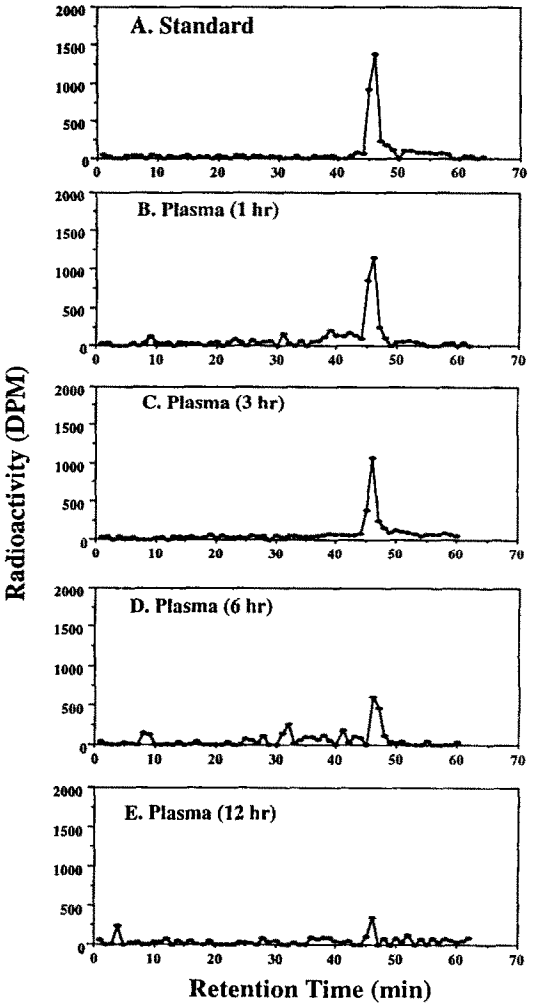

Fig. 4. HPLC analysis of radioactivity in plasma. The radioactivities in plasma samples were analyzed by ion-paired HPLC, using a Microsorb MV-C4 column and gradient elution (see Materials and Methods). Under the conditions used in the present study, the retention time for the standard ^{35}S-labeled 25-mer hybrid oligonucleotide was 45.5 min (panel A). This figure shows representative chromatograms for plasma samples from animals killed at various times after dosing. Similar HPLC profiles were observed with each of the animals in the present study. Greater than 90% of the radioactivity was present as intact oligonucleotide up to 12 hr post-dosing.

RESULTS

Pharmacokinetics and in vivo *stability of hybrid oligonucleotide in plasma*

The mean plasma concentrations over time of the hybrid oligonucleotide equivalents following i.v. bolus administration of ^{35}S-labeled hybrid oligonucleotide are illustrated in Fig. 2. The concentrations were based on the quantitation of radioactivity. Pharmacokinetic analysis revealed that plasma disappearance curves for hybrid oligonucleotide-derived radioactivity could be described by the sum of two exponentials, with half-lives of 0.34 and 52.02 hr (Table 1). The chemical form of radioactivity in plasma was further examined by PAGE and HPLC analysis, demonstrating the presence of mainly intact hybrid oligonucleotide for up to 12 hr as well as minor metabolites with lower molecular weights (Figs. 3 and 4). The initial phase of the plasma concentration–time curve may represent the distribution of the hybrid oligonucleotide into various tissues, and the elimination phase (β phase) may represent the redistribution and elimination of the hybrid oligonucleotide and its metabolites.

Elimination of hybrid oligonucleotide through urine and feces

Following administration of ^{35}S-labeled hybrid

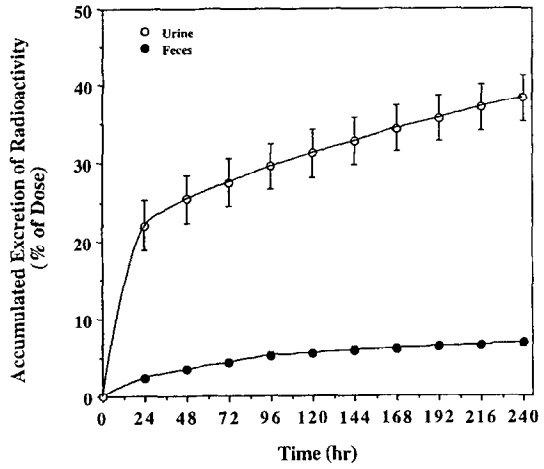

Fig. 5. Cumulative urinary and fecal excretion of radioactivity. Urinary and fecal excretions of hybrid oligonucleotide-derived radioactivity were expressed as means ± SD of the cumulative percentage of administered dose excreted over time (three animals per time point). ^{35}S-Labeled and unlabeled hybrid oligonucleotide were administered by i.v. bolus injection into rats at a dose of 30 mg/kg. Total excretion was based on the quantitation of radioactivity in urine and feces.

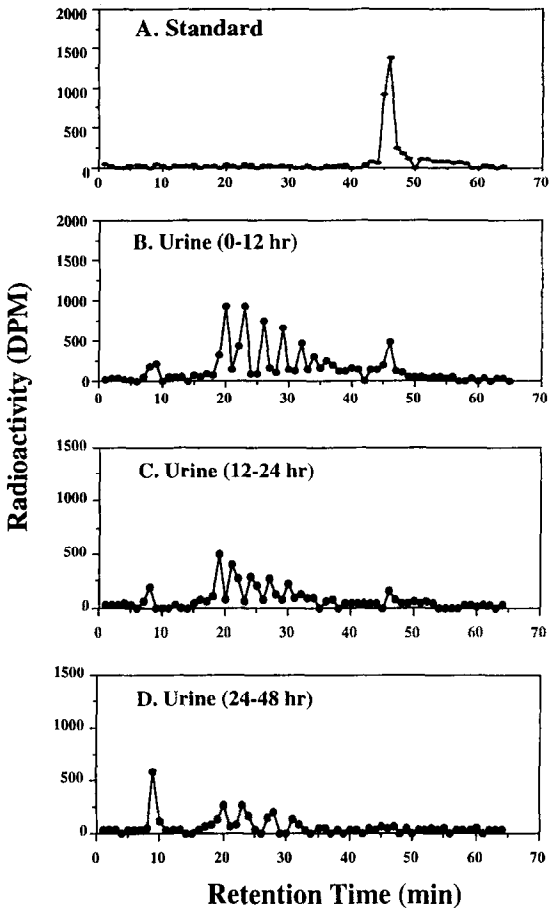

Fig. 6. HPLC analysis of radioactivity in urine. The radioactivities in urine samples were analyzed by ion-paired HPLC. This figure shows representative chromatograms for urine samples from one animal at various times. Similar HPLC profiles were observed with each of the animals in the present study. Both intact oligonucleotide and metabolites with lower molecular weights were detected in urine within 24 hr post-dosing.

oligonucleotide, urinary excretion represented the major pathway for elimination of hybrid oligonucleotide, with 21.98 ± 3.2% (mean ± SD) of the administered dose excreted within 24 hr, 25.37 ± 3.06% within 48 hr, and 38.13 ± 2.99% over 240 hr post-dosing (Fig. 5). The majority of the radioactivity in urine was present as degradative products of hybrid oligonucleotide; however, a trace of intact hybrid oligonucleotide was also detected in the urine by both HPLC (Fig. 6) and PAGE (data not shown). However, fecal excretion was a minor pathway of elimination of hybrid oligonucleotide with 2.34 ± 0.13% of the administered dose excreted over 24 hr and 6.74 ± 0.40% over 240 hr post-dosing (Fig. 5). The majority of radioactivity in feces was present as degradative products with lower molecular weights (data not shown).

Distribution and stability of hybrid oligonucleotide in various tissues

In the present study, stability and distribution of the hybrid oligonucleotide were determined in various tissues between 5 min and 240 hr after i.v. bolus administration of ^{35}S-labeled hybrid oligonucleotide. This hybrid oligonucleotide had a wide tissue distribution, with detectable radioactivity in all the tissues examined. To clearly describe the tissue distribution pattern of this hybrid oligonucleotide, the mean hybrid oligonucleotide-equivalent concentrations of radioactivity in various tissues were plotted along with corresponding plasma concentrations (Fig. 7). In the initial 30 min post-dosing, the hybrid oligonucleotide was distributed in kidneys, liver, spleen, bone marrow, and intestine. Most tissues examined had significantly higher concentrations compared with that observed in plasma 3 hr and longer after dosing.

In vivo stability of the hybrid oligonucleotide in various tissues was evaluated further by PAGE analysis, demonstrating that mainly intact hybrid oligonucleotide was present in most tissues examined, e.g. the kidneys (Fig. 8), liver (Fig. 9), and small intestine (Fig. 10). Based on analyses by HPLC and gel electrophoresis of extracts from plasma and tissue samples, the hybrid oligonucleotide was shown to be more stable than the PS-oligonucleotide with the same nucleotide sequence [17].

Pharmacokinetic analysis

Based on the experimental data, pharmacokinetic parameters for hybrid oligonucleotide in each tissue and biological fluid were calculated using computer-based two-compartmental i.v. bolus or absorption models. Table 1 summarizes the results using a two-compartmental i.v. bolus model to fit

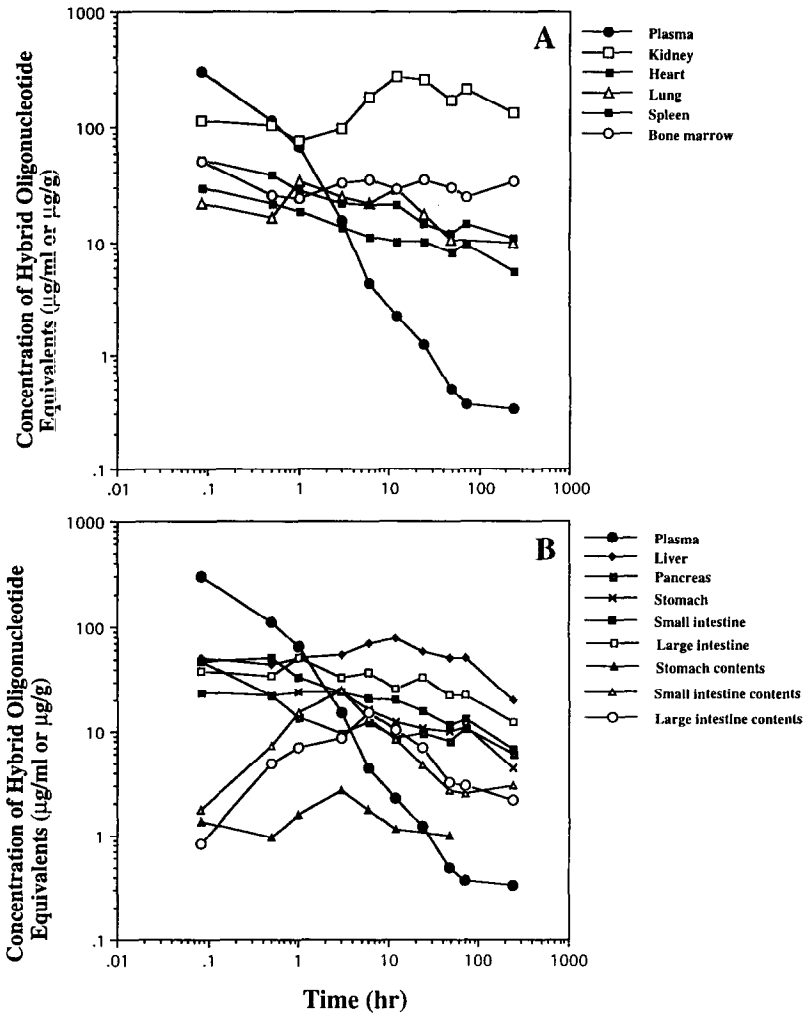

Fig. 7. Tissue concentration–time course of radioactivity. Mean tissue concentrations were expressed as micrograms of hybridized oligonucleotide equivalents per gram of tissue/organ after i.v. bolus administration of ^{35}S-labeled hybrid oligonucleotide to rats at a dose of 30 mg/kg. Three animals were used for each time point. Tissue concentration was based on the quantitation of radioactivity. To show the comparison of tissue concentrations and corresponding plasma levels, a plasma concentration–time curve was plotted in each panel.

the data for selected tissues/biological fluids. Table 2 summarizes the results using a two-compartmental absorption (first order) model to fit the data for selected tissues. As illustrated in Tables 1 and 2, elimination half-lives for most tissues examined were longer than that observed with plasma, further indicating accumulation of radioactivity in these tissues.

DISCUSSION

Antisense oligonucleotides represent a novel therapeutic strategy. PS-oligonucleotides are being widely used to modulate the expression of specific genes [1]. Compared with their phosphodiester oligonucleotide counterparts PS-oligonucleotides are more resistant to nuclease digestion, and their duplex with RNA is a substrate for RNase H. PS-oligonucleotides have been studied as antiviral agents, specifically inhibiting human immunodeficiency virus type-1, influenza virus, herpes simplex virus, human papilloma virus, and human cytomegalovirus [3]. PS-oligonucleotides have also been evaluated for modulating the expression of genes implicated in cancer [4] and parasites [5]. PS-oligonucleotides have been studied for their toxicity and safety in mice [23], rats [16], and monkeys [24] and found to be safe in mice, rats and dogs [25] but not in monkeys in which dose- and rate-dependent side-effects have been observed [24].

As required for traditional therapeutic agents, *in vivo* stability of an oligodeoxynucleotide is an important parameter related to its therapeutic effectiveness as well as possible side-effects. Pharmacokinetic studies of various PS-oligonucleotides have been carried out in mice [12,

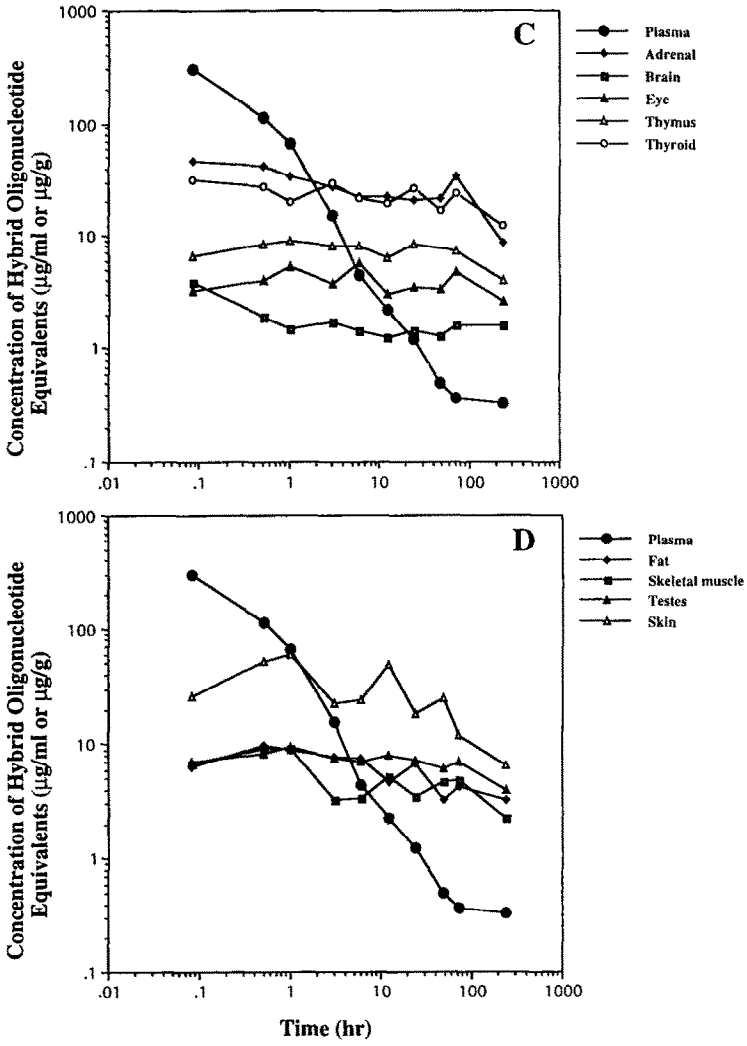

Fig. 7. continued.

14, 15], rats [13, 16, 17] and monkeys [25], and they show that PS-oligonucleotides have prolonged elimination half-lives and a wide tissue distribution. Analysis of the extracted PS-oligonucleotides at various time points after dosing shows that PS-oligonucleotides are degraded extensively in kidney, liver and other tissues [12–17]. In addition to degradation products, some high molecular weight metabolites were also observed [12]. *In vivo* degradation of PS-oligonucleotides was found to be primarily from the 3′-end [12–17], as modification of the 3′-end has been shown to increase the *in vivo* stability of PS-oligonucleotides [14].

The stability of PS-oligonucleotides has also been shown to be increased by incorporating segments of 2′-*O*-methylribonucleotide phosphorothioates at both the 3′- and 5′-ends, called hybrid oligonucleotides [18, 19]. This oligonucleotide design combines the favorable antisense properties of PS-oligonucleotides (RNase H activation) and 2′-*O*-methyloligoribonucleotide phosphorothioates (increased nuclease stability and duplex stability) [18, 19]. Hybrid oligonucleotides have been shown to be more potent antisense agents than PS-oligonucleotides [18, 19].

In the present report, we have studied *in vivo* stability, disposition and excretion of a hybrid oligonucleotide. This hybrid oligonucleotide is a 25-mer oligodeoxynucleotide phosphorothioate containing 2′-*O*-methylribonucleotide phosphorothioate (four internucleotide linkages) at both the 3′- and 5′-ends. Hybrid oligonucleotide (^{35}S-labeled at four contiguous intersect linkages at the 5′-end) was administered to rats by i.v. injection at a dose of 30 mg/kg. Plasma disappearance curves for the hybrid oligonucleotide-derived radioactivity could be described by a two-compartmental pharmacokinetic model. Urinary excretion represented the major pathway of elimination of the hybrid oligonucleotide, with fecal excretion being a minor pathway for elimination. A wide tissue distribution of the hybrid oligonucleotide-derived radioactivity was observed. In the present study, following analysis by HPLC and gel electrophoresis, greater biostability was

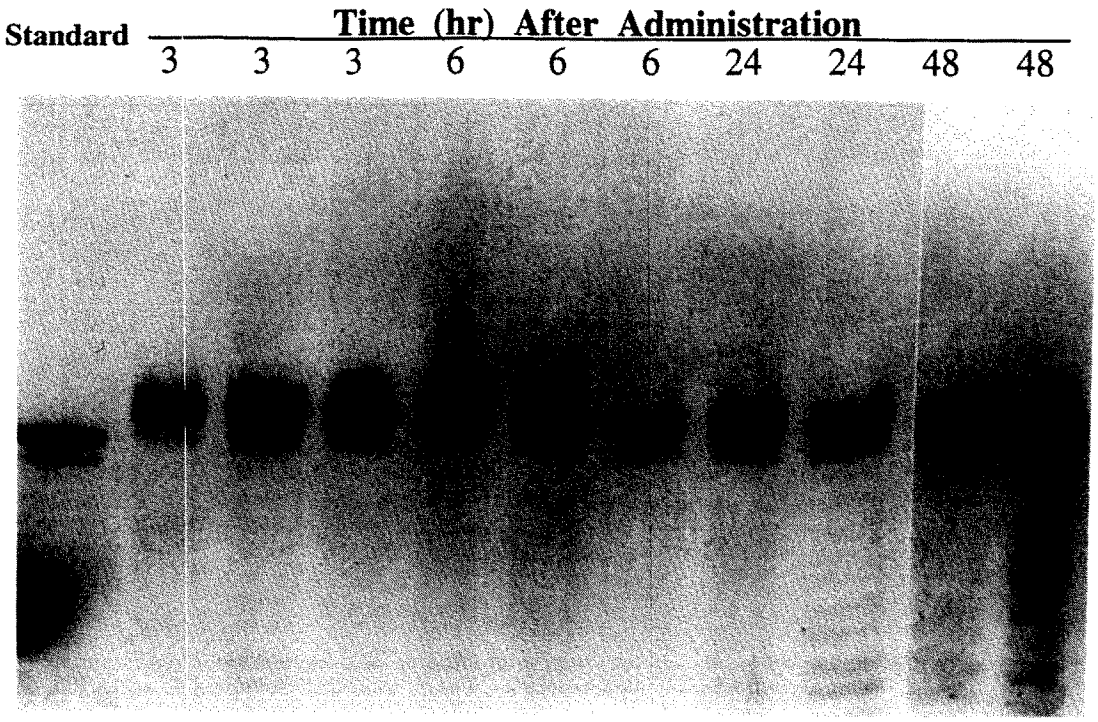

Fig. 8. Gel electrophoresis of radioactivity in kidneys. This figure shows a representative autoradiogram for kidney samples of animals killed at various times.

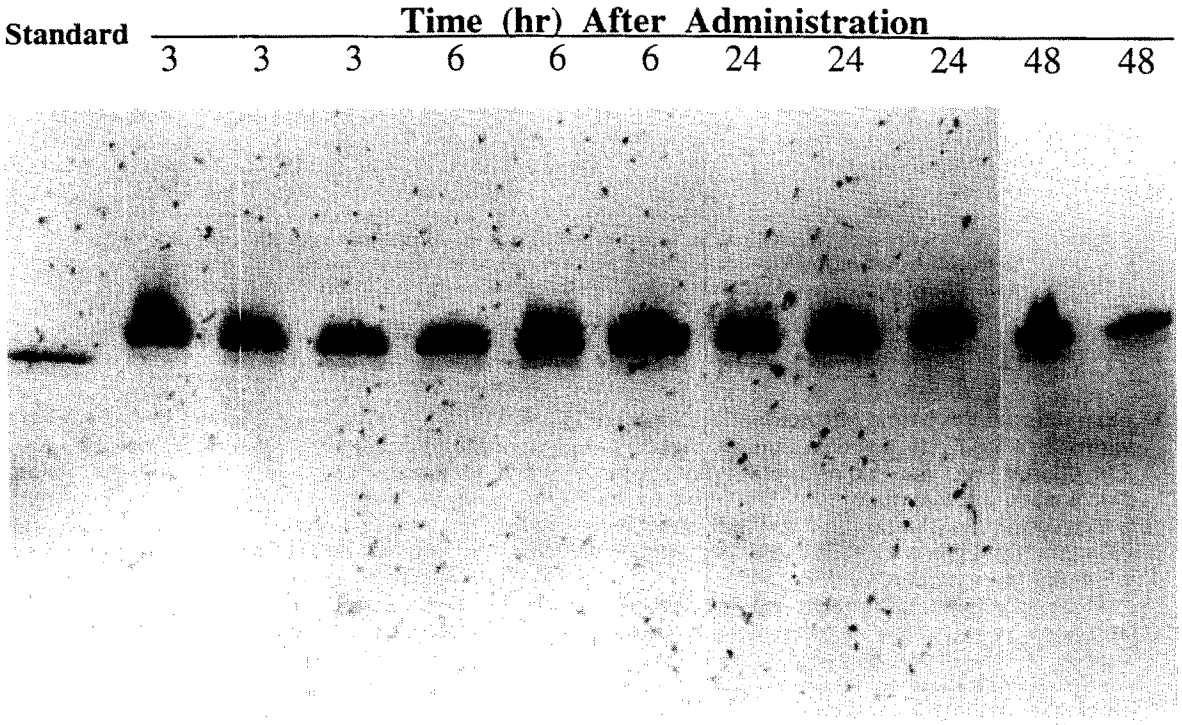

Fig. 9. Gel electrophoresis of radioactivity in liver. This figure shows a representative autoradiogram for liver samples of animals killed at various times.

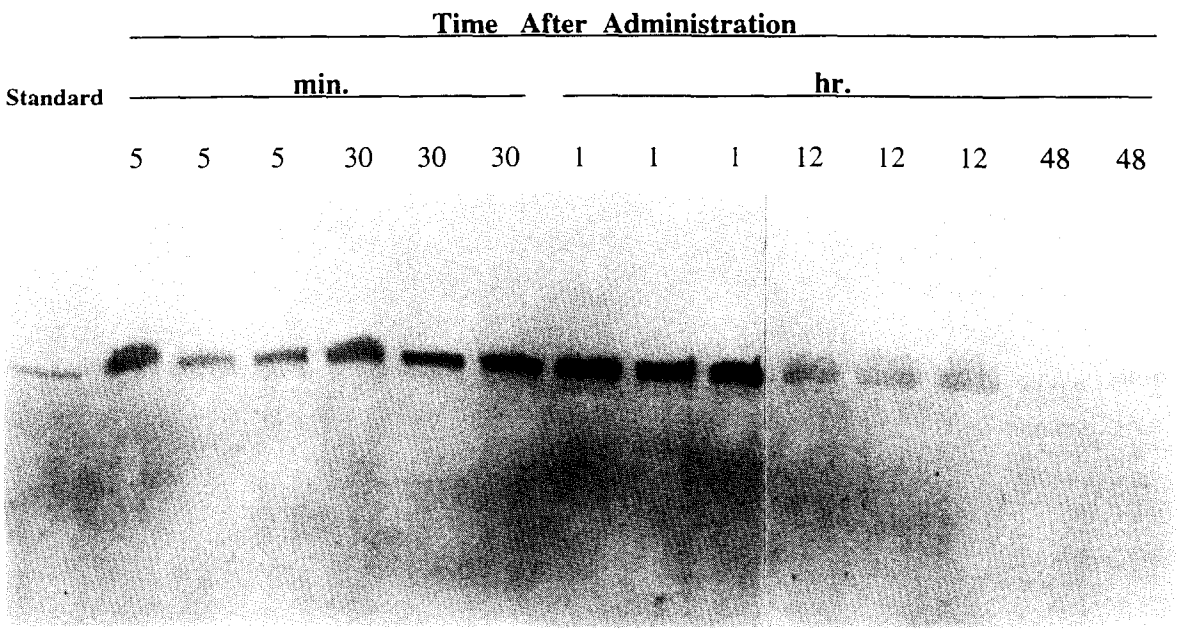

Fig. 10. Gel electrophoresis of radioactivity in small intestine. This figure shows a representative autoradiogram for small intestine samples of animals killed at various times.

Table 2. Pharmacokinetic parameters for hybrid oligonucleotide in various tissues (two-compartmental absorption model)*

Tissue	$T_{1/2A}$ (hr)	$T_{1/2\alpha}$ (hr)	$T_{1/2\beta}$ (hr)	AUC ($\mu g \cdot hr \cdot mL^{-1}$)	MRT (hr)
Bone marrow	0.01	0.01	141.29	7355.06	203.88
Eye	1.11	1.12	144.24	2000.94	208.75
Kidney	1.90	1.53	273.55	92483.46	398.64
Large intestine contents	1.04	1.04	115.37	3137.19	168.09
Liver	1.23	1.12	120.52	12705.51	175.25
Lung	0.35	0.60	177.75	5556.21	256.20
Small intestine contents	1.42	1.51	323.38	2124.12	440.87
Stomach contents	1.47	1.48	425.31	669.85	606.45
Thymus	1.06	1.07	103.12	2771.85	149.81

* Values are means based on the experimental data from 30 rats following administration of ^{35}S-labeled hybrid oligonucleotide. Concentrations in various tissues were based on the quantitation of radioactivity. $T_{1/2A}$ = absorption half-life. See the legend of Table 1 for the other abbreviations.

observed with this hybrid oligonucleotide compared with other PS-oligonucleotides examined earlier [12–17]. Based on the experimental data, pharmacokinetic parameters for the hybrid oligonucleotide in each tissue and biological fluid were calculated using computer-based two-compartmental i.v. bolus or absorption models.

Pharmacokinetic analysis of hybrid oligonucleotide in plasma is in general agreement with previous studies of PS-oligonucleotides [12, 14–17], which suggested that PS-oligonucleotides had prolonged elimination half-lives and a wide tissue distribution. Compared with a 25-mer PS-oligonucleotide (GEM 91) previously examined in our laboratory [17], the hybrid oligonucleotide has a shorter distribution half-life ($T_{1/2\alpha}$) and a longer elimination half-life ($T_{1/2\beta}$), suggesting that the hybrid oligonucleotide was taken up rapidly and retained in various tissues for a longer period. Analysis of the extracted radioactivity from plasma by HPLC and gel electrophoresis showed that most of the radioactivity was associated with the intact hybrid oligonucleotide up to 12 hr post-dosing. Initially, hybrid oligonucleotide was distributed mainly in highly perfused tissues including the liver, kidneys, heart, lungs and spleen. The prolonged elimination phase reflected retention of hybrid oligonucleotide (and its metabolites) in several tissues, especially the kidney, liver, lung, heart, bone marrow, and intestine. Analysis of the extracted radioactivity from kidney,

liver and small intestine by gel electrophoresis confirmed that the majority of the radioactivity was associated with the intact hybrid oligonucleotide. In contrast, we have observed significant degradation of PS-oligonucleotides in these tissues [12, 17]. Hybrid oligonucleotide was also found to be stable in other tissues examined in the present study (data not shown).

In the present study, urinary excretion represents the major route of elimination of hybrid oligonucleotide-derived radioactivity, qualitatively similar to what has been seen with GEM 91 [17]. Urinary excretion of hybrid oligonucleotide-derived radioactivity mainly occurred in the first 24 hr (approximately 22%). Over the subsequent 9-day period, 1–3% of the administered dose was excreted into urine in each 24-hr period. Analysis of urinary samples by HPLC (Fig. 6) and gel electrophoresis indicated that the degradation of the hybrid oligonucleotide was a slow process *in vivo*: within 12 hr after dosing, radioactivity in urine was associated with different lengths of oligonucleotides; however, the intact hybrid oligonucleotide was also detected. The degradation pattern showed that the 3′-end segment of 2′-*O*-methyloligoribonucleotide of the hybrid oligonucleotides was cleared off by enzymes, thereby generating an oligonucleotide population of 18-, 19-, 20-mer, etc. No 24-, 23-, or 22-mers were detected (see Figs. 3, 4, 6 and 8). Fecal excretion was less than 10% of the administered dose over 10 days following administration of hybrid oligonucleotide.

Since relatively high concentrations of hybrid oligonucleotide-derived radioactivity were observed in the liver and the intestinal tissue and contents, but not in the feces, an enterohepatic circulation of this hybrid oligonucleotide was indicated. Both intact and metabolic forms of hybrid oligonucleotide were found in the liver, intestinal tract and intraluminal contents. Further studies are needed to determine the biliary excretion, transport of the hybrid oligonucleotide across the intestinal wall, chemical forms at each site of the enterohepatic system, and lastly, the significance of the enterohepatic circulation on the pharmacology and toxicology of hybrid oligonucleotides.

Following administration of the hybrid oligonucleotide, there was a wide distribution of radioactivity in various tissues. Concentrations in various tissues were significantly higher than the corresponding plasma concentration 3 hr and thereafter following administration of the hybrid oligonucleotide. Accumulation of the hybrid oligonucleotide in these tissues may be important in its biological effects. As shown in Tables 1 and 2, for most tissues and biological fluids, the half-lives of the elimination phase of hybrid oligonucleotide-derived radioactivity were significantly longer than that observed in plasma.

In summary, the present study demonstrates that the 25-mer hybrid oligonucleotide was significantly more stable than other PS-oligonucleotides, which may be important in the future studies of hybrid oligonucleotides as therapeutic agents. More stable oligonucleotides at least have two advantages: one is that less frequency of dosing will be needed; another will be that less degradative metabolites will avoid potential unwanted side-effects from these metabolites.

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