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Short Communication

ABSORPTION, TISSUE DISTRIBUTION AND *IN VIVO* STABILITY IN RATS OF A HYBRID ANTISENSE OLIGONUCLEOTIDE FOLLOWING ORAL ADMINISTRATION

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Abstract—*In vivo* stability and oral bioavailability of an oligodeoxynucleotide phosphorothioate containing segments of 2′-*O*-methyloligoribonucleotide phosphorothioates at both the 3′- and 5′-ends (hybrid oligonucleotide) were studied. A 25-mer ^{35}S-labeled hybrid oligonucleotide was administered to rats by gavage at a dose of 50 mg/kg body weight. HPLC analysis revealed that this hybrid oligonucleotide was stable in the gastrointestinal tract for up to 6 hr following oral administration. Radioactivity associated with the hybrid oligonucleotide was detectable in portal venous plasma, systemic plasma, various tissues, and urine. Intact hybrid oligonucleotide was detected, by HPLC analysis, in portal venous plasma, systemic plasma, and various tissues. The majority of the radioactivity in urine was associated with degradative products with lower molecular weights, but the intact form was also detected. In summary, the hybrid oligonucleotide was absorbed intact through the gastrointestinal tract, indicating the possibility of oral administration of oligonucleotides, a finding that may be important in the development of antisense oligonucleotides as therapeutic agents.

Key words: antisense oligonucleotides; HIV; oral bioavailability

The use of antisense oligonucleotides has been shown to be a promising approach in the development of therapeutic agents for the treatment of viral infections (including HIV§ infection) and cancer [1]. In fact, several unmodified and modified antisense oligodeoxynucleotides have been demonstrated to have antisense activity, both *in vitro* and *in vivo* [1]. Thus far, most *in vivo* biological and pharmacokinetic studies have been carried out with PS-oligonucleotides [2–10]. In general, PS-oligonucleotides have a short distribution half-life and a longer elimination half-life in plasma, and are distributed widely into all major tissues following i.v. injection [2–8, 10]. Varying *in vivo* degradation of PS-oligonucleotides has been observed [2–10]. Several antisense oligonucleotides recently have entered early clinical evaluations including human pharmacokinetic studies [9, ‖]. Compared with the results from animal studies, PS-oligonucleotides have a similar pharmacokinetic profile in humans [9, ‖].

Attempts have been made recently to stabilize PS-oligonucleotides to avoid degradation *in vivo*. It has been observed that 2′-*O*-methyloligoribonucleotide phosphorothioates are more resistant to nucleases than the PS-oligonucleotide *in vitro* [11–13]. By incorporating segments of 2′-*O*-methyloligoribonucleotide phosphorothioate at the ends of PS-oligonucleotide, a significant increase in protection against exonuclease has been observed *in vitro*. In various assay models, oligodeoxynucleotide phosphorothioate containing segments of 2′-*O*-methyloligoribonucleotide phosphorothioates at both the 3′- and 5′- ends (hybrid oligonucleotide) has been shown to have greater stability *in vitro* and *in vivo*, as well as improved antisense activity over PS-oligonucleotides [11, 12].

More recently, *in vivo* biostability, disposition, and excretion of a 25-mer hybrid oligonucleotide were determined in rats after i.v. bolus administration of ^{35}S-labeled hybrid oligonucleotide at a dose of 30 mg/kg [14]. 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. The majority of radioactivity in plasma was associated with the intact hybrid oligonucleotide. Urinary excretion represented the major pathway of elimination, and fecal excretion was a minor pathway of elimination. A wide tissue distribution of hybrid oligonucleotide was observed based on radioactivity levels, and analysis by HPLC showed that the majority of the radioactivity was associated with the intact hybrid oligonucleotide. Compared with other oli-

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§ Abbreviations: HIV, human immunodeficiency virus; PS-oligonucleotide, oligodeoxynucleotide phosphorothioate; and hybrid oligonucleotide, hybrid oligonucleotide phosphorothioate.

‖ Zhang R, Yan J, Shahinian H, Amin G, Lu Z, Saag MS, Jiang Z, Temsamani J, Schechter PJ, Martin RR, Agrawal S and Diasio RB, Human pharmacokinetics of an anti-HIV antisense oligonucleotide phosphorothioate (GEM 91) in HIV-infected individuals. *The 34th Interscience Conference on Antimicrobial Agents and Chemotherapy*, Orlando, FL, Oct. 4–7, 1994. Later Breaker Session A5, p. 5.

godeoxynucleotide phosphorothioates previously examined which have a similar nucleotide sequence [5], the hybrid oligonucleotide has been found to have a shorter distribution half-life and a longer elimination half-life, and is more stable in various tissues [14]. More interestingly, following i.v. injection of this hybrid oligonucleotide to rats, intact compound was detected in the liver and intestinal tissue and contents, indicating that there is an enterohepatic circulation of this oligonucleotide [14]. These results suggest the possibility of alternative delivery of antisense oligonucleotides as therapeutic agents in the future. The purpose of the present study was to determine absorption, tissue distribution, and *in vivo* stability of the 25-mer hybrid oligonucleotide in rats following oral administration.

Materials and Methods

Synthesis of unlabeled and ^{35}S-labeled hybrid oligonucleotide. The 25-mer hybrid oligonucleotide (5'-CUCU-CGCACCCATCTCTCTCC-UUCU-3') contains segments of four 2'-*O*-methyloligoribonucleotide phosphorothioates at both the 3'- and 5'-ends. Synthesis of the hybrid oligonucleotide was carried out using deoxynucleoside phosphoramidites (Milligen, Milford, MA) and 2'-*O*-methylribonucleoside phosphoramidites (Glen Research, Sterling, VA) on an automated synthesizer (Biosearch 8800). The synthesis was carried out on a 1 mM scale by using the protocols reported earlier [11, 14, *]. The purity of the hybrid oligonucleotide was shown to be greater than 90% by analyses of ^{31}P NMR, ion exchange HPLC, polyacrylamide gel electrophoresis and melting temperature (both UV and circular dichroism), with the remainder being n-1 and n-2 products. To prepare the ^{35}S-labeled hybrid oligonucleotide, synthesis was carried out in a way similar to that described above except that the last five couplings were carried out using 2'-*O*-methylribonucleoside *H*-phosphonate. The 2'-*O*-methylribonucleoside *H*-phosphonates, U and C, were synthesized by following the PCl_3/triazole method,* 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 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 assembly, the controlled pore glass (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 [11]. Purification of the ^{35}S-labeled hybrid oligonucleotide was carried out by PAGE (20%, 7 M urea). The visualized band product under UV light was 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. The purity of the ^{35}S-labeled hybrid oligonucleotide was shown to be greater than 98% by analyses of ^{31}P NMR, ion exchange HPLC, and PAGE, with the remainder being n-1 and n-2 products.

Animals and treatment. The protocol for 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. The animals were given commercial diet and water *ad lib.* for 1 week prior to the study. Unlabeled and ^{35}S-labeled oligonucleotides were dissolved in physiological saline (0.9% NaCl) to a concentration of 25 mg/mL, and were administered to the animals via gavage at a dose of 50 mg/kg (sp. act. 7.7 μCi/mg). Doses were based on the pretreatment body weight and rounded to the nearest 0.01 mL. After dosing, each animal was placed in a metabolism cage and fed a commercial diet and water *ad lib.* Total voided urine was collected, and each metabolism cage was washed following the collection intervals. Total excreted feces was collected from each animal at various time points, and feces samples were homogenized prior to quantitation of radioactivity. Blood samples were collected and the animals were killed at various times (i.e. 1, 3, 6, 12, 24, and 48 hr; 2 rats/time point). Plasma was separated by centrifugation. Following removal, tissues/organs were trimmed of extraneous fat or connective tissue, emptied and cleaned of all contents, and individually weighed prior to homogenization. To determine the chemical form(s) of radioactivity in the portal venous blood, a separate group of animals was subjected to the same treatment procedure as above. Portal venous blood samples (1/rat) were taken at scheduled times (1, 3, and 6 hr), and plasma was separated by centrifugation.

To quantitate the total absorption of the hybrid oligonucleotide, two additional groups of animals (3/group) were treated using the same procedure as above. Animals were killed at 6 or 12 hr post-dosing, and the gastrointestinal tract was then removed. Radioactivities in the gastrointestinal tract, feces, urine, plasma, and the remainder of the body were determined separately. Total recovery of radioactivity was also determined to be 95 ± 6%. The percentage of the absorbed hybrid oligonucleotide-derived radioactivity was determined by the following calculation:

$$\frac{\text{(total radioactivity in the remainder of the body and plasma + total radioactivity in urine)}}{\text{(total radioactivity in the gastrointestinal tract, feces, urine, plasma, and the remainder of the body)}}.$$

Sample preparation and total radioactivity measurements. The total radioactivities in tissues and body fluids were determined by liquid scintillation spectrometry (LS 6000TA; Beckman, Irvine, CA), using a method described previously [5, 14]. In brief, biological fluids (plasma, 50–100 μL; urine, 50–100 μL) were mixed with 6 mL of scintillation solvent (Beckman) to determine total radioactivity. Feces were ground and weighed prior to being homogenized in a 9-fold volume of 0.9% NaCl. Following their removal, tissues were blotted immediately on Whatman No. 1 filter paper and weighed prior to being homogenized in 0.9% NaCl (3–5 mL/g wet weight). An aliquot of the homogenate (100 μL) was mixed with solubilizer (TS-2; RPI, Mt. Prospect, IL) and then with scintillation solvent (6 mL) to permit quantitation of total radioactivity.

HPLC analysis. The radioactivities in plasma and urine samples were analyzed by ion-paired HPLC using a modification of a method described previously [4]. Urine samples were centrifuged and passed through a 0.2-μm Acro filter (Gelman, Ann Arbor, MI) prior to analysis. Hybrid oligonucleotide and metabolites in plasma and tissue samples were extracted by methods described previously [14]. Using the extraction methods, the extraction efficiency was approximately 75% for 18–25 mer and 60% for 5–10 mer. Monomer cannot be extracted using this method. A Microsorb MV-C4 column (Rainin Instruments, Woburn, MA) was employed in HPLC using a Hewlett–Packard 1050 HPLC with a quaternary pump for gradient making. The 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

* 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.

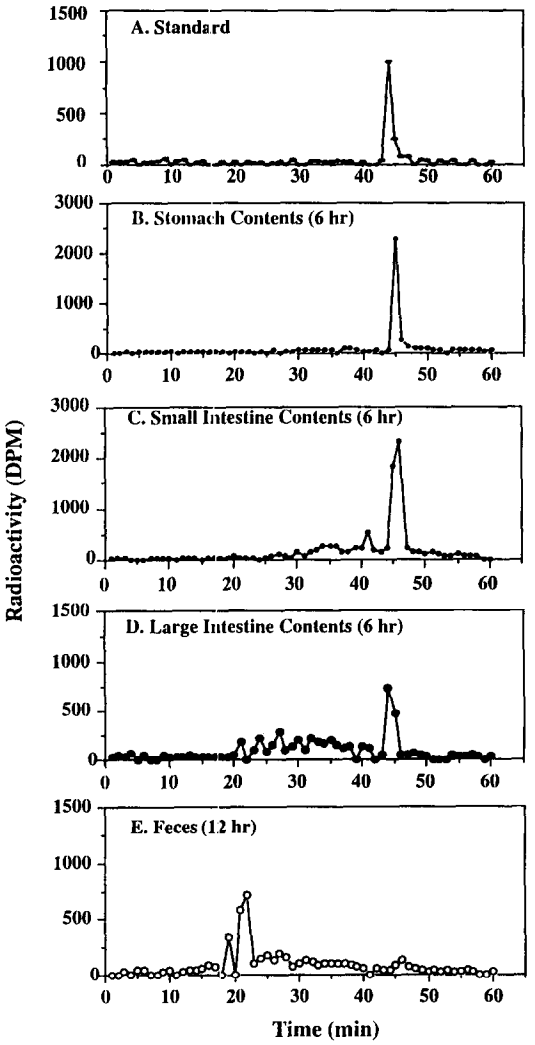

Fig. 1. HPLC analysis of radioactivity in the contents of stomach, small intestine, large intestine, and feces. The radioactivities in plasma samples were analyzed by ion-paired HPLC, using a Microsorb MV-C4 column and gradient elution (see Materials and Methods). Under the conditions used in the present study, the retention time for the standard ^{35}S-labeled 25-mer hybrid oligonucleotide was 45.0 min (panel A). This figure shows representative chromatograms for samples from animals killed at 6 hr after dosing. Similar HPLC profiles were observed with each of the animals in the present study.

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 of scintillation solvent to determine the radioactivity in each fraction.

Results

In vivo *stability of hybrid oligonucleotide in the gastrointestinal tract.* The hybrid oligonucleotide was shown to be stable in the stomach and in the small and large intestines (Fig. 1), as analyzed by HPLC. Up to 6 hr following administration, the hybrid oligonucleotide remained intact in the stomach, and its contents and the majority of radioactivity in the small intestine were associated with the intact oligonucleotide. Extensive degradation of this oligonucleotide was observed in the large intestine 6 hr after dosing (Fig. 1).

Absorption of hybrid oligonucleotide following oral administration. Radioactivity was detectable in various tissues following oral administration of the radiolabeled hybrid oligonucleotide. Figure 2A illustrates the plasma, liver, kidney and spleen concentration–time course of the oligonucleotide equivalents after oral administration of radiolabeled oligonucleotide. The hybrid oligonucleotide-derived radioactivity was detected in all tissues examined. Figure 2B illustrates the concentration–time courses for several representative tissues. An accumulation of radioactivity was observed in these tissues.

Urinary excretion of hybrid oligonucleotide following oral administration. Radioactivity was detected within 1 hr following oral administration of the hybrid oligonucleotide. Following a complete urine collection, the mean cumulative excretion of urinary radioactivity was determined to be 2.5% of the administered dose over 24 hr and 3.8% over 48 hr post-dosing. The majority of radioactivity in the urine was associated with the degradative products of the hybrid oligonucleotide. However, trace intact hybrid oligonucleotide was also detected (Fig. 3).

Chemical forms of radioactivity in portal venous blood, systemic plasma, and various tissues following oral administration. The chemical forms of radioactivity in portal venous plasma, systemic plasma, liver, and kidneys were examined further by HPLC, demonstrating the presence of both intact and degraded products of the hybrid oligonucleotide in these samples (Fig. 4).

Bioavailability of hybrid oligonucleotide following oral administration. No significant degradative products were detected in the stomach contents. The majority of the radioactivity in the small intestine contents was present as the intact compound for up to 12 hr post-dosing. The radioactivity in the large intestine was present as both the intact form and degradative products. Based on the quantitation of total radioactivity in the gastrointestinal tract, feces, urine, plasma, and the remainder of the body in two groups of animals (3/group), the total absorption of the hybrid oligonucleotide-derived radioactivity was determined to be 10.2 ± 2.5% over 6 hr and 25.9 ± 4.7% over 12 hr following oral administration of the radiolabeled hybrid oligonucleotide. Total recovery of radioactivity in the study was 95 ± 6%.

Discussion

The rationale of the present study is to demonstrate oral bioavailability of antisense oligonucleotides, which may facilitate the development of this class of compounds as therapeutic agents. The hybrid oligonucleotide was chosen as a test compound because it has shown greater *in vitro* and *in vivo* stability over other PS-oligonucleotides [11–14, *]. The present study established three major points regarding the oral availability of oligonucleotides: (1) the hybrid oligonucleotide is stable in stomach and small intestine tissues and contents, (2) the hybrid oligonucleotide is absorbed through the portal venous blood system, and (3) the absorbed oligonucleotide-derived radioactivity is associated with the intact oligonucleotide and distributed into various tissues, and excreted into urine, as seen previously with i.v. administration [14]. The above data demonstrate oral absorption of oligonucleotide, providing

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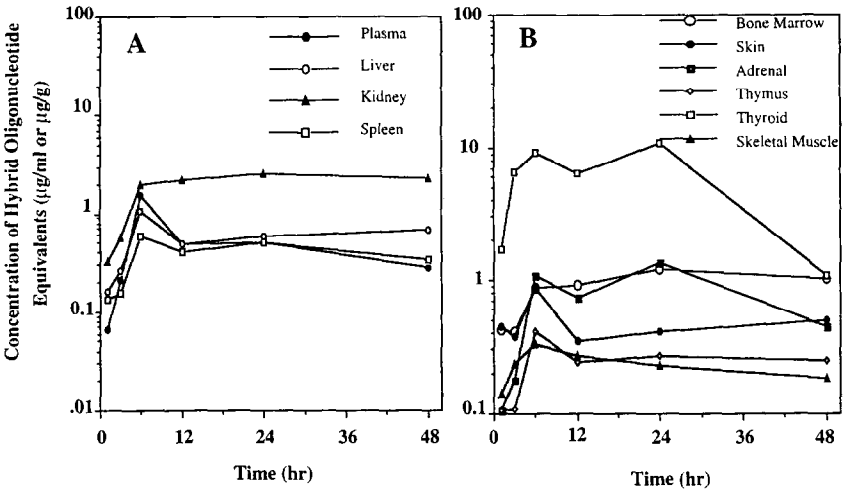

Fig. 2. Plasma and tissue concentration–time courses of radioactivity. Mean plasma and tissue concentrations were expressed as micrograms of hybridized oligonucleotide equivalents per milliliter of plasma or per gram of tissue/organ. Two animals were used for each time point. Tissue concentration was based on the quantitation of radioactivity.

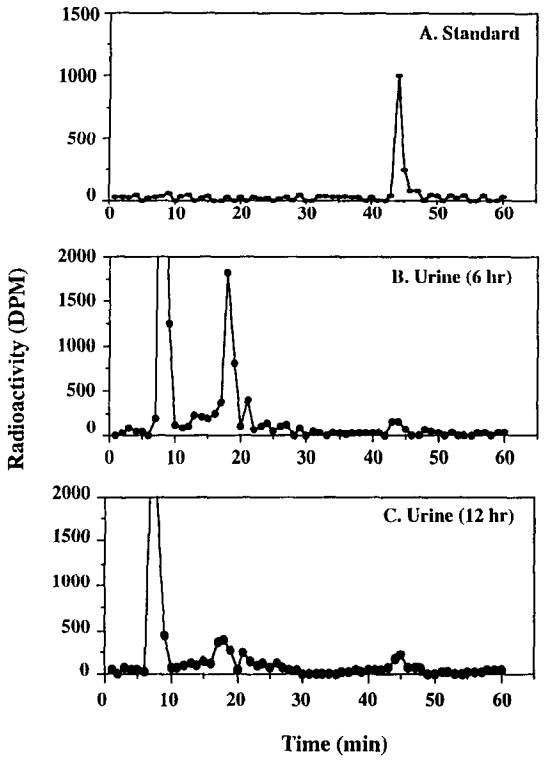

Fig. 3. HPLC analysis of radioactivity in urine. The radioactivities in urine samples were analyzed by HPLC. This figure shows representative chromatograms for urine samples from one animal at various times. Similar HPLC profiles were observed with each of the animals in the present study. Both intact oligonucleotide and metabolites with lower molecular weights were detected in urine with 24 hr post-dosing.

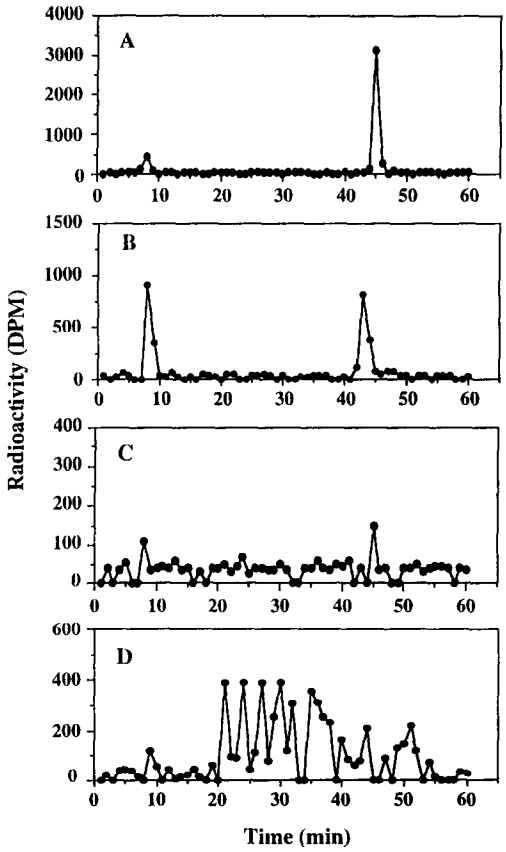

Fig. 4. HPLC analysis of radioactivity in the portal venous plasma (A), systemic plasma (B), liver (C), and kidney (D). This figure shows representative chromatograms for biological samples from one animal at 6 hr post-dosing. Similar HPLC profiles were observed with other animals in the present study.

the basis for future studies of oligonucleotides as orally available therapeutic agents.

Support for the oral availability of oligonucleotides derives from several observations. First, the stability of the oligonucleotide in the gastrointestinal tract was demonstrated by HPLC analysis. Polyacrylamide gel electrophoresis of extracts from these tissues confirmed the results (data not shown). Second, the majority of the radioactivity in the portal venous plasma was associated with the intact oligonucleotide, although degradative products were also detected. Third, radioactivity in the liver was associated with intact oligonucleotide as well as metabolites, as seen with i.v. injection. Fourth, intact oligonucleotide was found in systemic plasma, indicating that intact oligonucleotide is available to extrahepatic tissues. Fifth, a wide tissue distribution of radioactivity was observed with the intact oligonucleotide detected. Finally, a significant amount of radioactivity was excreted through urine, with intact oligonucleotide detected in the urine samples.

Despite extensive studies on the chemistry, molecular biology, and *in vitro* biological activity, limited studies on the pharmacology of antisense oligonucleotides have been conducted. To date, most *in vivo* animal studies have been carried out with PS-oligonucleotides [2–10]. In a preliminary study [2], we reported the pharmacokinetics of a ^{35}S-labeled 20-mer PS-oligonucleotide targeted to the HIV *tat* gene in mice via i.p. and i.v. injection. Radioactivity could be detected in most tissues up to 48 hr post-dosing. Similar results were reported by Iversen and colleagues [7, 8] following a study of ^{35}S-labeled 27-mer PS-oligonucleotide complementary to the HIV *rev* gene in mice at an i.p. dose of 50 mg/kg. Cossum *et al.* [3] reported a study of a ^{14}C-labeled 20-mer PS-oligonucleotide in female rats at an i.v. dose of 3.6 mg/kg. High radioactivities were found in the liver, kidney, bone marrow, skin and skeletal muscle. In plasma, the radioactivity was extensively bound to proteins. This PS-oligonucleotide was rapidly absorbed after intradermal injection [6], and tissue distribution of the PS-oligonucleotide was comparable to i.v. injection. A preliminary study in rabbits and monkeys by Iversen [7] suggested potential species differences in the pharmacokinetics of PS-oligonucleotides. Several PS-oligonucleotides and modified analogs have been studied recently in our laboratories for comprehensive pharmacologic analyses [5, 10, 14].

Pharmacologic study in humans is extremely limited, although several clinical studies of antisense oligonucleotides have been initiated. Recently, Crooke *et al.* [9] injected a 20-mer ^{14}C-labeled PS-oligonucleotide into patients' genital warts at a dose of 1 mg/wart. Radioactivity was present in wart tissues for 72 hr. Systemic absorption was also observed, and 30% of the radioactivity was eliminated through expiratory air. Human pharmacokinetics of a ^{35}S-labeled 25-mer PS-oligonucleotide was performed recently in our laboratory.* ^{35}S-Labeled GEM 91 was administered to 6 individuals by 2-hr i.v. infusion at a dose of 0.1 mg/kg body weight. Plasma disappearance curves for GEM 91-derived radioactivity could be described by the sum of two exponentials, with half-lives (mean ± SEM) of 0.18 (± 0.04) and 26.71 (± 1.67) hr. The chemical forms of radioactivity in plasma were further evaluated by PAGE and HPLC, demonstrating the presence of both intact GEM 91 and lower molecular weight metabolites. Urinary excretion represented the major pathway of elimination of GEM 91, with 49.15 ± 6.80% of the administered dose excreted within 24 hr and 70.37 ± 6.72% over 96 hr after GEM 91 administration. The radioactivity in urine was analyzed by HPLC and shown to be mainly lower molecular weight metabolites, indicating an extensive degradation of the PS-oligonucleotide.

The mechanisms responsible for the metabolism of oligonucleotides remain unclear. It is believed that the degradation of antisense oligonucleotides may be structure-, length-, dose-, administration route-, species-, and tissue-dependent. Compared with other oligonucleotides, PS-oligonucleotides are more stable *in vivo*. The degradation of PS-oligonucleotides is mainly by 3′-exonucleases [2, 4, 5, 7, 8, 10, 14]. After i.v. injection of a 20-mer PS-oligonucleotide into mice, there are no significant degradative products in urine [2]. In contrast, after i.p. injection, 75–90% of the PS-oligonucleotide in urine is degraded [2]. Interestingly, chain length extension of the administered PS-oligonucleotide was observed in the kidney, liver, and intestine. Recently, Cossum *et al.* [3, 6] reported that ^{14}C-labeled PS-oligonucleotide is eliminated mainly through expiratory air, and no intact PS-oligonucleotide is found in the urine or feces. However, other studies have shown that urinary excretion represents the major pathway of elimination following administration of ^{3}H- or ^{35}S-labeled oligonucleotides to animals [2, 4, 5, 7, 8, 10, 14] and humans.*

In conclusion, the present study provides initial evidence for the possibility of alternative delivery of antisense oligonucleotides as therapeutic agents. The mechanisms responsible for absorption of the hybrid oligonucleotide have not been defined in the present study. Further studies examining the mechanisms of transport of oligonucleotides in the gastrointestinal tract and liver, first-pass effects of the liver, enterohepatic circulation, and formulation of oligonucleotides are currently underway.

* Zhang R, Yan J, Shahinian H, Amin G, Lu Z, Saag MS, Jiang Z, Temsamani J, Schechter PJ, Martin RR, Agrawal S and Diasio RB, Human pharmacokinetics of an anti-HIV antisense oligonucleotide phosphorothioate (GEM 91) in HIV-infected individuals. *The 34th Interscience Conference on Antimicrobial Agents and Chemotherapy*, Orlando, FL, Oct. 4–7, 1994. Later Breaker Session A5, pp. 5.

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