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Biodistribution of phosphodiester and phosphorothioate siRNA

Dwaine A. Braasch,[a,†] Zain Paroo,[a,†] Anca Constantinescu,[b,†] Gang Ren,[b] Orhan K. Öz,[b] Ralph P. Mason[b] and David R. Corey[a,*]

[a]*Departments of Pharmacology and Biochemistry, University of Texas Southwestern Medical Center, Dallas, TX 75390-9041, USA*
[b]*Department of Radiology, University of Texas Southwestern Medical Center, Dallas, TX 75390-9058, USA*

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Abstract—Short interfering RNAs (siRNAs) are valuable tools for analyzing protein function in mammalian cell culture. This success has led to high expectations for in vivo and therapeutic applications. However, the pharmacokinetic properties of siRNA are not known. Here we report the biodistribution of a phosphodiester (PO) siRNA duplex and examine the effect of phosphorothioate (PS) linkages. Our findings indicate that biodistribution of siRNA is similar to that for single-stranded antisense oligonucleotides and offer insights for use of siRNA in vivo.

1. Introduction

Antisense oligonucleotides are significant tools for controlling cellular processes and have the potential to make important contributions to basic science and medicine.[1,2] The impact of antisense lies in its generality. Examination of an mRNA sequence immediately suggests the design of complementary oligonucleotides that have the potential to be high affinity ligands. Efficient synthetic protocols make it possible to obtain hundreds or thousands of oligonucleotides to elucidate the function of specific genes. Once active oligonucleotides are identified they can be used as tools for basic research in cell biology, to validate proteins as targets for drug discovery, or as lead compounds for the development of therapeutics.

Recently, small interfering RNAs (siRNAs) have proven to be robust agents for analysis of biological function in cell culture.[3–7] Early success has led to high expectations for in vivo and therapeutic applications. However, the behavior of siRNA in vivo is not known. Key unanswered questions are whether the strengths of siRNAs can be translated into general strategies for controlling gene expression in animals and whether siRNA can become a new class of therapeutic agent.

Recent reports have demonstrated that siRNA can decrease expression of target genes in the liver.[8–10] It is not clear, however, whether siRNA can be effectively distributed to other organs or how biodistribution might be improved. Single-stranded antisense oligonucleotides have been shown to reduce gene expression in animals and chemical modifications, in particular the introduction of phosphorothioate (PS) linkages, improve biodistribution and pharmacokinetic properties.[11–13] Here, we characterize the biodistribution and pharmacokinetic properties of synthetic duplex RNA in mice and determine the effects of PS substitutions on the behavior of siRNA in vivo.

2. Synthesis and characterization of siRNAs

We synthesized two duplex RNAs for these studies. One contained two phosphodiester (PO/PO) RNA strands, the design commonly used for siRNA. The other siRNA contained one phosphodiester strand and one phosphorothioate strand (PO/PS) to test the hypothesis that PS linkages would improve the pharmacokinetic properties of the duplex. We have previously shown that siRNA duplexes that contain PS linkages retain the ability to reduce gene expression in mammalian cell culture.[5] The duplexes used here had similar thermal stabilities as measured by melting temperature (T_m) values, 76 °C for the PO/PO duplex and 72 °C for the PO/PS duplex (Fig. 1A).

Previously, we had shown that phosphodiester and phosphorothioate RNA duplexes were stable for up to 72 h when incubated in 5% fetal bovine serum.[5] These

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* Corresponding author. Tel.: +1-214-648-5096; fax: +1-214-648-5095; e-mail: david.corey@utsouthwestern.edu
† Contributed equally to this work.

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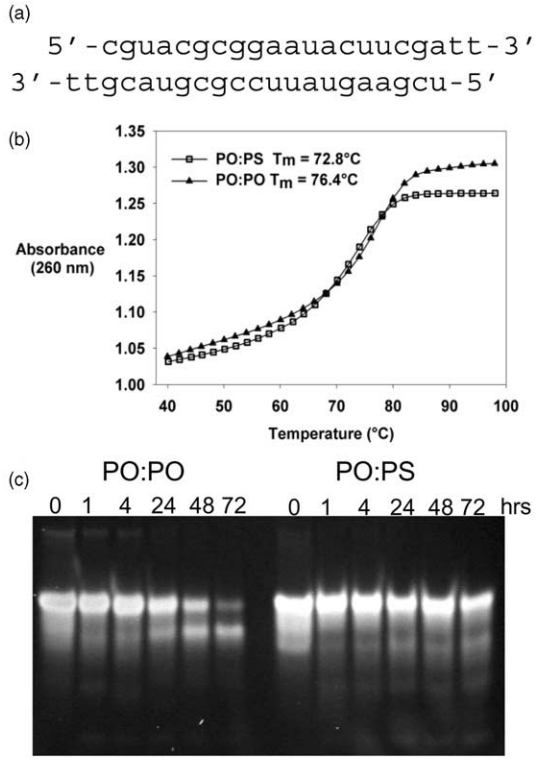

Figure 1. Thermal and serum stability of PO:PO and PO:PS RNA duplexes. (A) Thermal stability profiles for PO/PO and PO/PS duplex RNAs. (B) Serum stability of PO/PO and PO/PS duplex RNAs. Samples were incubated in 50% (v/v) mouse serum at 37 °C.

conditions cause single-stranded RNA to be degraded within seconds. Such serum conditions reflect those used in tissue culture experiments and the stability of duplex RNA in low serum concentrations probably contributes to the robustness of siRNA in cell culture.

To determine stability to cleavage by nucleases under conditions more similar to those in vivo, we incubated the PO/PO homoduplex and the PO/PS heteroduplex at 37 °C in 50% murine serum for up to 72 h. In contrast to our previous results in bovine serum, addition of PS linkages to one strand significantly stabilized the PO/PS duplex relative to the PO/PO duplex. Degradation of the PO/PO duplex was observed after 24 h, while the analogous PO/PS duplex remained intact for up to 72 h (Fig. 1B). The lower serum stability of the PO/PO duplex in the experiments reported here relative to our previous work[5] is probably due to use of a 10-fold higher concentration of serum.

3. Labeling of siRNA with ^{123}I and ^{125}I

To investigate biodistribution siRNA was labeled with with either ^{123}I or ^{125}I. Iodine labels were added on the 3′-terminus of the sense strand through a C7 amide linkage modified with tyrosine. For gamma camera planar imaging studies, the PO/PO homoduplex was labeled with ^{123}I. ^{123}I was chosen for this purpose because its emission properties are well suited for commonly available gamma cameras.

For tissue distribution studies, both duplexes were labeled with ^{125}I (Fig. 2A). ^{125}I was chosen because its half-life is long enough to allow biodistribution studies over a 72 h period and because ^{125}I has been used to examine the biodistribution of single-stranded antisense oligonucleotides.[14] The presence of PS linkages led to inefficient iodination, therefore, the PO strand of the PO/PS heteroduplex was labeled. It is possible that inefficient iodination of PS-containing duplexes was due to greater interactions between iodine and sulfur relative to iodine and oxygen. When analyzed by gel electrophoresis after labeling with iodine, duplex RNA (Fig. 2B) was intact. We note that it is possible that the iodine modification improves stability of the siRNA duplex to degradation by nucleases.

4. Imaging the early distribution of siRNA

In vivo imaging offers the ability to visualize early events following the administration of siRNA, allowing observations that would not have been possible otherwise. We used planar imaging of ^{123}I labeled PO/PO siRNA to evaluate distribution during the first 10 min after intravenous injection. Serial images were taken at one min intervals and revealed that distribution to the liver and kidney occurred within the first min after injection and peaked within the first 5 min (Fig. 3). By 5 min, some radiolabel was apparent in the bladder, suggesting that some duplex RNA is rapidly excreted.

5. Biodistribution of siRNA

To further analyze the tissue distribution of siRNA, we administered the ^{125}I labeled PO/PO and PO/PS

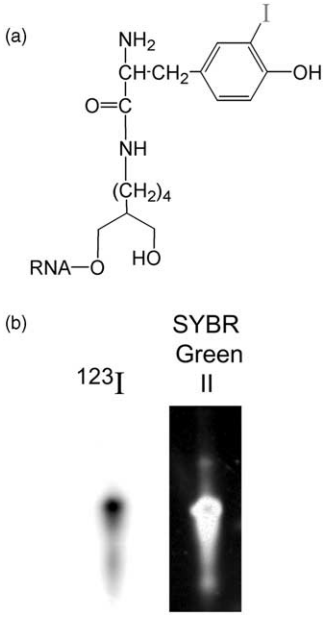

Figure 2. Synthesis and analysis of labeled RNA. (A) Chemical structure of an RNA strand labeled at the 3′ position with a seven carbon linker and iodinated tyrosine. (B) Autoradiograph of 1 μCi of ^{123}I-labeled PO/PO homoduplex siRNA and corresponding SYBR Green II staining after characterization by denaturing gel electrophoresis.

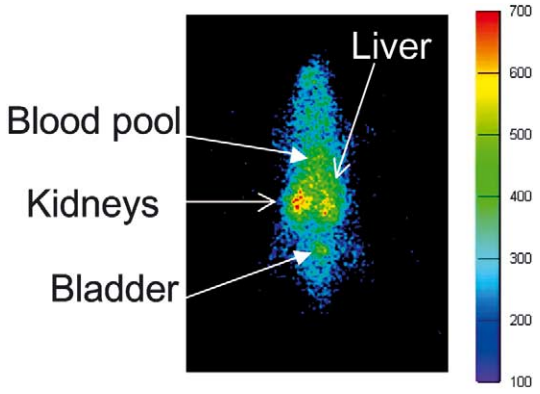

Figure 3. Planar imaging of uptake of the PO/PO homoduplex five min after intravenous adminstration. Shown is a representative pseudocolor image. Relative activity per pixel is depicted on the scale to the right.

duplexes to mice by intravenous injection. Animals were divided into groups of four and sacrificed at 1, 4, 24, 48, and 72 h. The lung, blood, brain, liver, lungs, kidney, and spleen were collected and the radioactivity in each organ was measured by scinitillation counting (Fig. 4).

^{125}I-labeled siRNA preferentially accumulated in the kidney and liver at all time points. These findings are similar to observations with single stranded DNA and 2′-*O*-meRNA oligonucleotides labeled with ^{125}I,[14] ^{18}F,[14] ^{35}S,[12,13] or ^{3}H.[11] Lesser concentrations were observed in the lung, spleen, and heart. Very low concentrations were detected in the brain, presumably reflecting a poor ability to penetrate the blood–brain barrier. Levels of siRNA decreased markedly after 24 h, however, detection persisted for up to 72 h.

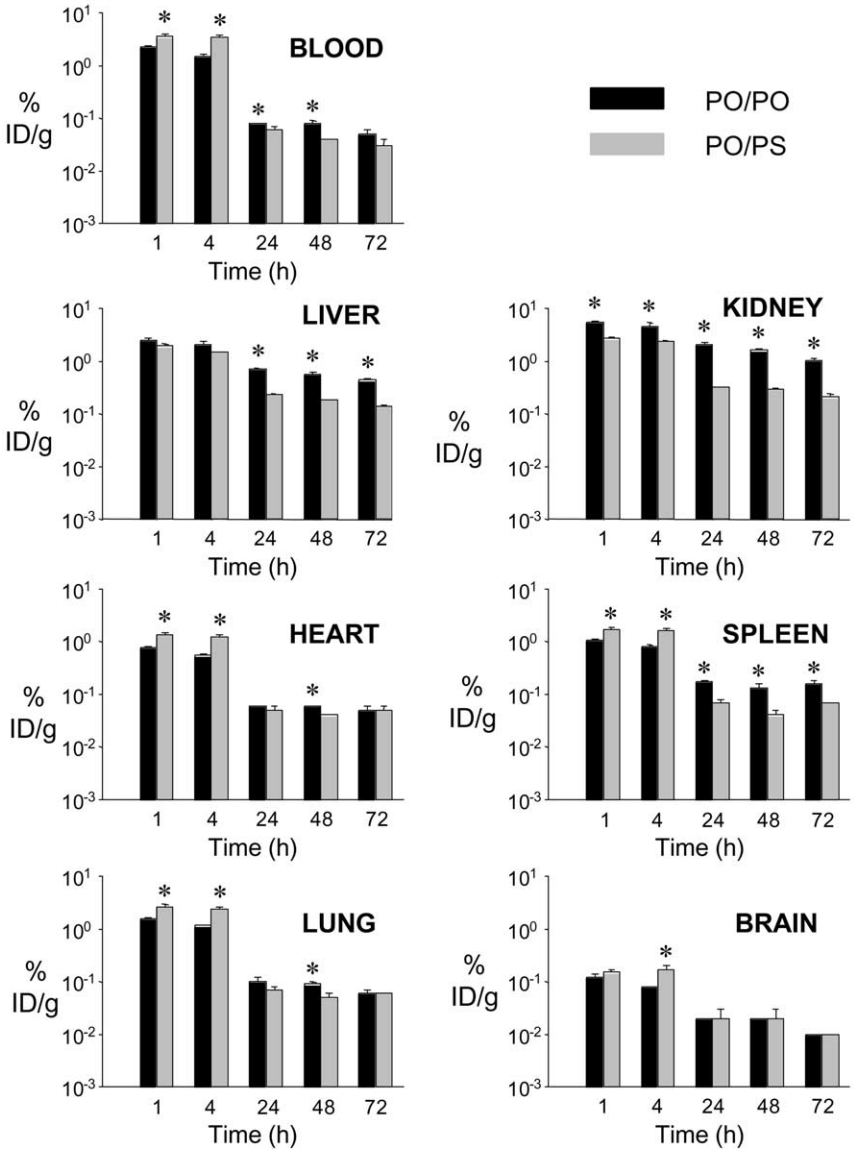

Figure 4. Biodistribution of PO/PO (black) and PO/PS (grey) siRNA duplexes in mice. At 1, 4, 24, 48, and 72 h following administration of oligonucleotide animals were sacrificed. Activity was calculated as percentage of injected dose per gram (% ID/g). Data are expressed as means ± SE of four animals per time point per siRNA treatment group. Statistical comparisons between PO/PO and PO/PS were performed by Student's *t* tests. * Indicates a statistically significant difference between PO/PO and PO/PS ($P < 0.05$). The Y axes are logarithmic.

Because of the paucity of information regarding delivery of siRNA in vivo, we performed preliminary experiments, using the PO/PO duplex, comparing intraperitoneal and intravenous injections. A similar pattern of biodistribution between the methods of delivery was observed (data not shown), suggesting that the route of administration was not a critical variable for tissue uptake.

During the first four h post-injection the concentration of the PO/PS heteroduplex in the blood was significantly higher than the concentration of the PO/PO homoduplex. Single stranded PS DNA or PS 2′-*O*-methoxyethyl RNA oligomers have yielded similar results relative to analogous PO oligomers.[12,13] Higher circulating concentrations of the PO/PS duplex may reflect the superior serum stability of the PO/PS duplex relative to the PO/PO duplex (Fig. 1) and may suggest an advantage for the incorporation of PS linkages into siRNA.

We note that in Figure 1, superior serum stability for the PO/PS duplex versus the PO/PS duplex did not become apparent until 24 h, whereas differences in the concentration in the blood were apparent after 1 h. One explanation for this discrepancy is that the conditions used for the in vitro experiment (50% serum) were substantially different from those encountered in vivo. We also note that these biodistribution experiments indicate the location of the iodine label. It is not certain that the siRNA is intact and differences between amounts of the intact PS/PO duplex and the intact PO/PO duplex may be larger than they appear.

The introduction of PS linkages yielded mixed results for distribution of siRNA to various organs. We observed increases in distribution to the spleen, heart and lung at one and four h, possibly reflecting the higher circulating concentrations at these times. Introduction of PS linkages reduced the level of siRNA delivered to the liver and kidney. Overall, the effects were modest; suggesting that introduction of PS linkages may not play a major role in determining the distribution of siRNA.

6. Conclusion

siRNA is an efficient and convenient tool for controlling gene expression in cultured mammalian cells. Use of synthetic siRNA for inhibiting gene expression in animals would extend the range of research applications for RNAi and provide the basis for new therapeutic strategies. Our data are significant because they indicate that siRNA has favorable pharmacokinetic properties and can be delivered to a wide range of organs. Antisense compounds are progressing through clinical trials.[1,2] The similarity between biodistribution of siRNA and antisense oligonucleotides encourages the belief that siRNA will also have substantial potential in human trials and that chemical modifications may have a significant influence on in vivo potency.

7. Experimental

7.1. Synthesis and analysis of iodine-labeled duplex RNA

RNA strands (plus-strand 5′-cguacgcggaauacuucgatt-3′ minus-strand 5′-ucgaaguauuccgcguacgtt-3′), were synthesized to include either PO or PS linkages.[5] The duplexes were targeted to firefly luciferase and reduced luciferase activity in cultured CV-1 cells transfected with the gene encoding luciferase (data not shown). An siRNA against luciferase was chosen because luciferase is not present in mice and the anti-luciferase siRNA is not likely to exert sequence-specific physiological effects. It is possible that an siRNA against a target mRNA expressed in a specific tissue might lead to greater distribution to that tissue, but more research will be needed to determine whether this outcome is plausible. Serum stability studies were performed as described[5] except that 50% mouse serum was substituted for 5% bovine serum.

Desalting and purification of dimethoxytrityl-protected oligomers was performed using Oasis HLB (3 cc) sample extraction columns (Waters Corporation, Milford, MA). Oligomers intended for iodination were synthesized on a 3′-amino modified C7 CPG resin (Glen Research Sterling, VA). After synthesis and before cleavage and deprotection, the F-moc group on the C7 linker was removed and a tyrosine residue (Advanced ChemTech, St. Louis, MO) added using an Expedite 8909 synthesizer (Applied Biosystems, Foster City CA). Following the addition of the tyrosine residue, the RNA strands were cleaved from the solid support and deprotected.

^{125}I-labeling of the tyrosine residue on the plus-strand was performed by the Iodo-Gen method.[15] Labeling was preceded by the addition of 50 μL of 0.5 M PB (sodium phosphate, pH 7.4) to the side of the tube opposite the Iodo-Gen pellet. The RNA–tyrosine compound was dissolved in 75 μL of diethylpyrocarbonate treated water and a final concentration of 0.05 M PB was added, followed immediately by the addition of ~500 μCi of Na-^{125}I (carrier free, Perkin Elmer, Shelton, CT) or ^{123}I (carrier free, MDS Nordion, Vancouver, BC). PO-homoduplex was preannealed in 0.05 M PB prior to labeling. The PO-RNA strand intended for PO/PS heteroduplex formation were labeled as a single-strand and then annealed with the PS-RNA minus-strand. Two labeling reactions were performed per molecule to maximize the overall efficiency of labeling. Each labeling was performed with 50 μg of Iodo-Gen in a 12×75 cm tube for 5 min on ice in 0.05 M Sodium Phosphate buffer pH 7.4. Iodinated RNAs were purified from non-incorporated isotope with MicroSpin G-25 Sephadex size exclusion columns (Amersham) which had been washed 5 times with DEPC (diethyl pyrocarbonate, 0.1%) treated water.

An aliquot equal to 1 μCi of labeled RNA was mixed with 8 μL of formamide and subjected to electrophoresis on a 15% denaturing gel containing 8 M urea (National Diagnostics, Atlanta, GA) in TBE buffer

(0.089 M Tris base, 0.089 M boric acid (pH 8.3) and 2 mM Na_2EDTA). Gels were exposed to film and then stained with a 1:20,000 dilution of SYBR Green II (Amresco Inc., Solomon OH) in TBE.

8. Imaging in vivo localization

All animal procedures were performed in accordance with the University of Texas Southwestern Medical Center Institutional Animal Care and Research Advisory Committee. Male Balb/c mice (~25 g) were obtained from Harlan Laboratories (Indianapolis, IN). Radiolabeled siRNA (^{123}I, 10 μCi) was intravenously administered to anesthetized mice. Animals were immediately placed on one head of a triple headed Toshiba 9300 gamma camera equipped with a low energy high resolution collimator. Serial one min images were acquired for 30 min. Experiments were repeated three times. A 20% window was centered on the 159 keV peak. Data was collected in a 128 by 128 matrix. Images were generated using a standard filtered back projection technique.

9. Biodistribution

Syringes containing ^{125}I-siRNA with either double stranded PO/PO RNA or a heteroduplex of PO/PS RNA were prepared. Animals were lightly anesthetized by inhalation of isoflurane (Abbott Laboratories; Chicago, IL) and then injected iv with ^{125}I-labeled siRNAs. Activity of syringes was measured before and after injection to determine the net dose administered (~3.5 μCi per mouse). At 1, 4, 24, 48 and 72 h following administration of oligonucleotide, organs were harvested, rinsed with PBS, blotted dry, weighed and radioactivity counted with a Wizard Automatic γ-Counter (Perkin–Elmer). Activity was calculated as percentage of injected dose per gram (% ID/g). Data are expressed as means ± SE of four animals per time point per siRNA treatment group.

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