Pharmacokinetic Profiling of Conjugated Therapeutic Oligonucleotides: A High-Throughput Method Based Upon Serial Blood Microsampling Coupled to Peptide Nucleic Acid Hybridization Assay

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Therapeutic oligonucleotides, such as small interfering RNAs (siRNAs), hold great promise for the treatment of incurable genetically defined disorders by targeting cognate toxic gene products for degradation. To achieve meaningful tissue distribution and efficacy *in vivo*, siRNAs must be conjugated or formulated. Clear understanding of the pharmacokinetic (PK)/pharmacodynamic behavior of these compounds is necessary to optimize and characterize the performance of therapeutic oligonucleotides *in vivo*. In this study, we describe a simple and reproducible methodology for the evaluation of *in vivo* blood/plasma PK profiles and tissue distribution of oligonucleotides. The method is based on serial blood microsampling from the saphenous vein, coupled to peptide nucleic acid hybridization assay for quantification of guide strands. Performed with minimal number of animals, this method allowed unequivocal detection and sensitive quantification without the need for amplification, or further modification of the oligonucleotides. Using this methodology, we compared plasma clearances and tissue distribution profiles of two different hydrophobically modified siRNAs (hsiRNAs). Notably, cholesterol-hsiRNA presented slow plasma clearances and mainly accumulated in the liver, whereas, phosphocholine-docosahexaenoic acid-hsiRNA was rapidly cleared from the plasma and preferably accumulated in the kidney. These data suggest that the PK/biodistribution profiles of modified hsiRNAs are determined by the chemical nature of the conjugate. Importantly, the method described in this study constitutes a simple platform to conduct pilot assessments of the basic clearance and tissue distribution profiles, which can be broadly applied for evaluation of new chemical variants of siRNAs and micro-RNAs.

Keywords: pharmacokinetics, therapeutic oligonucleotides, siRNA, RNA interference, biodistribution

Introduction

GENE SILENCING USING small interfering RNAs (siRNAs) holds great promise for the treatment of incurable genetically defined disorders by preventing the expression of toxic gene products. This class of therapeutic oligonucleotides targets cognate disease-causing messenger RNA (mRNA) for degradation [1]. The inherent sequence specificity, potency, and lasting activity make RNA interference (RNAi)-based drugs an ideal therapeutic strategy for disease targets that have been deemed “undruggable” [2,3]. Although historically restricted

by their poor *in vivo* stability and biodistribution, recent advancements in oligonucleotide chemistry have greatly improved the pharmacokinetic (PK) and pharmacodynamic (PD) properties of synthetic siRNAs. Indeed, the major breakthrough in the field consists of fully modified siRNAs conjugated to branched *N*-acetylgalactosamine (GalNAc), which efficiently mediates targeted delivery to hepatocytes in humans [4]. Based on the success achieved by GalNAc-conjugated siRNAs, several other promising ligands are expected to emerge with the goal of enabling targeted delivery beyond the liver. Clear understanding of plasma clearances and tissue distribution profiles

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of these compounds will be of great importance to establish their potential for modulation of gene expression *in vivo*.

Determining PK/PD relationships is an essential step in the characterization of novel gene silencing technologies. Indeed, assessment of the PK properties of new siRNA and/or micro-RNA (miRNA) variants at early stages of discovery is key to enable optimization of dosing regimens and prioritization of lead compounds [5]. Since oligonucleotides do not readily diffuse through the plasma membrane, *in vitro* assays traditionally employed for screening of small molecules, such as the parallel artificial membrane permeability assay, have limited applicability in this case [5,6]. Thus, an initial determination of PK profiles is preferentially performed *in vivo* using rodents, and typically require >3 animals/compound, per route of administration and per time point [7–9]. The large number of animals necessary in these studies and the amount of starting compound required to conduct such investigations are not logistically practical for high-throughput assessment in small laboratories.

Furthermore, most preclinical biodistribution studies published to date have been conducted using oligonucleotides modified with fluorescent [10–12] or biotin tags [13,14], or using radioisotopes [15–18]. Although these labels are undeniably useful for microscopic visualization of cellular distribution of therapeutic oligonucleotides [19,20], they present several limitations as surrogate measures of oligonucleotide concentrations *in vivo*. Rapid cleavage by exonucleases, or other enzymes, may render these labels free in the systemic circulation to be distributed and cleared independently from the parent siRNA compound [21,22]. These moieties, which are covalently attached to siRNAs, may largely interfere with the biodistribution of the parent siRNA, hence reducing their predictive utility in PK profiling studies.

Alternatively, highly sensitive noncompetitive hybridization-ligation enzyme-linked immunosorbent assays have been developed and are widely used as a strategy for assessing PK and tissue distribution profiles of antisense oligonucleotides (ASOs) [23–25]. Although these assays are a remarkable tool for ASO characterization [23,24], their application for siRNA quantification has been limited, possibly due to the requirement for double labeling of these compounds [14]. Therefore, there is an unmet need for a broadly applicable quantitative methodology that supports preliminary evaluation of plasma PK and tissue distribution profiles of therapeutic oligonucleotides, using small quantities of oligonucleotides and low animal numbers.

In this study, we describe a high-throughput method for PK profiling of conjugated siRNAs that couples serial blood microsampling with a highly sensitive assay for quantification based on peptide nucleic acid (PNA) hybridization. The PNA hybridization assay uses an appropriate fluorescently labeled PNA probe that fully hybridizes with the target guide strand of the siRNA in tissue lysates, and antisense strand-PNA hybrids are subsequently resolved and analyzed by high-performance liquid chromatography [26]. The required reagents are easy to maintain compared to other methods, and yet the remarkable sensitivity of the assay enables very small samples of blood and tissue to be collected for quantification. Therefore, using three to five animals per test compound, preliminary PK and tissue distribution profiles can be generated. Importantly, the method described in this study is compatible with high-throughput sample processing in a 96-

well plate format, which accelerates sample turnaround time and facilitates screening of multiple compounds.

To validate the utility of this method, we selected two chemically distinct conjugated asymmetric hydrophobically modified siRNA (hsiRNAs): cholesterol-hsiRNA (Chol-hsiRNA) and phosphocholine-docosahexaenoic acid-hsiRNA (PC-DHA-hsiRNA). Except for the conjugated ligand, these oligonucleotides contained the same backbone and sugar modifications, previously described in Nikan *et al.* [27]. Earlier studies with these compounds have shown that they display significantly different biodistribution profiles in the brain, after intrastriatal stereotaxic injections [27,28]. In these studies, the degree of distribution strongly and inversely correlated with the hydrophobicity. Although highly hydrophobic Chol-hsiRNAs presented limited spread from the site of injection, PC-DHA-hsiRNAs diffused to other brain regions further away from the striatum, such as the cortex [27,28]. Hence, to demonstrate the suitability of the method described for the analysis of these chemically distinct hsiRNAs, we decided to investigate the blood/plasma PK and tissue distribution profiles of these compounds after intravenous (IV) and subcutaneous (SC) administrations.

Materials and Methods

Synthesis and preparation of conjugated hsiRNAs

hsiRNAs consist of fully modified 20-nucleotide antisense (guide) strand and a 15-nucleotide sense (passenger) strand that anneal forming an asymmetric duplex. Backbone and sugar modifications contained within the nucleotide structure of each strand have been previously described [27]. For the purpose of this method validation, both hsiRNA conjugates were synthesized using a previously identified sequence targeting peptidylprolyl isomerase B (PPIB) mRNA [29] (Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/nat).

Cholesterol and phosphocholine docosahexaenoic acid moieties were conjugated to the 3'-end of the sense strand as described in Nikan *et al.* (2016) [27] and Nikan *et al.* (2017) [28]. Briefly, functionalized O-DMTr TEG linked cholesterol was succinated and coupled to the amine-bearing controlled pore glass (CPG) to obtain cholesterol-conjugated solid support. In the case of PC-DHA-hsiRNAs, phosphocholine functionality was first coupled to a protected L-serine, deprotected, and then attached to a succinate conjugated C7 amine linker CPG. Both functionalized CPG were used for the solid-phase oligonucleotide synthesis of the sense strand and a Unylinker[®] terminus (ChemGenes, Wilmington, MA) for the antisense strand. Oligonucleotides were synthesized on an Expedite ABI DNA/RNA Synthesizer following established protocols.

Cleavage and deprotection were carried out using 40% aqueous methylamine at 45°C for 1 h for sense strands containing phosphocholine docosahexaenoic acid, and at 65°C for 15 min for sense strands containing cholesterol and antisense strands. Oligonucleotides were then cooled at room temperature, and subsequently frozen and dried under vacuum in a speed-vac. The resulting pellets were suspended in water. The final purification of oligonucleotides was performed on an Agilent Prostar System (Agilent, Santa Clara, CA) equipped with a Hamilton HxSil C18 column (150×21.2) using the following conditions: buffer A: 50 mM sodium acetate in

water with 5% acetonitrile (ACN); buffer B: ACN; gradient: 90% A, 10% B to 10% A, 90% B in 20 min; temperature: 70°C; and flow rate: 5 mL/min for sense strands; and with a Dionex NucleoPac PA-100 9×250 using the following conditions: buffer A: 30% ACN in water; buffer B: 30% ACN in 1 M sodium perchlorate (NaClO₄); gradient: 100% A, 100% A to 30% A in 7 min, 80% B in 30 min; temperature: 65°C; and flow rate: 10 mL/min for the antisense strand.

The pure oligonucleotides were collected, desalted by size-exclusion chromatography using a Sephadex G25 column (GE Healthcare Life Sciences, Marlborough, MA), and lyophilized. Antisense and sense strands were annealed and duplex formation validated through gel electrophoresis. Finally, duplexed Chol-hsiRNAs and PC-DHA-hsiRNAs were lyophilized using a speed-vac and resuspended in sterile 0.9% sodium chloride (NaCl).

In vivo dosing and serial blood microsampling

In vivo experiments were conducted in FVB/N female mice ~12-week old (~22–25 g) obtained from Charles River. Experiments were performed only in females to facilitate housing and remove the interference of gender variability. Due to the discovery nature of this study, whereby we aim to screen the PK/distribution profiles of conjugated hsiRNAs, gender variability may dilute meaningful differences between compounds, rendering the results unusable for the selection of candidates. Although males were not considered in this study, subsequent studies that aim thorough PK and toxicological characterization of selected compounds will require inclusion of both sexes. In this study, mice were given ~1 week to habituate to the animal facility and maintained on a 12-h light/12-h dark cycle with temperature (22°C ± 1°C)- and humidity (~55%)-controlled conditions. All animal procedures were approved by the University of Massachusetts Medical School Institutional Animal Care and Use Committee (IACUC, protocol no. A-2411).

Forty-eight hours before the initiation of the study, animals were briefly anesthetized and hair was removed from both hind legs using Nair[®] hair removal cream. Hair removal is essential as it minimizes interference with blood collection. The animals included on the IV arm of the study were placed on a heat blanket to enable vasodilation of the tail vein and simplify injections. Chol-hsiRNAs and PC-DHA-hsiRNAs (20 mg/kg, ~200 µL) were administered intravenously through the tail vein, or subcutaneously between the shoulder blades (*n* = 3–6/group/route of administration).

Microsamples of blood (~20 µL) were serially collected from the saphenous vein at given time points (in a similar manner as described in Ref. [30]). To this end, animals were restrained, the hind leg immobilized, and slight pressure applied gently above the knee joint to dilate the lateral saphenous vein. Using a 30G1/2 sterile needle or a lancet, the vein was punctured and the blood droplet collected using Microvette[®] CB300 K2R tubes (Cat. No. 16.444.100; Sarstedt), which allow collection of small droplets of blood through capillary action [30]. Slight pressure is applied on the punctured site to stop the bleeding, and the animal was returned to its cage. Subsequently, 10 µL of blood was pipetted into a 96-well plate containing tissue lysis solution (Cat. No. MTC096H; MasterPure, EpiCentre). The remainder of the blood was centrifuged at 10,000 *g* at 4°C for 10 min, to isolate plasma (similar

protocols for plasma isolation found in Refs. [11,30]). Plasma was then transferred to a 96-well plate with the same format/template to simplify sample processing. Lysed blood and plasma samples were stored at –80°C until analysis.

After collection of the last blood sample at 48 h, animals were euthanized and perfused with phosphate-buffered saline for at least 3 min at 7 mL/min to eliminate the interference of blood. Tissues, including liver, kidney, spleen, and skin (between the shoulder blades) were harvested and placed in RNAlater[®] (Cat. No. R0901; Sigma) overnight. RNAlater was then removed and tissues frozen at –80°C until processed for quantification. Two millimeter tissue punches (Cat. No. MTP-33 31; Braintree Scientific) with ~10 mg were taken and lysed as described in the PNA hybridization assay section.

PNA hybridization assay

Levels of the hsiRNA guide (antisense) strand in blood, plasma, and other tissues were measured by PNA Hybridization Assay. The assay was originally developed by Roehl *et al.* [26] and is now being offered as a bioanalytical service in a regulated GLP/GCP environment (Axolabs, Germany). In this assay, a fully complementary Cy3-labeled PNA probe was used to hybridize the antisense strand present on hsiRNA duplexes (Supplementary Table S1). Briefly, blood and plasma samples were further diluted in lysis solution to a total of 200 µL (Cat. No. MTC096H; MasterPure, EpiCentre) containing 1 µL proteinase K (20 mg/mL) (Cat. No. 25530-049; Invitrogen). Alternatively, liver, kidney, and spleen samples were lysed and digested in 300 µL of QG Homogenizing Solution (Cat. No. QG0517; Affymetrix) containing 1–2 µL proteinase K (20 mg/mL).

All samples were processed in QIAGEN Collection Microtubes holding 3 mm tungsten beads, and homogenized using a QIAGEN TissueLyser II for 2 × 3 min at frequency 30/s. Samples were then centrifuged at ~1,000 *g* for 10 min, incubated for 1 h at 55°C–60°C, and then transferred to a 96-well plate. At this time, if mRNA quantification is intended, a sample of the tissue lysate may be separated to perform QuantiGene[®] branched DNA (bDNA) assay (following manufacturer's instructions).

For PNA quantification, sodium dodecyl sulfate was precipitated using 20 µL potassium chloride (3 mol/L) per sample (blood, plasma, urine, etc.) and centrifuged at 4,000 *g* for 15 min. Supernatants were then diluted in 150 µL of hybridization buffer (50 mM Tris 10% ACN, pH 8.8) containing ~33 nM (5 pmol/150 µL of total sample) of PPIB Cy3-labeled PNA (PNABio, Thousand Oaks, CA) and transferred to a PCR 96-well plate (Cat. No. AB-0800/W; Thermo Scientific). Annealing was carried out on a Biorad c1000 thermal cycler using the following protocol: 90°C for 15 min and 50°C for 15 min.

Plates were then loaded into an Agilent Technologies 1260 Infinity Quad-pump High-Performance Liquid Chromatography system with a 1260 HiP ALS autosampler. Samples were programmed to run through a DNAPac PA100 anion exchange column (Thermo Fisher Scientific) and detected by a 1260 FLD fluorescent detector. The mobile phase consisted of buffer A [50% water, 50% ACN, 25 mM Tris-HCl (pH 8.5), and 1 mM ethylenediaminetetraacetate] and buffer B (800 mM NaClO₄ in buffer A). A steep gradient of buffer B (10%–100% within 2.5 min) was used for elution of guide strand-PNA hybrids. The Cy3 signal (550ex/570em) was

monitored and recorded, and peaks were integrated to obtain the area under the curve (AUC). Final concentrations were ascertained by correlating AUCs obtained from experimental samples with a corresponding calibration curve. Calibration curves were generated by spiking known amounts of the respective hsiRNA conjugate into tissue lysates derived from an untreated animal (Supplementary Fig. S1). Spiked samples for calibration and experimental samples were processed and analyzed side by side under the same laboratorial conditions.

Pharmacokinetic parameter analysis

All data points represent mean $\pm$ standard deviation of the observed values at each time point. PK parameters were calculated based on a model-independent analysis using PKSolver [31]. Absorption ($t_{1/2\text{abs}}$) and distribution ($t_{1/2\alpha}$) half-lives were calculated based on the method of residuals. Blood/plasma bioavailability (F) following SC administration was calculated by the ratio of AUC_{SC} and AUC_{IV} for both Chol-hsiRNAs and PC-DHA-hsiRNAs.

Results

Tandem serial blood microsampling/PNA hybridization assay allows for high-throughput PK profiling of conjugated hsiRNAs

The method described in this study consists of a 15-step protocol, which allows high-throughput characterization of blood/plasma PK properties of synthetic RNAi-based drugs (Fig. 1). Briefly, the method is based on serial blood microsampling from the saphenous vein upon compound administration, followed by PNA hybridization assay for quantification.

Serial blood microsampling from the saphenous vein allowed for small droplets of blood to be obtained from the same animal over the course of 48 h in a controlled manner. This sampling technique reduced the inherent variability of injecting multiple animals for assessment at different time points, yet allowed for full characterization of the plasma PK of two chemically different hsiRNAs. For the purpose of this study, a significantly lower number of animals was required ($n = \sim 3\text{--}6/\text{compound}$ per route of administration) than what is traditionally used in PK studies for therapeutic oligonucleotides ($n \geq 3/\text{compound}$ per route of administration, per time point) [7,8]. Limiting the total amount of blood to $\sim 200\ \mu\text{L}$ per animal, this protocol is applicable to both mice and rats. The lower animal usage is important, not only from the ethical standpoint but also from the perspective of small laboratories, since reduced quantities of test compounds are required.

Highly selective and sensitive detection of the antisense strand of the tested hsiRNAs, from blood and plasma samples, were achieved by hybridization to a complementary Cy3-labeled PNA probe without the need for RNA extraction and amplification. Sample processing was carried out in a 96-well plate format, which allowed simultaneous analysis of multiple samples and organs. Anion exchange chromatography was used to efficiently resolve unhybridized PNA and autofluorescent tissue remnants, which elute in the void volume of the column, from hybridized antisense-PNA (Supplementary Fig. S1). Corresponding peaks were integrated and all results presented were at least ~ 3 -fold above the limit of quantification and ~ 10 -fold above the limit of

detection. Furthermore, the data demonstrate that this highly versatile PNA-based method may be used to assess tissue retention in peripheral organs, such as liver, kidney, and others. Indeed, others have also successfully used this tool to not only characterize and quantify biodistribution of sense and antisense strands of siRNA oligonucleotides but also their metabolites, in one single measurement [26,32–34]. The ability to identify and quantify metabolites in different biological matrixes makes this assay a particularly attractive method for follow-up mass-balance studies. Although not aimed for initial screening of PK proprieties, mass-balance studies are aimed at characterizing routes and extent of elimination, and should therefore be part of a more comprehensive PK evaluation.

It is important to note that this method facilitates selective detection of conjugated hsiRNAs without requiring prior modification of the parent hsiRNA molecule with an appropriate dye (eg, cyanine dye) or biotin, or using radioisotopes. Although dye conjugation is one of the most commonly used strategies to study biodistribution of oligonucleotides [36], several studies have demonstrated that the addition of such molecules may substantially change the PK/PD behavior of the parent molecule [36,37]. Rapid bleaching, quenching, or cleavage of dyes and/or radioisotopes from the parent oligonucleotide within the *in vivo* setting may limit the use of these labels as surrogate measures of oligonucleotide concentration [38]. In contrast, the high-throughput method described in this study allows accurate determination of the blood/plasma profile of novel conjugated hsiRNAs in their native form, as they would be used in the clinical setting. Thus, this strategy may support fast development of lead compounds in an effective and inexpensive manner, which can be carried out in most academic laboratories and is compatible with the Quantigene bDNA assay that enables high-throughput assessment of gene silencing [39]. Together, these two assays may be used to fully characterize tissue distribution and gene silencing, key aspects required to evaluate *in vivo* performance of therapeutic oligonucleotides.

To validate this methodology and ascertain its suitability to assess a broad range of chemically diverse siRNAs, we have successfully tested its applicability to determine the PK profiles of Chol-hsiRNAs and PC-DHA-hsiRNAs. Compounds were administered either IV or SC and concentrations were monitored in blood/plasma at different time points. These two routes of administration were selected since they are the most widely used for therapeutic oligonucleotides for systemic applications. Finally, at the endpoint of the study, other organs, such as liver, kidney, spleen, and skin, were also analyzed.

Chol-hsiRNAs and PC-DHA-hsiRNAs present distinct blood and plasma profiles after IV and SC administration

Chol-hsiRNAs and PC-DHA-hsiRNAs showed a biexponential decline in blood/plasma concentration, particularly evident after IV administration (Fig. 2a, b). Both conjugated hsiRNAs presented an initial rapid distribution phase followed by a slower terminal elimination phase (Fig. 2a, b). Similar pattern of concentration decline was observable for PC-DHA-hsiRNAs after peak time in SC injected animals, but seemed to be less apparent for Chol-hsiRNAs after SC

FIG. 1. High-throughput 15-step method for detection of blood/plasma concentration of therapeutic oligonucleotides. Oligonucleotide injection (1). Serial blood sampling ($\sim 20 \mu\text{L}$) through the saphenous vein (2). Blood ($10 \mu\text{L}$) transfer to 96-well plate (stored at -80°C) (3). Plasma isolation (4). Plasma ($10 \mu\text{L}$) transfer to 96-well plate (stored at -80°C) (5). Addition of $200 \mu\text{L}$ of lysis buffer (with proteinase K) and transfer to QIAGEN collection tubes (6). QIAGEN Tissue Lyser II processing (7). Centrifugation (8). Proteinase K digestion (55°C , 1 h) (9). 3 M KCl precipitation to remove detergents (10). Centrifugation (11). Transfer to 96-well PCR plate and addition of Cy3-labeled PNA probe (12). Hybridization (95°C , 15 min, slow cool down) (13). Separation of hybrid duplexes by HPLC, DNAPac PA100 (14). Integration and quantification of peaks (15). HPLC, high-performance liquid chromatography; PCR, polymerase chain reaction; PNA, peptide nucleic acid.

dosing (Fig. 2c, d). Although blood and plasma concentration vs. time profiles were overall comparable, plasma concentrations were on average $\sim 48\%$ higher than what was detected in the blood. The respective blood to plasma concentration ratios were 0.77 for PC-DHA-hsiRNAs and 0.59 for Chol-hsiRNAs, indicating low binding to red blood cells (RBCs) for both compounds. Thus, as conjugated hsiRNAs are not significantly sequestered in RBCs, plasma values were used to calculate the relevant PK parameters. Differences between both conjugated hsiRNAs and the routes of administration tested are depicted in their distinct PK parameters in Tables 1 and 2.

Focusing on IV administrations (Fig. 2a, b and Table 1), PC-DHA-hsiRNAs showed a more rapid initial distribution/elimination half-life ($t_{1/2\alpha}$) ~ 18 min than Chol-hsiRNAs ~ 33 min. Further investigations into this initial distribution phase revealed that PC-DHA-hsiRNAs are found at relatively high concentrations in the urine within 20 min after IV administration ($\sim 2,358 \mu\text{g/mL}$), and at peak time after SC dosing ($\sim 297 \mu\text{g/mL}$). Alternatively, Chol-hsiRNAs had been excreted to a much lesser extent at comparable time

points ($\sim 49 \mu\text{g/mL}$ for IV and $\sim 20 \mu\text{g/mL}$ for SC). Thus, the 15–48-fold higher concentrations of PC-DHA-hsiRNAs that were found in the urine suggest that this compound is more actively excreted than Chol-hsiRNAs at this early distribution/elimination phase (Supplementary Fig. S2). However, the inverse was true for terminal elimination half-life ~ 850 min (~ 14.2 h) in the case of PC-DHA-hsiRNAs and 516 min (~ 8.5 h) in the case of Chol-hsiRNAs. Furthermore, Chol-hsiRNAs presented a total exposure over time, determined by the AUC, 3.6-fold higher than what was observed for PC-DHA-hsiRNAs. The mean residence time (MRT) of Chol-hsiRNAs was on average ~ 50 min longer than what was detected for PC-DHA-hsiRNAs. Conversely, PC-DHA-hsiRNAs depicted higher values for clearance (Cl) and volume of distribution (V_z) than what was found for Chol-hsiRNAs.

Analyzing results from SC administrations (Fig. 2c, d and Table 2), PC-DHA-hsiRNAs seemed to be rapidly absorbed from the site of injection, depicted by a quick absorption half-life (~ 16 min) and a relatively short time to peak (~ 30 – 60 min). On the other hand, Chol-hsiRNAs showed a slower

FIG. 2. Chol-hsiRNAs and PC-DHA-hsiRNAs present distinct blood and plasma clearance kinetics after IV and SC injection. Mice were injected with Chol-hsiRNA and PC-DHA-hsiRNA conjugates (20 mg/kg) IV (**a, b**) or SC (**c, d**). Serial blood sampling was performed through the lateral saphenous vein. Antisense strands were quantified using PNA hybridization assay. $n=5-6$, SD. Chol-hsiRNA, cholesterol-hsiRNA; IV, intravenous; PC-DHA-hsiRNA, phosphocholine-docosahexaenoic acid-hsiRNA; SC, subcutaneous; SD, standard deviation.

absorption half-life (~ 60 min) and a relatively longer time to peak ($\sim 180-240$ min). While the differences of AUCs between both conjugated hsiRNAs were reduced when compared to IV (most likely due to differences in absorption), the differences between MRT increased to ~ 172 min

(~ 2.9 h). Furthermore, on average, the MRTs for both conjugated hsiRNAs were 3.5-fold higher after SC administration than after IV administration. Correspondingly, plasma bioavailability after SC administration was 0.56 for PC-DHA-hsiRNAs and 0.21 for Chol-hsiRNAs, which reiterates

TABLE 1. PLASMA PHARMACOKINETIC PARAMETERS FOR CHOL-HSI RNA AND PC-DHA-HSI RNA CONJUGATES AFTER INTRAVENOUS ADMINISTRATION

Parameter	Unit	PC-DHA-hsiRNA	Chol-hsiRNA
k	1/min	0.0008	0.0013
$t_{1/2\beta}$	min	849.8	515.8
$t_{1/2\alpha}$	min	17.8	33.2
C_{max}	µg/mL	572.3	753.4
AUC_{0-48h}	µg/(mL·min)	15249.2	54532.5
AUC_{0-inf}	µg/(mL·min)	15381.0	54807.5
MRT_{0-inf}	min	107.4	156.9
V_z	mL	42.3	6.8
Cl	mL/min	0.0345	0.0091

AUC, area under the curve; Chol-hsiRNA, cholesterol-hsiRNA; MRT, mean residence time; PC-DHA-hsiRNA, phosphocholine-docosahexaenoic acid-hsiRNA.

TABLE 2. PLASMA PHARMACOKINETIC PARAMETERS FOR CHOL-HSI RNA AND PC-DHA-HSI RNA CONJUGATES AFTER SC ADMINISTRATION

Parameter	Unit	PC-DHA-hsiRNA	Chol-hsiRNA
k_a	1/min	0.0431	0.0116
$t_{1/2}$ absorption	min	16.1	59.8
k	1/min	0.0006	0.0016
$t_{1/2\beta}$	min	1075.4	428.1
T_{max}	min	30-60	180-240
C_{max}	µg/mL	50.5	14.0
AUC_{0-48h}	µg/(mL·min)	8322.5	11517.8
AUC_{0-inf}	µg/(mL·min)	8653.2	11654.0
MRT_{0-inf}	min	377.9	549.6
V_z/F	mL	95.2	26.5
Cl/F	mL/min	0.0614	0.0429

FIG. 3. Chol-hsiRNAs and PC-DHA-hsiRNAs display dose-dependent increases in blood concentrations after SC injection. (a, b) Mice were injected subcutaneously with Chol-hsiRNA or PC-DHA-hsiRNA conjugates 10 or 20 mg/kg. Serial blood sampling was performed through the lateral saphenous vein at predetermined time points. Antisense strands were quantified using PNA hybridization assay. $n=3-6$, SD.

that the former compound is more extensively absorbed from the injection site.

Dose-dependent increase in concentration was observed in the blood for both compounds after SC injection of 10 and 20 mg/kg (Fig. 3). In this experiment, conjugated hsiRNAs maintained their characteristic blood concentration-time pro-

file and T_{Max} times, but the maximum concentration (C_{Max}) after dosing approximately doubled. Although AUCs for both compounds increased with the dose, the fold increase was only of 1.5 and 1.8 for PC-DHA-hsiRNAs and Chol-hsiRNAs, respectively. It is worth noting that for both conjugated hsiRNAs and administration routes tested, AUC_{0-48} corresponded to

FIG. 4. Chol-hsiRNAs and PC-DHA-hsiRNAs show distinct tissue retention and accumulation 48 h postdosing. Mice were injected with Chol-hsiRNA and PC-DHA-hsiRNA conjugates (20 mg/kg) IV or SC. Liver (a), kidney (b), spleen (c) and (d) skin were collected after 48 h and antisense strands quantified using PNA hybridization assay. Skin biopsies were collected between the shoulder blades, at the site for SC injections. $n=5-6$, SD.

>96% of total $AUC_{0-\infty}$, indicating that by 48 h, most compounds have been cleared from the blood/plasma compartment. The high volume of distribution for both IV and SC also corroborates the fact that these conjugated hsiRNAs are highly distributed and cleared from the systemic circulation.

Chol-hsiRNAs and PC-DHA-hsiRNAs show distinct tissue retention and accumulation

Chol-hsiRNAs and PC-DHA-hsiRNAs displayed different blood/plasma PK profiles when administered both SC and IV, and not surprisingly, they also presented different tissue accumulation and retention profiles (Fig. 4). After 48 h upon IV or SC administration of a 20 mg/kg dose, Chol-hsiRNAs were extensively retained in the liver (Fig. 4a), representing ~35%–37% of the initial dose (Supplementary Table S2). On the other hand, PC-DHA-hsiRNAs preferentially accumulated in the kidney (Fig. 4b), where ~15%–22% of the dose was retained. Although to a lesser extent when compared to the liver, Chol-hsiRNAs were also retained in the spleen (~0.42% of initial dose) to a larger degree than PC-DHA-hsiRNAs (~0.09% of initial dose) (Fig. 4c and Supplementary Table S2). In this study, the route of administration did not affect the biodistribution pattern of these conjugated hsiRNAs; however, it affected the total concentration of oligonucleotide found in the organs.

No significant accumulation was observed in skin biopsies (obtained between the shoulder blades) after IV administration; however, significant amounts of both conjugated hsiRNAs were found at the injection site 48 h after SC injections. The concentration of Chol-hsiRNAs at the site of injection was twofold higher than what was found for PC-DHA-hsiRNAs (Fig. 4d). Slow release from the skin and subsequent rapid association with the liver may justify the lower C_{Max} obtained with Chol-hsiRNAs when compared with PC-DHA-hsiRNAs (Figs. 2 and 3). In a separate study, we have confirmed that the maximum retention of Chol-hsiRNAs was almost achieved by 20 min after IV dosing, or 4 h (240 min) after SC dosing (Supplementary Fig. S2). PC-DHA-hsiRNAs on the other hand were rapidly absorbed into the blood/plasma compartment and from there, rapidly redistributed to the kidney and/or excreted. However, the fact that concentrations in the kidney 20 min after IV administration (~82 ng/mg) or at peak times after SC (~117 ng/mg) did not reach the maximum achieved by 48 h (~274 ng/mg for IV and ~407.8 ng/mg for SC) suggests that the distribution process is still in progress.

Together these data show that PC-DHA-hsiRNAs present a much faster blood and plasma kinetics when compared to Chol-hsiRNAs. Provided that the same siRNA sequence and chemical modifications were used in these oligonucleotides, we can conclude that the conjugated modality is a strong determinant of the PK profile and biodistribution of these hsiRNAs.

Discussion

In this study, we establish, for the first time, a high-throughput method suitable for PK profiling of conjugated hsiRNAs at discovery stages. The procedure greatly reduces the number of animals conventionally used for characterization of blood/plasma PK profiles of therapeutic oligonucleotides, and the amount of starting compound required for PK char-

acterization. Furthermore, the method removes the need for introducing fluorophores, biotin labels, or radioisotopes, commonly used to visualize and/or quantify the amount of oligonucleotide in the tissue [10–13,15,16]. Alternatively, the PNA hybridization assay uses a selective PNA probe that allows for unequivocal detection and quantification of the target antisense strand contained in hsiRNAs [26,32,33]. This highly sensitive method of detection was developed by Roehl *et al.* [26], and has also been used by others for quantification of oligonucleotides in several tissue matrixes [32,33]. In this study, we have adapted the protocol to allow for high-throughput screening of systemically administered hsiRNAs by combining it with serial blood microsampling and devising an accelerated sample processing format. Altogether, this approach may constitute a valid and inexpensive platform to rapidly screen PK profiles of different conjugated siRNAs in academic laboratories. As miRNAs are structurally similar to siRNAs, this methodology may be readily applied for the evaluation of plasma PK and tissue accumulation profiles of novel miRNA compounds.

If very large numbers of conjugated oligonucleotides are being screened using this methodology, a cassette-accelerated analysis approach may be used to reduce the number of samples to be processed. As described in Liu *et al.* [5], (i) samples from the same time points within the same treatment group may be pooled together and analyzed; or (ii) samples from different treatment groups, but from same time points, may be pooled together and analyzed. The first option is likely to be more broadly applicable to most laboratories; however, it removes the possibility of determining inter-animal variability since a total pooled amount will be determined. The latter option will require the use of different targeting sequences on the siRNA design, which in turn allow detection using specific PNA probes tagged with different fluorescent dyes. In this case, analysis will involve the use of a multichannel fluorescent detector for quantification, thus maintaining the possibility to evaluate interanimal variability. Since it is now generally accepted that tissue disposition of conjugated siRNAs occurs independent of sequence [12,40,41], and provided that the pattern of chemical modifications is kept constant, the above-mentioned strategy may be conceptually feasible. Although some optimization will be required to minimize the interference of the different fluorophores, this approach would be of great value to further accelerate screening of valuable moieties for targeted delivery of therapeutic oligonucleotides.

Applying the method described in this article, we have successfully characterized the blood/plasma PK and tissue distribution profiles of two chemically distinct hsiRNAs: Chol-hsiRNAs and PC-DHA-hsiRNAs. The former containing a cholesterol and the latter a phosphocholine docosahexaenoic acid moiety at the 3'-end of the sense strand, Chol-hsiRNAs and PC-DHA-hsiRNAs presented a multi-exponential decline in blood/plasma concentrations over time, which was characterized by a rapid distribution phase ($t_{1/2\alpha}$ = 18–33 min) and a slow elimination phase ($t_{1/2\beta}$ = 8–14 h). These findings represent a major improvement over naked unmodified siRNAs, which have been previously shown to rapidly degrade and clear from the blood compartment within 20 min after IV administration ($t_{1/2\beta}$ = 2–5 min) [7,17,42]. In agreement with our findings, others have also demonstrated that chemically modified siRNAs and

ASOs present enhanced blood circulating times and tissue retention profiles [8,43].

Chemically modifying the oligonucleotide structure not only confers resistance against enzymatic degradation but may also promote binding to plasma proteins, resulting in longer half-lives [44]. The data showed that after the initial distribution phase, Chol-hsiRNAs and PC-DHA-hsiRNAs were preferentially retained in the liver and kidney and were slowly released to the systemic circulation, with less than 1%–4% of the dose found in the blood compartment after 48 h. Similar postdistribution phases and relatively long terminal half-lives have been described for second-generation ASOs containing fully phosphorothioated backbones [36,37]. These PS backbones have been shown to enable low affinity binding to plasma albumin, preventing rapid loss of the unbound fraction through glomerular filtration, yet enhancing tissue distribution and retention [40]. Due to the relatively high content in PS linkages in Chol-hsiRNAs and PC-DHA-hsiRNAs, we hypothesize that an analogous mechanism may be involved in defining distribution and postdistribution phases of these conjugated hsiRNAs. Thus, these results add to the growing body of knowledge that chemical modifications within the oligonucleotide structure play a critical role not only in plasma PK profiles but also in tissue distribution and accumulation of therapeutic oligonucleotides [21].

By maintaining the same pattern of backbone and ribose modifications, we were able to gain valuable insights on the influence of the conjugated ligands in tissue disposition of these oligonucleotides. In this study, Chol-hsiRNAs consistently exhibited higher AUCs and MRTs and lower plasma clearances than what was observed for PC-DHA-hsiRNAs, after both IV and SC administrations. We hypothesize that this is the result of strong binding of Chol-hsiRNAs to serum proteins, with subsequent increase in blood residency times and high level of tissue accumulation in the liver. Preliminary *in vivo* binding studies in the laboratory have shown that Chol-hsiRNAs primarily bind to low-density lipoprotein (LDL) particles.* Similarly, Wolfrum *et al.* previously described that cholesterol-conjugated siRNAs associate with LDL, high-density lipoproteins (HDL), and albumin to different extents [45]. In these studies, binding to LDL and HDL lipid particles was demonstrated to result in high levels of accumulation of conjugated siRNA in the liver [46]. Although the mechanism of cellular uptake of Chol-hsiRNAs warrants further investigations, it may be mediated, in part, by LDL receptors, which are highly expressed in the liver.

Despite that levels of retention in the liver did not seem to be affected by the route of administration, Chol-hsiRNAs showed low plasma bioavailability upon SC dosing. This may be explained by the high level of retention of these compounds in the skin. Highly lipophilic Chol-hsiRNAs are believed to associate with the SC fat at the site of injection, perhaps, forming an oligonucleotide depot that slowly releases the compound to the systemic circulation over time. Absorption of Chol-hsiRNAs from the SC site of injection was followed by fast retention in the liver. In fact, by 20 min after IV injection or 4 h (peak time in the plasma) after SC injection, Chol-hsiRNAs had almost achieved the maximum concentration observed in this organ by 48 h. Finally, it is

worth noting that the concentrations of Chol-hsiRNAs detected in liver and kidney biopsies at 48 h in this study were comparable to what was previously found in an independent study in the laboratory [34].

PC-DHA-hsiRNAs were more rapidly and extensively absorbed from the SC site of administration than Chol-hsiRNAs, consequently displaying greater bioavailability in the plasma than the latter. This result is in line with the distribution patterns previously observed in the brain, with PC-DHA-hsiRNAs diffusing from the site of injection to a greater extent than observed with Chol-hsiRNAs [27,28]. Systemically, after IV or SC administrations, PC-DHA-hsiRNAs were highly retained in the kidney, to a similar extent observed for unconjugated fully phosphorothioated ASOs [23,24,46]. PC-DHA-hsiRNAs show significantly lower affinity to LDL,[†] which might explain differences in clearance rates and tissue distribution profiles. Mechanistic studies ought to be conducted to thoroughly dissect the roles of the conjugated moiety (PC-DHA) and the PS tail in the processes of cellular uptake in the kidney. Despite that Chol-hsiRNA also contained a highly phosphorothioated tail, this compound did not distribute to the kidney extensively, suggesting that the backbone chemistry is not the major determinant for uptake in this particular case. Although data clearly demonstrate that the conjugated modality can be a strong determinant in the systemic distribution of these compounds, additional studies are required to systematically identify binding partners in the blood and tissues, which may play a crucial role in the PK properties of these compounds.

The recent developments and successes with GalNAc-conjugated siRNAs have undoubtedly demonstrated that drug disposition of therapeutic oligonucleotides is strongly determined by the conjugated chemical modality [2,3,47]. GalNAc-conjugated siRNAs and ASOs rapidly distribute to hepatocytes in the liver by receptor-mediated uptake through asialoglycoprotein receptor [48]. In this case, the conjugated modality enables targeted delivery to the liver with minimal uptake in secondary tissues, thus reducing the likelihood of undesired side effects. This powerful paradigm has encouraged several research groups to explore conjugate-based strategies to modulate oligonucleotide delivery to specific tissues and cellular subpopulations [27,48,49]. Indeed, in years to come, a wide variety of novel ligands are likely to emerge as potential conjugates for therapeutic oligonucleotides. Therefore, developing tools that help understanding and systematically determining the plasma PK and tissue distribution profiles of these conjugated siRNAs and miRNAs becomes essential.

The high-throughput method described in this study constitutes a useful platform that may simplify screening and characterization of large panels of novel conjugated oligonucleotides, providing sufficient information for dose selection and design of efficacy studies, in the context of an academic laboratory. Naturally, a more comprehensive PK and toxicological characterization would be required for selected compounds that progress toward formal preclinical development. This includes conducting relevant mass-balance studies for accurately ascertaining drug elimination routes and extent, as

*Unpublished data, Osborn *et al.* manuscript in preparation.

[†]Unpublished data, Osborn *et al.* manuscript in preparation.

well as major metabolic pathways with respective identification and quantification of metabolites [50].

Conclusion

The high-throughput method described allowed for the characterization of the blood/plasma PK and biodistribution profiles of two chemically distinct hsiRNA modalities: Chol-hsiRNAs and PC-DHA-hsiRNAs. The method enabled fast sample processing using 96-well plate format and greatly reduced animal usage and overall cost of determining the PK and biodistribution profiles of these conjugated hsiRNAs. Furthermore, the use of sequence specific PNA probes for detection, permitted to overcome the need of specifically modifying hsiRNA molecules to contain adequate molecular labels for the assessment PK and biodistribution. This may not only facilitate initial compound screening tasks but also development of selected lead compounds to the clinic. Finally, the compatibility of this method with other high-throughput assays routinely used for the evaluation of gene silencing efficacy represents a great advantage of this method for screening of therapeutic oligonucleotides.

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Author Disclosure Statement

A.K. owns stock at RXi Pharmaceuticals and Advirna LLC, which holds a patent on asymmetric, hydrophobically modified siRNAs. Other authors do not have any competing financial interest to disclose.

References

- Scherman D, A Rousseau, P Bigey and V Escriou. (2017). Genetic pharmacology: progresses in siRNA delivery and therapeutic applications. *Gene Ther* 24:151–156.
- Khvorova A. (2017). Oligonucleotide therapeutics—a new class of cholesterol-lowering drugs. *N Engl J Med* 376:4–7.
- Khvorova A, MF Osborn and MR Hassler. (2014). Taking charge of siRNA delivery. *Nat Biotech* 32:1197–1198.
- Zimmermann TS, V Karsten, A Chan, J Chiesa, M Boyce, BR Bettencourt, R Hutabarat, S Nochur, A Vaishnav and J Gollob. (2017). Clinical proof of concept for a novel hepatocyte-targeting GalNAc-siRNA conjugate. *Mol Ther* 25:71–78.
- Liu B, J Chang, WP Gordon, J Isbell, Y Zhou and T Tuntland. (2008). Snapshot PK: a rapid rodent in vivo preclinical screening approach. *Drug Discov Today* 13:360–367.

- Li C, B Liu, J Chang, T Groessl, M Zimmerman, Y-Q He, J Isbell and T Tuntland. (2013). A modern in vivo pharmacokinetic paradigm: combining snapshot, rapid and full PK approaches to optimize and expedite early drug discovery. *Drug Discov Today* 18:71–78.
- Morrissey DV, JA Lockridge, L Shaw, K Blanchard, K Jensen, W Breen, K Hartsough, L Machemer, S Radka and V Jadhav. (2005). Potent and persistent in vivo anti-HBV activity of chemically modified siRNAs. *Nat Biotechnol* 23:1002–1007.
- Solano EC, DJ Kornbrust, A Beaudry, JW-D Foy, DJ Schneider and JD Thompson. (2014). Toxicological and pharmacokinetic properties of QPI-1007, a chemically modified synthetic siRNA targeting caspase 2 mRNA, following intravitreal injection. *Nucleic Acid Ther* 24:258–266.
- Yu RZ, T-W Kim, A Hong, TA Watanabe, HJ Gaus and RS Geary. (2007). Cross-species pharmacokinetic comparison from mouse to man of a second-generation antisense oligonucleotide, ISIS 301012, targeting human apolipoprotein B-100. *Drug Metab Dispos* 35:460–468.
- Bienk K, ML Hvam, MM Pakula, F Dagnæs-Hansen, J Wengel, BM Malle, U Kragh-Hansen, J Cameron, JT Bukrinski and KA Howard. (2016). An albumin-mediated cholesterol design-based strategy for tuning siRNA pharmacokinetics and gene silencing. *J Control Release* 232:143–151.
- Chen S, YYC Tam, PJ Lin, AK Leung, YK Tam and PR Cullis. (2014). Development of lipid nanoparticle formulations of siRNA for hepatocyte gene silencing following subcutaneous administration. *J Control Release* 196:106–112.
- Huang Y, Q Cheng, J-L Ji, S Zheng, L Du, L Meng, Y Wu, D Zhao, X Wang and L Lai. (2016). Pharmacokinetic behaviors of intravenously administered siRNA in glandular tissues. *Theranostics* 6:1528.
- Malhotra M, C Tomaro-Duchesneau, S Saha and S Prakash. (2013). Systemic siRNA delivery via peptide-tagged polymeric nanoparticles, targeting PLK1 gene in a mouse xenograft model of colorectal cancer. *Int J Biomater* 2013:252531.
- Kim E-J, TG Park, Y-K Oh and C-K Shim. (2010). Assessment of siRNA pharmacokinetics using ELISA-based quantification. *J Control Release* 143:80–87.
- Lozac'h F, J Christensen, T Faller, E Kerkhof, J Krauser, M Garnier, K Litherland, A Catoire, F Natt and J Hunziker. (2016). ADME studies of [5-3H]-2'-O-methyluridine nucleoside in mice: a building block in siRNA therapeutics. *Pharmacol Res Perspect* 4:e00209.
- Van de Water FM, OC Boerman, AC Wouterse, JG Peters, FG Russel and R Masereeuw. (2006). Intravenously administered short interfering RNA accumulates in the kidney and selectively suppresses gene function in renal proximal tubules. *Drug Metab Dispos* 34:1393–1397.
- Christensen J, K Litherland, T Faller, E Van de Kerkhof, F Natt, J Hunziker, J Krauser and P Swart. (2013). Metabolism studies of unformulated internally [3H]-labeled short interfering RNAs in mice. *Drug Metab Dispos* 41:1211–1219.
- Viel T, R Boisgard, B Kuhnast, B Jego, K Siquier-Pernet, F Hinnen, F Dolle and B Tavittian. (2008). Molecular imaging study on in vivo distribution and pharmacokinetics of modified small interfering RNAs (siRNAs). *Oligonucleotides* 18:201–212.
- Ly S, DM Navaroli, M-C Didiot, J Cardia, L Pandarathan, JF Alterman, K Fogarty, C Standley, LM Lifshitz,

- et al.* (2017). Visualization of self-delivering hydrophobically modified siRNA cellular internalization. *Nucleic Acids Res* 45:15–25.
20. Alterman JF, LM Hall, AH Coles, MR Hassler, M-C Didiot, K Chase, J Abraham, E Sottosanti, E Johnson and E Sapp. (2015). Hydrophobically modified siRNAs silence Huntingtin mRNA in primary neurons and mouse brain. *Mol Ther Nucleic Acids* 4:e266.
 21. Geary RS, TA Watanabe, L Truong, S Freier, EA Lesnik, NB Sioufi, H Sasmor, M Manoharan and AA Levin. (2001). Pharmacokinetic properties of 2'-O-(2-methoxyethyl)-modified oligonucleotide analogs in rats. *J Pharmacol Exp Ther* 296:890–897.
 22. Park J, J Park, Y Pei, J Xu and Y Yeo. (2016). Pharmacokinetics and biodistribution of recently-developed siRNA nanomedicines. *Adv Drug Deliv Rev* 104:93–109.
 23. Yu RZ, MJ Graham, N Post, S Riney, T Zanardi, S Hall, J Burkey, CS Shemesh, TP Prakash and PP Seth. (2016). Disposition and pharmacology of a GalNAc3-conjugated ASO targeting human lipoprotein (a) in mice. *Mol Ther Nucleic Acids* 5:e317.
 24. Yu RZ, R Gunawan, N Post, T Zanardi, S Hall, J Burkey, T-W Kim, MJ Graham, TP Prakash and PP Seth. (2016). Disposition and pharmacokinetics of a GalNAc3-conjugated antisense oligonucleotide targeting human lipoprotein (a) in monkeys. *Nucleic Acid Ther* 26:372–380.
 25. Rosie ZY, B Baker, A Chappell, RS Geary, E Cheung and AA Levin. (2002). Development of an ultrasensitive non-competitive hybridization–ligation enzyme-linked immunosorbent assay for the determination of phosphorothioate oligodeoxynucleotide in plasma. *Anal Biochem* 304:19–25.
 26. Roehl I, M Schuster and S Seiffert. (2011). Oligonucleotide detection method. US Patent US20110201006A1:1–9.
 27. Nikan M, MF Osborn, AH Coles, BM Godinho, LM Hall, RA Haraszti, MR Hassler, D Echeverria, N Aronin and A Khvorova. (2016). Docosa-hexaenoic acid conjugation enhances distribution and safety of siRNA upon local administration in mouse brain. *Mol Ther Nucleic Acids* 5:e344.
 28. Nikan M, MF Osborn, AH Coles, A Biscans, B Godinho, R Haraszti, E Sapp, D Echeverria, M DiFiglia and N Aronin. (2017). Synthesis and evaluation of parenchymal retention and efficacy of a metabolically stable, O-phosphocholine-N-docosa-hexaenoyl-L-serine siRNA conjugate in mouse brain. *Bioconjug Chem* 28:1758–1766.
 29. Reynolds A, D Leake, Q Boese, S Scaringe, WS Marshall and A Khvorova. (2004). Rational siRNA design for RNA interference. *Nat Biotechnol* 22:326–330.
 30. Peng SX, BA Rockafellow, TM Skedzielewski, ND Huebert and W Hageman. (2009). Improved pharmacokinetic and bioavailability support of drug discovery using serial blood sampling in mice. *J Pharm Sci* 98:1877–1884.
 31. Zhang Y, M Huo, J Zhou and S Xie. (2010). PKSolver: an add-in program for pharmacokinetic and pharmacodynamic data analysis in Microsoft Excel. *Comput Methods Programs Biomed* 99:306–314.
 32. Trubetskoy VS, JB Griffin, AL Nicholas, EM Nord, Z Xu, RM Peterson, CI Wooddell, DB Rozema, DH Wakefield, DL Lewis and SB Kanner. (2017). Phosphorylation-specific status of RNAi triggers in pharmacokinetic and biodistribution analyses. *Nucleic Acids Res* 45:1469–1478.
 33. Tian Q, J Rogness, M Meng and Z Li. (2017). Quantitative determination of a siRNA (AD00370) in rat plasma using peptide nucleic acid probe and HPLC with fluorescence detection. *Bioanalysis* 9:861–872.
 34. Haraszti RA, L Roux, AH Coles, AA Turanov, JF Alterman, D Echeverria, BM Godinho, N Aronin and A Khvorova. (2017). 5'-Vinylphosphonate improves tissue accumulation and efficacy of conjugated siRNAs in vivo. *Nucleic Acids Res* 45:7581–7592.
 35. Järve A, J Müller, I-H Kim, K Rohr, C MacLean, G Fricker, U Massing, F Eberle, A Dalpke and R Fischer. (2007). Surveillance of siRNA integrity by FRET imaging. *Nucleic Acids Res* 35:e124.
 36. Harborth J, SM Elbashir, K Vandenburgh, H Manninga, SA Scaringe, K Weber and T Tuschl. (2003). Sequence, chemical, and structural variation of small interfering RNAs and short hairpin RNAs and the effect on mammalian gene silencing. *Antisense Nucleic Acid Drug Dev* 13:83–105.
 37. Grünweller A, C Gillen, VA Erdmann and J Kurreck. (2003). Cellular uptake and localization of a Cy3-labeled siRNA specific for the serine/threonine kinase Pim-1. *Oligonucleotides* 13:345–352.
 38. Dunne J, B Drescher, H Riehle, P Hadwiger, BD Young, J Krauter and O Heidenreich. (2003). The apparent uptake of fluorescently labeled siRNAs by electroporated cells depends on the fluorochrome. *Oligonucleotides* 13:375–380.
 39. Coles AH, MF Osborn, JF Alterman, AA Turanov, BM Godinho, L Kennington, K Chase, N Aronin and A Khvorova. (2016). A high-throughput method for direct detection of therapeutic oligonucleotide-induced gene silencing in vivo. *Nucleic Acid Ther* 26:86–92.
 40. Geary RS, D Norris, R Yu and CF Bennett. (2015). Pharmacokinetics, biodistribution and cell uptake of antisense oligonucleotides. *Adv Drug Deliv Rev* 87:46–51.
 41. Geary RS. (2009). Antisense oligonucleotide pharmacokinetics and metabolism. *Expert Opin Drug Metab Toxicol* 5:381–391.
 42. Godinho BM, JR Ogier, A Quinlan, R Darcy, BT Griffin, JF Cryan and CM O'Driscoll. (2014). PEGylated cyclodextrins as novel siRNA nanosystems: correlations between polyethylene glycol length and nanoparticle stability. *Int J Pharm* 473:105–112.
 43. Thompson JD, DJ Kornbrust, JW Foy, EC Solano, DJ Schneider, E Feinstein, BA Molitoris and S Erlich. (2012). Toxicological and pharmacokinetic properties of chemically modified siRNAs targeting p53 RNA following intravenous administration. *Nucleic Acid Ther* 22:255–264.
 44. Khvorova A and JK Watts. (2017). The chemical evolution of oligonucleotide therapies of clinical utility. *Nat Biotechnol* 35:238–248.
 45. Wolfrum C, S Shi, KN Jayaprakash, M Jayaraman, G Wang, RK Pandey, KG Rajeev, T Nakayama, K Charrise and EM Ndungo. (2007). Mechanisms and optimization of in vivo delivery of lipophilic siRNAs. *Nat Biotechnol* 25:1149–1157.
 46. Geary RS, ZY Rosie, T Watanabe, SP Henry, GE Hardee, A Chappell, J Matson, H Sasmor, L Cummins and AA Levin. (2003). Pharmacokinetics of a tumor necrosis factor- α phosphorothioate 2'-O-(2-methoxyethyl) modified antisense oligonucleotide: comparison across species. *Drug Metab Dispos* 31:1419–1428.

47. Prakash TP, MJ Graham, J Yu, R Carty, A Low, A Chappell, K Schmidt, C Zhao, M Aghajan and HF Murray. (2014). Targeted delivery of antisense oligonucleotides to hepatocytes using triantennary N-acetyl galactosamine improves potency 10-fold in mice. *Nucleic Acids Res* 42: 8796–8807.
48. Bortolozzi A, A Castañé, J Semakova, N Santana, G Alvarado, R Cortés, A Ferrés-Coy, G Fernández, MC Carmona and M Toth. (2012). Selective siRNA-mediated suppression of 5-HT_{1A} autoreceptors evokes strong anti-depressant-like effects. *Mol Psychiatry* 17: 612–623.
49. Ferrés-Coy A, M Galofré, F Pilar-Cuéllar, R Vidal, V Paz, E Ruiz-Bronchal, L Campa, Á Pazos, J Caso and J Leza. (2016). Therapeutic antidepressant potential of a conjugated siRNA silencing the serotonin transporter after intranasal administration. *Mol Psychiatry* 21: 328–338.
50. Penner N, LJ Klunk and C Prakash. (2009). Human radiolabeled mass balance studies: objectives, utilities and limitations. *Biopharm Drug Dispos* 30:185–203.

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