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Fatty Acid-Modified Gapmer Antisense Oligonucleotide and Serum Albumin Constructs for Pharmacokinetic Modulation

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Delivery technologies are required for realizing the clinical potential of molecular medicines. This work presents an alternative technology to preformulated delivery systems by harnessing the natural transport properties of serum albumin using endogenous binding of gapmer antisense oligonucleotides (ASOs)/albumin constructs. We show by an electrophoretic mobility assay that fatty acid-modified gapmer and human serum albumin (HSA) can self-assemble into constructs that offer favorable pharmacokinetics. The interaction was dependent on fatty acid type (either palmitic or myristic acid), number, and position within the gapmer ASO sequence, as well as phosphorothioate (PS) backbone modifications. Binding correlated with increased blood circulation in mice ($t_{1/2}$ increased from 23 to 49 min for phosphodiester [PO] gapmer ASOs and from 28 to 66 min for PS gapmer ASOs with 2\textsuperscript{14} palmitic acid modification). Furthermore, a shift toward a broader biodistribution was detected for PS compared with PO gapmer ASOs. Inclusion of 2\textsuperscript{14} palmitoyl to the ASOs shifted the biodistribution to resemble that of natural albumin. This work, therefore, presents a novel strategy based on the proposed endogenous assembly of gapmer ASOs/albumin constructs for increased circulatory half-life and modulation of the biodistribution of gapmer ASOs that offers tunable pharmacokinetics based on the gapmer modification design.

INTRODUCTION

Gene silencing by antisense oligonucleotides (ASOs) offers great potential as a therapeutic strategy. Gapmer ASOs are short single-stranded ASOs containing a central DNA sequence commonly flanked by a locked nucleic acid (LNA) sequence that interrupt mRNA expression by induction of RNase H activation, and exhibit cellular entry without the necessity of a transfection agent by a process termed gymnosis. Stability and blood half-life extension technologies, however, are needed because of oligonucleotides susceptibility to serum nuclease degradation and rapid limited distribution into organs like the liver and kidney, resulting in a short blood circulatory half-life of $\sim$2-10 min. Great efforts have been made to improve the plasma stability of ASOs, with a wide range of different chemical sugar modifications such as 2'-O-methyl (2'O-Me), 2'-O-methoxyethyl (2'MOE), and constrained ethyl (CEt; LNA containing a methyl substituent), and backbone modifications from a phosphodiester (PO) to phosphorothioate (PS) design used. The low molecular weight of ASOs, however, still results in rapid elimination from the blood that limits the potential to accumulate at target tissue sites.

A vast array of viral and non-viral vectors has been developed to improve the effectiveness of nucleic acid therapeutics; however, many vectors are based on complex designs, with cost and safety considerations that reduce the likelihood of clinical applications. Previous work has implicated the involvement of HSA in the pharmacokinetics of PS ASOs because of non-specific interactions. Furthermore, a shift toward a broader biodistribution was detected for PS compared with PO gapmer ASOs. Inclusion of 2\textsuperscript{14} palmitoyl to the ASOs shifted the biodistribution to resemble that of natural albumin. This work, therefore, presents a novel strategy based on the proposed endogenous assembly of gapmer ASOs/albumin constructs for increased circulatory half-life and modulation of the biodistribution of gapmer ASOs that offers tunable pharmacokinetics based on the gapmer modification design.
counterparts. A recent study suggested strong binding between PS ASOs and albumin; however, the binding affinity of PS ASOs with albumin has previously been reported to be low (370–480 μM). Thus, fatty-acid-modified gapmer ASOs may offer a greater likelihood to form an endogenous assembled construct with HSA for altering the pharmacokinetics of the ASO. This work introduces a delivery technology platform based on endogenous self-assembly of gapmer ASOs and HSA for tuning pharmacokinetics.

RESULTS

The chemical structure of the N2’-functionalized amino-LNA monomers presented in Table 1 show the fatty acid and backbone modification sites. The gapmer ASOs modified with either one or two palmitic or myristic acid residues at different locations within the ASO sequence were performed utilizing 2’-amino-LNA nucleosides as the modification site (Figure 1). The interaction with recombinant HSA (rHSA) of a range of different fatty acid-modified gapmer ASO designs was investigated using an electrophoretic mobility shift assay (Figure 2). It was found that the number, position, and fatty acid modification influenced the binding affinity to albumin based on a modification screen of the gapmer ASOs. Increased binding affinity was observed with increased number of fatty acid modifications. Introduction of PS backbone linkages increased the binding affinity of fatty acid-modified ASOs to albumin; however, the increase was much less significant compared with that of fatty acid modifications.

<table>
<thead>
<tr>
<th>ASO</th>
<th>Design</th>
<th>Modification</th>
<th>Backbone</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td></td>
<td>-</td>
<td>PO</td>
</tr>
<tr>
<td>#2</td>
<td></td>
<td>1 × 5’ palmitoyl</td>
<td>PO</td>
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<tr>
<td>#3</td>
<td></td>
<td>1 × 5’-palmitoyl, 1 × 3’-palmitoyl</td>
<td>PO</td>
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<tr>
<td>#4</td>
<td></td>
<td>2 × 3’-palmitoyl</td>
<td>PO</td>
</tr>
<tr>
<td>#5</td>
<td></td>
<td>1 × 5’-myristic acid</td>
<td>PO</td>
</tr>
<tr>
<td>#6</td>
<td></td>
<td>1 × 5’-myristic acid, 1 × 3’-myristic acid</td>
<td>PO</td>
</tr>
<tr>
<td>#7</td>
<td></td>
<td>-</td>
<td>PS</td>
</tr>
<tr>
<td>#8</td>
<td></td>
<td>1 × 5’-palmitoyl</td>
<td>PS</td>
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<tr>
<td>#9</td>
<td></td>
<td>1 × 5’-palmitoyl, 1 × 3’-palmitoyl</td>
<td>PS</td>
</tr>
<tr>
<td>#10</td>
<td></td>
<td>2 × 3’-palmitoyl</td>
<td>PS</td>
</tr>
<tr>
<td>#11</td>
<td></td>
<td>1 × 5’-myristic acid</td>
<td>PS</td>
</tr>
<tr>
<td>#12</td>
<td></td>
<td>1 × 5’-myristic acid, 1 × 3’-myristic acid</td>
<td>PS</td>
</tr>
</tbody>
</table>

Gapmer ASOs with 0× and 2× 3’ palmitoyl modifications (referred to as 0× Pal and 2× Pal, respectively), with either PO or PS backbone linkages, were then used to investigate correlation between the binding data and blood circulatory half-life and biodistribution in mice. Addition of a Cy5.5 fluorescent tag to the 5’ end of the gapmer ASOs was shown not to affect the binding affinity to albumin for the 0× Pal and 2× Pal ASOs with either PO or PS backbone linkages. For blood circulatory half-life and biodistribution studies, Cy5.5 fluorescent-labeled 0× Pal and 2× Pal gapmer ASOs were tracked using live fluorescent bioimaging. Gapmer ASOs (3.5 mg/kg in a total volume of 200 μL of either the 0× Pal gapmer ASOs and equivalent moles of 2× Pal PO, 0× Pal PS, or 2× Pal PS gapmer ASOs) were administered by a single bolus tail-vein intravenous injection into 8- to 9-week-old healthy female C57BL/6 mice (n = 5). Blood was collected from the tongue after 1 min by sublingual vein puncture, and by tail-nicking after 30 min, 2 hr, 4 hr, and 24 hr. Plasma was isolated and the fluorescence determined by an In Vivo Imaging Service (IVIS) scanner. Quantification of plasma fluorescence revealed a more than 2-fold increase in blood circulatory half-life for the 2× Pal gapmer ASO (t1/2 23 min and t1/2 49 min for PO ASOs with 0× and 2× Pal, respectively) most probably because of endogenous self-assembly of gapmer ASOs/albumin constructs. Inclusion of PS linkages resulted in a 20% increased circulatory half-life for the 0× Pal PS and a further 35% increase for the 2× Pal PS compared with the PO gapmer ASOs (t1/2 28 min and t1/2 66 min for PS gapmer ASOs with 0× and 2× Pal, respectively) (Figure 3).

The biodistribution of the different modified gapmer ASOs (0× Pal PO, 2× Pal PO, 0× Pal PS, and 2× Pal PS) was determined by measurement of fluorescence in the individual organs, 24 hr post-injection of 3.5 mg/kg in C57BL/6 mice. From the ex vivo organ scan, a clear shift in biodistribution was observed dependent on modifications (Figure 4). The non-palmitoylated PS gapmer ASOs showed a distinct superior accumulation within the kidney, in comparison with the non-palmitoylated PO gapmer ASOs (Figure 4). For the
Palmitoylated PO gapmer ASOs, an 8-fold increase in signal from the liver and a 4-fold decrease in the signal in the kidney were observed compared with non-palmitoylated PO. The palmitoylated PS gapmer ASOs, however, showed a 2-fold decrease in the signal from the kidney but a 2- to 8-fold increase in signal for the rest of the organs compared with non-palmitoylated PO gapmer ASOs. The biodistribution of the palmitoylated ASOs/albumin assemblies shifted to a biodistribution resembling that of albumin, supporting endogenous ASO/HSA binding and involvement of albumin in the transport of palmitoylated gapmer ASOs.

Figure 2. Polyacrylamide Gel Electrophoresis of Gapmer ASO/HSA Constructs
Upper panels show recombinant human serum albumin (rHSA)-bound gapmer antisense oligonucleotides (ASOs); lower panels show naked gapmer ASOs. Gapmer ASOs without (left) or with (right) phosphorothioate (PS) backbone linkages are shown. (A) No palmitoylated modifications. (B) From top to bottom: 1×3’-palmitoylated modification, 1×3’- and 1×5’-palmitoylated modifications, 2×3’-palmitoylated modifications, 1×5’-myristoylated modification, and 1×3’- and 1×5’-myristoylated modifications myristoylated modifications. Gapmer ASOs are visualized by SYBR gold staining. Illustrations depict the different gapmer ASO designs: phosphodiester (PO) backbone is represented by a thin black line, whereas PS backbone is represented by a thick black line. LNA is depicted by black dots, palmitic acid is represented by black ovals, and myristic acid is represented by hollow ovals. For an example of a whole gel, refer to Figure S1.
DISCUSSION
Site-specific delivery is a challenge to the clinical translation of nucleic-acid-based therapeutics. In this work we modulate binding to HSA to control gapmer ASOs pharmacokinetics. Utilization of an endogenous assembly process allows harnessing the natural transport properties of albumin \(^{17,18}\) without the necessity for elaborate preformulated designs and associated adverse effects with many delivery systems. Previous work has shown a rapid exponential clearance from peak plasma concentrations of ASOs following intravenous injection, with a serum half-life of \(\sim 2-40\) min and similar pharmacokinetic properties across species. \(^{19}\) This is thought to be due to renal clearance and a rapid distribution into tissue, followed by a slower elimination phase proposed to be caused by an equilibrium between post-distribution phase plasma concentrations and tissue concentrations. \(^{20}\) Inclusion of PS linkages within the backbone of ASOs has shown to increase the blood circulatory half-life, attributed to a non-specific binding with serum proteins such as albumin that may act as an endogenous vector carrying ASOs. \(^{12,21,22}\) Initial biodistribution following systemic administration may be facilitated by binding of PS ASOs to serum proteins, and that lack of serum protein interaction results in kidney filtration. \(^{15,21,23,24}\) Among the three main plasma proteins studied (albumin, \(\alpha_2\)-macroglobulin, and \(\alpha_1\)-acid glycoprotein), albumin exhibited the strongest binding to PS ASOs using a radiolabeled method, implicating a major role in PS ASOs transport in vivo. \(^{15,23}\) PS backbone-mediated binding to plasma proteins has been suggested to be nonspecific low affinity to hydrophilic sites, with no competition displacement shown by a range of albumin binding drugs with known albumin binding sites. \(^{23}\) Piao et al. \(^{24}\) showed by fluorescence correlation spectroscopy (FCS) analysis and a gel shift assay the importance of a PS backbone for binding of ASOs to albumin. However, despite the reported binding between PS-modified ASOs and albumin, PS-modified ASOs still exhibit a short circulatory half-life of \(\sim 2-40\) min. \(^{7}\) Marketed non-oligonucleotide drugs such as fatty acid-modified Levemir and Victoza aim to utilize albumin’s intrinsic transport properties and long circulatory half-life for long-lasting therapeutics by fatty acid-mediated binding of endogenous albumin. Advancing the gapmer ASO designs by inclusion of a fatty acid moiety, therefore, offers the possibility to develop a more stable gapmer ASO/HSA construct to facilitate an extended circulatory half-life.

In our work both palmitoyl and myristoyl amino-modified gapmer ASOs bind with HSA as shown by an electrophoretic method. Furthermore, the binding affinity of the gapmer ASOs toward HSA was strongly dependent on number and position of the modifications, and the binding toward HSA increased when replacing PO linkages with PS linkages in the backbone of the gapmer ASOs. We investigated the albumin binding of gapmer ASOs with one or two palmitoyl or myristic acid modifications at varying positions in the gapmer ASOs. Inclusion of two fatty acid modifications such as \(2 \times\) palmitoyl or \(2 \times\) myristic acids, either two in one wing or one in each of the two wings, exhibited a stronger binding to albumin than a single modification on the same ASOs. Therefore, the modification numbers play a crucial role in binding affinity rather than modification positions. We
show by Nanoparticle Tracking Analysis (NTA) a high particle concentration of $7.5 \times 10^8$ particles/mL for palmitoylated gapmer ASOs that decreased to $2.5 \times 10^8$ particles/mL when incubated with HSA (Figure S2) that suggests interaction and gapmer solubilization. A low $1.9 \times 10^8$ particles/mL was observed for gapmer ASOs without fatty acid or backbone modification, similar to $2.4 \times 10^8$ particles/mL of the HSA alone group. To investigate the likelihood of palmitoyl-mediated gapmer ASOs/albumin endogenous assembly for pharmacokinetic modulation, a co-localization gel shift assay was performed following gapmer ASO incubation with mouse serum (Figure S3). A distinct gapmer band that co-localized with the predominant serum protein, most likely albumin, was found for the 2× palmitoyl gapmer ASOs (both 1× 3′-palmitoyl and 2× 3′-palmitoyl) in contrast with no restricted migration or co-localization observed for the non-modified gapmer ASO.

In vivo experiments in mice were performed to investigate correlation between serum albumin binding and pharmacokinetics. We showed an increased circulatory half-life of the gapmer ASOs modified with 2× palmitoyl at the 3′ end ($t_{1/2}$ 23 min and $t_{1/2}$ 49 min for 0× and 2× 3′-palmitoylated PO gapmer ASOs, respectively). Inclusion of PS linkages further increased the circulatory half-life ($t_{1/2}$ 28 min and $t_{1/2}$ 66 min for 0× and 2× 3′-palmitoylated gapmer ASOs with PS linkages, respectively), suggesting that the effects are additive, which may be attributed to an increased binding to serum albumin, as was observed for the in vitro binding experiments. Rapid binding ($<1$ min) was found between the 2× 3′-palmitoyl gapmer ASO and recombinant human serum albumin (rHSA) in a gel shift assay (Figure S4), which supports an instant endogenous assembly process needed to facilitate pharmacokinetic modulation.

Previous studies have shown that gapmer ASOs rapidly transfer from blood into tissues and distribute with highest concentrations in the liver, kidney, bone marrow, adipose tissue, and lymph nodes. We showed that the non-functionalized gapmer ASOs accumulate primarily within the kidney, with trace amounts detected in the liver,
siRNA. We have previously shown that cholesteryl modification of the binding domain to be more processive. The type of modification was shown to increase blood circulatory half-life (from <5 min to >30 min) compared with unmodified siRNA. We have previously shown that cholesteryl modifications on siRNA extend the plasma half-life and biodistribution more significantly than LNA modification. Correlation was found between albumin binding and modification number, with two cholesteryl modifications showing a plasma half-life of 71 min and 45 min for one cholesteryl modification preformed with albumin.

The gapmer ASO designs described in this work were focused on optimal binding rather than gene silencing activity design considerations. RNase H activity was demonstrated using an in vitro RNase H assay. Escherichia coli RNase H is widely used to compare the effect of nucleotide modifications on RNase H activity and is very structurally similar to the mammalian one, in which the only difference is that the mammalian one contains an N-terminal hybrid binding domain to be more processive. The type of modification was shown to influence the level of target mRNA cleavage (Figure S5). The inclusion of two palmitoyls at the 5' end lowered the activity compared with inclusion of a single palmitoyl at both the 3' and 5' ends, which suggests a necessity to screen and select designs with combined binding and activity functionality for optimal therapeutic effects for each particular mRNA target. The strong albumin binding observed for the 3'- and 5'-palmitoyl gapmers identifies potential candidate designs.

In this work, wild-type mice (C57BL/6) were used for ASO pharmacokinetics studies, and HSA was used for investigation of ASO binding. Albumin from different species share a high sequence homology above 70%; however, we cannot discount interspecies binding differences that may influence interpretation of in vitro binding and in vivo performance correlation. The availability of a double-transgenic humanized HSA and human neonatal Fc receptor mouse model that better mimics the human physiological conditions could be used for future pharmacokinetics studies.

This work introduces a technology platform for a design-based fatty acid modification approach to potentiate endogenous albumin binding for tunable pharmacokinetics of gapmer ASOs. Further development and optimization may offer an attractive alternative to preformulated molecular medicine drug delivery systems.

MATERIALS AND METHODS

ASOs

The gapmer ASOs (ASO 1–12) were prepared by automated oligonucleotide synthesis using commercial DNA and LNA phosphoramidites and palmitoyl- and myristoyl-amino-LNA phosphoramidites using standard methods for oligonucleotide synthesis, workup, purification, and isolation with minor modifications as published for oligonucleotides containing palmitoyl-amino-LNA nucleotide monomers. Sequences of all ASOs are listed: ASO 1: 5'-TAGccgtcactt CTC-3', ASO 2: 5'-PEGccgtcacttCTC-3', ASO 3: 5'-PEGccgtcactt CPC-3', ASO 4: 5'-TAGccgtcacttCPP*-3', ASO 5: 5'-MAGccgtcactt CTC-3', ASO 6: 5'-MAGccgtcacttCMC-3', ASO 7: 5'-TAGccgt caacttCTC-3', ASO 8: 5'-PEGccgtcacttCTC-3', ASO 9: 5'-PEGccgtca cttCPC3-3', ASO 10: 5'-TAGccgtcacttCPP*-3', ASO 11: 5'-MAG ccgtcacttCTC-3', ASO 12: 5'-MAGccgtcacttCMC-3'. P and M denote palmitoyl-amino-LNA and myristoyl-amino-LNA thymine monomers, respectively; P* and M* denote palmitoyl-amino-LNA and myristoyl-amino-LNA 5-methyl-cytosine monomers, respectively; A, C, G, and T denote LNA monomers, and a, c, g, and t denote DNA monomers. Underlined sequences are with PS linkages. Gapmer ASOs were dissolved in Nuclease-Free water (Ambion) to 200 μM, and concentrations were determined using an Implen NanoDrop (Thermo Scientific). Oligonucleotides were stored at −20°C.

Self-Assembly of Gapmer ASOs with HSA

rHSA (Sigma) was used in this experiment. Binding toward rHSA was carried out by titrating the amount of HSA (0.63–40 μM) to a constant amount of gapmer ASOs (1 μM) in a fixed volume (20 μL). Samples were left to incubate at room temperature for 4 hr before analysis by 8% native gel electrophoresis.

Electrophoretic Mobility Shift Assay

Gel experiments were carried out using the XCell SureLock Mine-Cell Electrophoresis System, using 8% polyacrylamide (National Diagnostics) gels 1× TBE (Tris-borate-ethylenediaminetetraacetic acid [EDTA]) buffer (from 10× stock; GibCO, Life Technologies). Samples were loaded using Novex TBE Running Buffer (5×) (Invitrogen). Gels were run in 1× TBE buffer. Staining for gapmer ASOs was performed using SYBR-Gold nucleic acid stain (Invitrogen) following standard protocol.

In Vivo Blood Circulatory Half-Life and Biodistribution of Fatty-Acid-Modified Gapmer ASOs

Seven- to eight-week-old female C57BL/6 mice were maintained for 2 weeks prior to the experiments. To each mouse a dose of 3.5 mg/kg in a total volume of 200 μL of Cy5.5-labeled gapmer ASOs with either 0× or 2× 3'-palmitoylated modifications, and either PO or PS backbone linkages were administered (n = 5 per gapmer ASO design) by tail-vein injection in a 200 μL volume PBS solution. After injection, blood samples of 10 μL were taken into capillary tubes (Hirschmann Laborgeräte) after 1 min from the tongue, by sublingual vein puncture, and after 30 min, 2 hr, and 4 hr by tail-nicking. At
24 hr the animals were terminated and the organs collected. Blood and organs were analyzed using the IVIS Spectrum Biotagger (PerkinElmer). Spectral unmixing and subsequent data analysis was carried out using the Living Image software, version 4.3.1 (PerkinElmer).

The experiments were approved by the Danish Experimental Inspectorate (J.No. 2013-15-2934-00789), and housing of the mice was carried out according to Danish legislation and the Directive 2010/63 on the protection of animals used for scientific purposes.

SUPPLEMENTAL INFORMATION
Supplemental Information includes six figures and can be found with this article online at http://dx.doi.org/10.1016/j.ymthe.2017.05.009.

AUTHOR CONTRIBUTIONS
K.A.H., J.W., and J.K. provided conception of the research. K.A.H., J.W., M.L.H., and F.D.-H. planned the studies. M.L.H., Y.C., and J.S.N. performed experiments, and analyzed and interpreted data. K.A.H. and M.L.H. drafted the manuscript. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST
J.W. is inventor on a patent application covering the palmitoyl-gamper technology platform presented herein, and this patent application is owned by RiboTask ApS, of which he is co-owner.

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