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Lipid Nanoparticles Improve Activity of Single-Stranded siRNA and Gapmer Antisense Oligonucleotides in Animals

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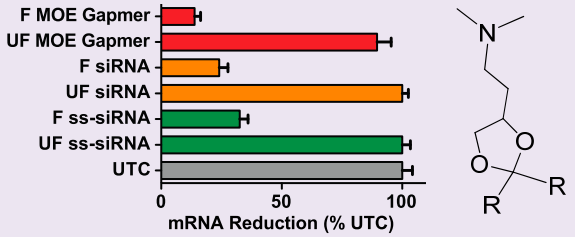
ABSTRACT: We evaluated the abilities of an antisense oligonucleotide (ASO), a small interfering RNA (siRNA), and a single-stranded siRNA (ss-siRNA) to inhibit expression from the *PTEN* gene in mice when formulated identically with lipid nanoparticles (LNPs). Significantly greater reductions in levels of *PTEN* mRNA were observed for LNP-formulated agents compared to unformulated drugs when gene silencing was evaluated after a single dose in the livers of mice. An unformulated ss-siRNA modified with a metabolically stable phosphate mimic 5′-(*E*)-vinylphosphonate showed dose-dependent reduction of *PTEN* mRNA in mice, albeit at doses significantly higher than those observed for formulated ss-siRNA. These results demonstrate that LNPs can be used to deliver functional antisense and ss-siRNA therapeutics to the liver, indicating that progress in the field of siRNA delivery is transferable to other classes of nucleic acid-based drugs.



F: formulated; UF: unformulated; UTC: untreated control

Oligonucleotide-based agents that work through antisense and RNA interference (RNAi) mechanisms show great promise in the clinic, particularly against targets that are otherwise not targetable with small-molecule drugs.[1−3]

Antisense oligonucleotides (ASOs) may induce RNase H cleavage of target mRNA or may modulate splicing or induce translational arrest.[1,4] RNase H-mediated cleavage of mRNA is directed by single-stranded DNA oligonucleotides that bind to complementary mRNA. In "gapmers", the central region of the ASO is made up of DNA residues to direct mRNA cleavage *via* RNase H, and the flanking regions are modified nucleotides that enhance the binding affinity or nuclease resistance.[1,4] Duplexes with RNA or RNA-like chemical modifications known as small interfering RNAs (siRNAs) mediate gene silencing through the RNA interference (RNAi) pathway.[5−8] Recent reports have shown that certain chemically modified single-stranded RNAs (ss-siRNAs) can also induce mRNA cleavage *in vitro* and *in vivo* through an RNAi-based mechanism.[9]

One of the main challenges facing developers of nucleic acid therapeutics is the delivery of functional nucleic acids to target tissues.[4] Transfection reagents are routinely utilized to enhance the delivery of nucleic acids for *in vitro* studies.[10] Identifying materials that mediate delivery of nucleic acids drugs to target tissues in animals has proved difficult.[11] A novel class of molecules, termed lipid nanoparticles (LNPs, Figure 1a), have been developed to deliver short RNAs to mammalian cells both *in vitro* and *in vivo*.[12−15] LNP-formulated siRNAs have been shown to effectively silence expression of several genes including *ApoB*, *Factor VII*, and *PCSK9 in vivo*.[13,16]

In this study, we evaluated the influence of LNP formulation on the *in vivo* activity of an antisense oligonucleotide and an ss-siRNA and compared their activities with that of an unoptimized siRNA targeting the same gene. We chose to target the tumor suppressor gene *phosphatase and tensin homologue* (*PTEN*)[17] as gapmer ASOs, ss-siRNAs, and siRNAs have been characterized that inhibit expression of this gene.[9,18−20] PTEN is expressed in many different tissues, including the liver,[21] an organ for which LNP-mediated delivery of siRNAs has previously been validated.[12]

ss-siRNA **1**, siRNA **2**, and ASO **3** (Table 1) were used for this study; activities of all oligonucleotides have been previously characterized in cell-based assays.[9,22,23] The ss-siRNA **1** is modified with both 2′-*O*-(2-methoxyethyl) (2′-MOE), 2′-*O*-methyl (2′-OMe), and 2′-fluoro (2′-F) nucleotide analogues (Figure 1b). The siRNA **2** was prepared by annealing ss-siRNA **1** with complementary RNA.[9,24] ASO **3** contains 2′-MOE residues that flank a central DNA region. Earlier studies showed that the siRNA **2** had *in vitro* potency higher than that of either ss-siRNA **1** or ASO **3**.[9] In agreement with the previous study, evaluation of these agents transfected into HeLa cells with Lipofectamine 2000 revealed the same trend (Table 1).

To determine the *in vivo* efficacy of ss-sRNA **1**, siRNA **2**, and the ASO **3** under identical formulation conditions, each agent was encapsulated in the LNP using DLin-KC2-DMA,[17] DSPC, cholesterol, and PEG-lipid in a molar ratio of 57.5/7.5/31.5/

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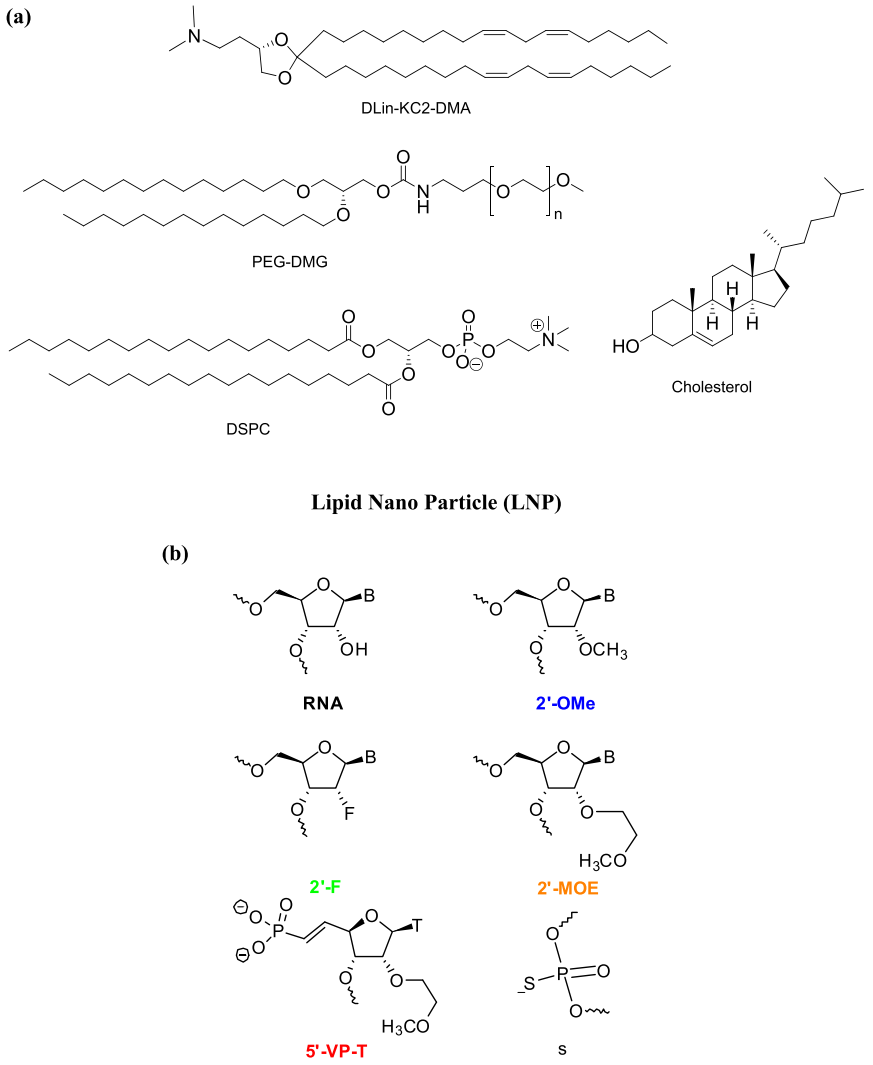

Figure 1. (a) Chemical structure of the LNP: 2,2-dilinoleyl-4-dimethylaminoethyl[1,3]dioxolane. (b) Structures of nucleotide analogues used to modify agents used in this study.

Table 1. PTEN ss-siRNA, siRNA, and ASO Sequences, Chemical Modifications, and Activity in Cell Culture[a]

No.	Sequence	Chemistry	Comment	IC_{50} nM
1	5'-P_o-$T_sU_sGU_sCU_sCU_sGG_sUC_sCU_sU_sA_sC_sU_sU_sA_sA$ -3'	2'-F, 2'-OMe, 2'-MOE, 5'-P	ss-siRNA	2 ± 0.3
2	5'-P_o-$T_sU_sGU_sCU_sCU_sGG_sUC_sCU_sU_sA_sC_sU_sU_sA_sA$ -3' 3'- r(AACAGAGACCAGGAAUGAA) -5'	2'-F, 2'-OMe, 2'-MOE,5'-P	siRNA	0.2 ±.01
3	5'-d($^mC_sT_sG_sG_s{}^mC_sT_sA_sG_s{}^mC_s{}^mC_sT_s{}^mC_sT_sG_sG_sA_sT_sT_sT_sG_sA$)-3'	2'-MOE, DNA	MOE gapmer ASO	4 ± 2
4	**5'-VP-** $T_sU_sGU_sCU_sCU_sGG_sUC_sCU_sU_sA_sC_sU_sU_sA_sA$ -3'	2'-F, 2'-OMe, 2'-MOE, 5'-VP-T	ss-siRNA	1 ± 0.4

[a]Modifications in each strand are indicated by color corresponding to that in the column headed "Chemistry", and structures are shown in Figure 1b. P = phosphate; s = phosphorothioate; 2′-F = 2′-flouro; 5′-VP-T = 5′-(*E*)-vinylphosphonate-2′-MOE T; ^{m}C = 5-methylcytidine.

3.5, shown in Figure 1a. Mice (n = 4/group) were dosed intravenously with 4.5 mg kg^{-1} of ss-siRNA **1**, siRNA **2**, or ASO **3** formulated with LNP. A second control group was dosed intravenously with 4.5 mg kg^{-1} of the unformulated ss-siRNA **1**, siRNA **2**, or ASO **3**. Following a single administration, LNP-formulated ss-siRNA **1**, siRNA **2**, and ASO **3** inhibited *PTEN* mRNA expression at similar levels (Figure 2a). The slight target reduction observed for unformulated ASO **3** was expected given its potency *in vitro* (IC_{50} of 4 nM) and pharmacokinetic properties of oligonucleotides with phosphorothioate backbone. In contrast, the unformulated siRNA and ss-siRNA were inactive (Figure 2a), presumably due to rapid clearance. Importantly the LNP formulation enhanced the potency of ss-siRNA **1**, siRNA **2**, and ASO **3**. Finally the formulated compounds proved to be well-tolerated as no changes in plasma transaminase levels or body and organ weights were observed (data not shown).

Next, we determined the concentrations in liver of full-length oligonucleotides and metabolites after a single dose of the LNP-formulated ss-siRNA **1**, siRNA **2**, or ASO **3**. The oligonucleo-

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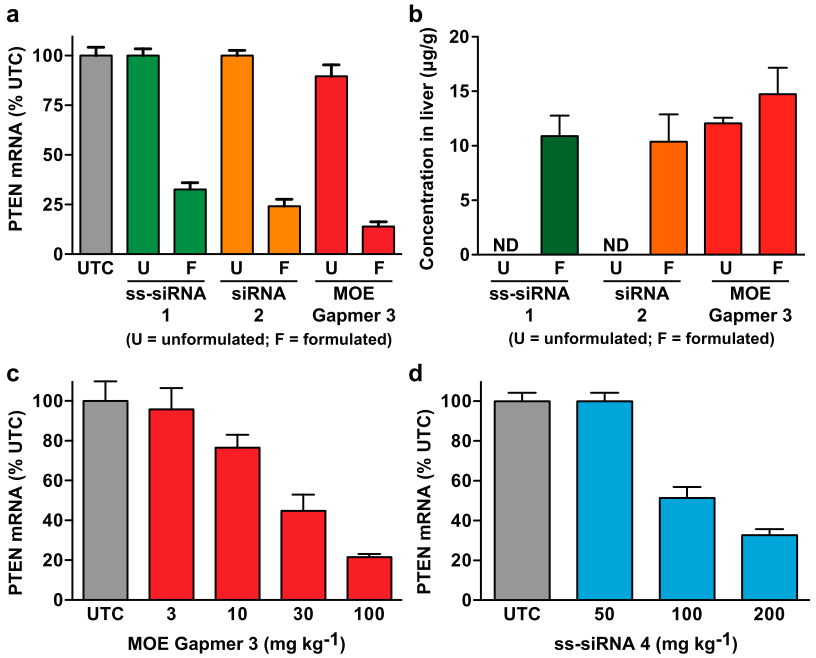

Figure 2. (a) Reduction of *PTEN* mRNA in liver of mice that received a single intravenous dose (4.5 mg kg^{-1}) of either LNP-formulated ss-siRNA **1**, siRNA **2**, or ASO **3** or unformulated ss-siRNA **1**, siRNA **2**, or ASO **3**. (b) Concentration of ss-siRNA **1**, siRNA **2**, or ASO **3** in the liver of mice treated with 4.5 mg kg^{-1} of either formulated or unformulated ss-siRNA **1**, siRNA **2** and ASO **3**. (c) *PTEN* mRNA inhibition in mice (n = 4) after single subcutaneous dose of 3, 10, 30, or 200 mg kg^{-1} of unformulated ASO **3**. (d) *PTEN* mRNA inhibition in mice (n = 4) after single subcutaneous dose of 50, 100, or 200 mg kg^{-1} of unformulated ss-siRNA **4**.

tides were extracted from the liver of treated mice and analyzed by liquid chromatography−mass spectrometry (LC−MS). Neither ss-siRNA **1** nor siRNA **2** extracted from mouse liver contained a 5′-terminal phosphate group (Figure 2b). No siRNA **2** full-length passenger strand was detected.[9] Only full-length ASO **3** was identified. Similar tissue levels were observed for formulated ss-siRNA **1**, the guide strand of siRNA **2**, and ASO **3** (Figure 2b). These results are in accordance with the known biodistribution properties of ASOs, unformulated siRNAs, and formulated siRNAs.[4,25]

The loss of the 5′-phosphate observed for unformulated ss-siRNA **1** in mice is important given that the 5′-phosphate is required for ss-siRNA activity.[9] That the formulated ss-siRNA **1** with a metabolically unstable 5′-phosphate showed good activity in mice suggests that LNP formulation protects the 5′-phosphate from phosphatase-mediated metabolism in the liver. ss-siRNA **1** and siRNA **2** extracted from mouse liver did not contain the 5′-terminal phosphate group (Figure 2b), suggesting that phosphatases rapidly removed the 5′-phosphate once the ss-siRNA or siRNA dissociated from the LNP complex.

Dose-dependent reductions of *PTEN* mRNA were observed for the unformulated ASO **3** (Figure 2c). In contrast, no target reduction was observed for unformulated ss-siRNA **1** and siRNA **2** even at 300 mg kg^{-1} dose (data not shown). To achieve ss-siRNA activity *in vivo* without the use of formulations, we developed a metabolically stable phosphate mimic 5′-(*E*)-vinylphosphonate (Figure 1b, 5′-VP-T) with conformation and stereoelectronic properties similar to those of the natural phosphate.[9] Consistent with the reported data,[9] unformulated ss-siRNA **4** (Table 1) modified with a 5′-VP showed dose-dependent reduction of *PTEN* mRNA in mice (Figure 2d), albeit at doses higher than those observed for formulated ss-siRNA **1**.

Our results show that LNP formulations significantly improved gene silencing by an MOE gapmer ASO and by an ss-siRNA *in vivo* relative to unformulated agents. This trend is similar to that previously described for siRNAs.[26] LNP formulations increased the ED_{90} of ASO **3** (ED_{90} 4.5 mg kg^{-1}) more than 20-fold relative to unformulated ASO **3** (ED_{90} 100 mg kg^{-1}). LNP formulations increased the ED_{75} of the ss-siRNA more than 45-fold (4.5 vs 200 mg kg^{-1}) of the ss-siRNA. LNPs have been shown to enhance distribution of siRNA to liver.[15] Without LNP formulation, no ss-siRNA was detected in liver, and the LNP formulation also increased the amount of MOE gapmer ASO detected in liver (Figure 2b). Importantly, the enhancement in activity observed for the LNP-formulated ASO was significantly greater than would be expected from the increase in target tissue accumulation, suggesting that other factors are likely contributing to the observed enhanced activity. Specifically, the LNP (Figure 1a) used in this study has a pK_a (which represents an average surface charge of the particle) of 6.4 and will become positively charged in endosome.[15] In addition, biophysical characterization of this LNP suggests that it has improved capacity for destabilizing lipid bilayers.[15] Consequently, the LNP may enhance the release of the nucleic acid-based drugs from the endosomes.

This study provides a direct comparison of the relative efficacies of LNP-formulated MOE gapmer ASO, ss-siRNA, and siRNA, each targeting the same region of the *PTEN* mRNA in livers of mice. While the potencies of the oligonucleotides toward the PTEN target were not individually optimized, these nucleic acid-based agents possess notable differences. The MOE gapmer ASO and ss-siRNA are single-stranded and amphipathic, whereas the siRNA is double-stranded and thus approximately twice the molecular weight of the other agents and more hydrophilic. The differences in size, chemical structures, and physical properties of molecules suggest that

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the single-stranded agents might exhibit biodistribution and pharmacokinetics different from those of the duplex siRNA. Nevertheless, this study indicates that materials used to deliver siRNA can enhance delivery of MOE gapmer ASOs and ss-siRNAs and suggests that progress in the field of siRNA delivery may be transferable to other nucleic acid-based drugs. Our results corroborate the potential utility of nonviral, lipid-like carriers for the efficient delivery of ASOs and ss-siRNA therapeutics.

METHODS

Synthesis of Oligonucleotides. MOE gapmer ASO and ss-siRNA were synthesized according to the previously reported procedure.[9,19] siRNA was synthesized as reported[15] earlier.

Potencies of ss-siRNA, siRNA, and MOE Gapmer ASO in HeLa Cells. HeLa cells were seeded in 96-well plates at 5,000−10,000 cells well^{-1} 16 h prior to treatment. Transfection was performed using Opti-MEM medium (Life Technologies) containing 4−6 μg mL^{-1} Lipofectamine 2000 (Life Technologies) for 4 h at 37 °C. HeLa cells were grown in DMEM at 37 °C in 5% CO_2. Cells were lysed 16 h post transfection, and total RNA was purified using RNeasy 3000 Bio Robot (Qiagen). Reduction of target mRNA was determined by qRT-PCR as previously described.[27] Target mRNA levels were normalized to total RNA using RiboGreen (Life Technologies). IC_{50} curves and values were generated using Prism 4 software (GraphPad Software, www.graphpad.com).

LNP Formulation. LNP formulations included an ionizable cationic lipid, cholesterol, poly(ethylene glycol)-lipid (PEG-lipid), and nucleic acid as previously described.[28] LNP systems were made using the preformed vesicle method.[29] DLin-KC2-DMA,[17] 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), cholesterol, and PEG-lipid (PEG-DMG) were solubilized in ethanol at a molar ratio of 57.5/7.5/31.5/3.5. The lipid mixture was added to an aqueous buffer (100 mM citrate, pH 3) with mixing to final ethanol and lipid concentrations of 35% (v/v) and 7.4 mg mL^{-1}, respectively, and allowed to equilibrate at RT for 2 min before extrusion. The hydrated lipids were extruded through two stacked 80-nm pore-sized filters (Nuclepore) at RT using a Lipex Extruder (Northern Lipids) until a vesicle diameter of 70−90 nm, as determined by dynamic light scattering analysis, was obtained. This generally required 1−3 passes. The nucleic acid (solubilized in a 100 mM citrate, pH 3, 35% ethanol) was added to the presized empty vesicles. After a nucleic acid/lipid ratio of 0.10 (w/w) was achieved, the mixture was incubated for 30 min at 35 °C to allow vesicle reorganization and encapsulation of the nucleic acid. The ethanol was then removed, and the external buffer was replaced with PBS (155 mM NaCl, 3 mM Na_2HPO_4, 1 mM KH_2PO_4, pH 7.5) by dialysis using a 10,000 molecular weight cutoff membrane. Particle size was determined using a Malvern Zetasizer NanoZS. The nucleic acid entrapment efficiencies were greater than 95% as determined by Ribogreen assay (Invitrogen).[30] The resulting particles had a mean diameter of approximately 70 nm.

Treatment of Mice. All animal experiments were conducted according to the American Association for the Accreditation of Laboratory Animal Care guidelines and were approved by the Animal Welfare Committee. Male Balb/c mice, aged 6−7 weeks, were obtained from Charles River Laboratories. Test samples were diluted to desired concentrations in phosphate-buffered saline (PBS), filter sterilized, and administered by injection into tail veins in a volume of 100−250 μL. Animals were maintained at a constant temperature of 23 °C and fed standard lab diet and given free access to water. Animal weights were monitored prior to dosing and throughout the live phase of the study. Immediately prior to sacrifice, mice were anesthetized with isoflurane and a terminal bleed was performed by cardiac puncture. Plasma or serum was isolated from whole blood. Alanine aminotransferase (ALT) levels were determined using an Olympus AU400e bioanalyzer (Beckman Coulter). Immediately following terminal blood draw, mice were sacrificed by cervical dislocation while under anesthesia. In conjunction with necropsy, liver and spleen weights were determined.

RNA Analysis. Liver tissue was homogenized in RLT buffer containing 0.01% β-mercaptoethanol (Qiagen) immediately following sacrifice. RNA was extracted using RNeasy columns (Qiagen) according to the manufacturer's protocol. RNA was eluted from the columns with water. RNA samples were analyzed by fluorescence-based quantitative RT-PCR using an Applied Biosystems 7700 sequence detector. Levels of target RNAs were normalized to total RNA levels using the Ribogreen method (Life Technologies).[30] Primers used for determination of *PTEN* RNA levels are as follows: forward primer, 5′-ATG ACA ATC ATG TTG CAG CAA TTC-30, RP 50 CGA TGC AAT AAA TAT GCA CAA ATCA-3′; and reverse primer, 5′-6FAM-CTG TAA AGC TGG AAA GGG ACG GAC TGG T-TAMRA-3′.

Determination of Liver Concentrations of Nucleic Acid Agents Using LC−MS. ss-siRNAs, siRNAs, and MOE gapmer ASO were extracted from liver samples along with five to eight standards of each analyte prepared in control liver and analyzed by LC−MS using previously described methods.[9] Briefly, a 27-mer MOE/DNA phosphorothioate oligonucleotide was added as an internal standard to each sample and analyte standard before extraction with phenol/chloroform and then solid-phase extraction (SPE) of the resulting aqueous extract using phenyl-functionalized silica sorbent (Biotage). Mass measurements were made using a single quadrupole mass spectrometer and selected ion monitoring (SIM) of ion m/z corresponding to the −4 charge state of ss-siRNA with and without the 5′ phosphate, siRNA guide strand, MOE gapmer ASO, and −5 charge state of the internal standard. Peak areas from extracted ion chromatograms were determined for each analyte and normalized to the peak area of the internal standard. Concentrations were determined using a trendline established with the extracted standards and reported as μg g^{-1} liver.

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Notes

The authors declare no competing financial interest.

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