

Efficient and Persistent Splice Switching by Systemically Delivered LNA Oligonucleotides in Mice

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Locked nucleic acid (LNA) oligomers were found to be very effective in their ability to modulate alternative splicing *in vivo* in transgenic mice that ubiquitously express a modified EGFP pre-mRNA containing an aberrantly spliced β -globin intron (IVS2-654). Following intraperitoneal injections, the splice-switching oligonucleotide LNA SSO-654 targeted to the aberrant 5' splice site in EGFP-654 pre-mRNA corrected aberrant splicing and increased production of repaired EGFP mRNA mainly in the liver, colon, and small intestine. Little or no effect was detected in heart, lung, or kidney, the organ where most of the oligonucleotide was distributed after four consecutive daily injections. In the liver, LNA SSO-654 had an EC₅₀ of 3 mg/kg, approximately 17-fold more potent than its 2'-O-methyl congener. Moreover, in the liver, colon, and small intestine oral doses of 50 mg/kg resulted in detectable levels of splice switching. The effects of four daily injections at 25 mg/kg persisted for up to 29 days but did not result in liver toxicity. The results indicate that the LNA backbone confers sequence- and organ-specific functional biodistribution of the oligonucleotides and that these potent compounds have the potential to be safe and long-acting modulators of diseases treatable by splicing manipulation.

Key Words: alternative splicing, inborn genetic diseases, antisense oligonucleotides, locked nucleic acid, transgenic mice, EGFP protein

INTRODUCTION

Macromolecular therapies [1] have emerged as powerful alternatives to classical small-molecule drugs. Rather than relying on the screening of approximately 10⁴–10⁶ randomly generated small molecules, larger polymeric molecules of nucleic acids and peptides can be designed to act specifically on the target of interest, such that many fewer oligomers (10¹–10²) must be screened to achieve a “hit.” Rationally designed antibodies and conjugates to specific signaling molecules have quickly made their way from the laboratory to clinical practice [2]. A nucleic acid drug, Pegaptanib, an RNA aptamer against VEGF, was newly approved by the FDA as the first antiangiogenic therapeutic for wet macular degeneration [3] and shows substantial clinical benefits providing much needed treatments for patients.

Completion of the human genome led to the realization that most of the human proteome diversity is accomplished by alternative splicing of gene transcripts, or pre-mRNA [4]. It is now apparent that many biological

processes such as apoptosis can be controlled by alternative splicing and that its deregulation can cause a wide range of diseases, including cancer [5,6]. Studying alternative splicing on a global scale may also lead to the gene targets of genetic diseases, which have thus far evaded combinatorial small molecule screens. One of the first practical uses for the emerging data will be the rational design and development of drugs that modulate alternative splicing, to which antisense oligonucleotides are well suited. Indeed, in both cell and animal models, splice switching oligonucleotides (SSO) are capable of shifting alternatively spliced transcripts toward the more therapeutically favorable isoforms in a variety of genes, including lamin A [7], dystrophin [8], β -globin, bcl-x, CFTR, interleukin-5 receptor, tau, and SMN (reviewed in [9,10]).

SSOs are chemically modified both to eliminate counterproductive degradation of target pre-mRNA in the duplex by RNase H and to improve their *in vivo* efficacy (reviewed in [11,12]). In cells, fully modified oligonucleotides, including 2'-O-methyl (2'OMe) and 2'-O-methoxy-

ethyl (2'OMOE) substitutions and morpholino, and PNA backbones have all shown activity as SSOs. In a mouse model for splice switching activity in which incorrect splicing of the enhanced green fluorescent protein (EGFP)-654 transgene transcript can be corrected, and proper EGFP translation upregulated by an RNase H-inactive oligonucleotide (Fig. 1A), we established the *in vivo* efficacy of 2'-OMOE and PNA oligomers and showed that the addition of up to four lysine residues to the latter increased their uptake and performance [13]. Here, in the same system, we show that oligonucleotides with alternating deoxyribose/locked nucleic acid (LNA) subunits have dramatically higher efficacy than 2'OMe oligonucleotides. We also show that they are nontoxic and that the sequence-specific antisense effects of a single-dose regimen of LNA SSO persists for more than 3 weeks. Furthermore, oral delivery of unformulated LNA SSO induced minimal but clearly detectable splice switching in a sequence-specific manner in targeted pre-mRNA. The results indicate that the LNA backbone may offer significant advantages as a potential gene-specific therapeutic.

RESULTS

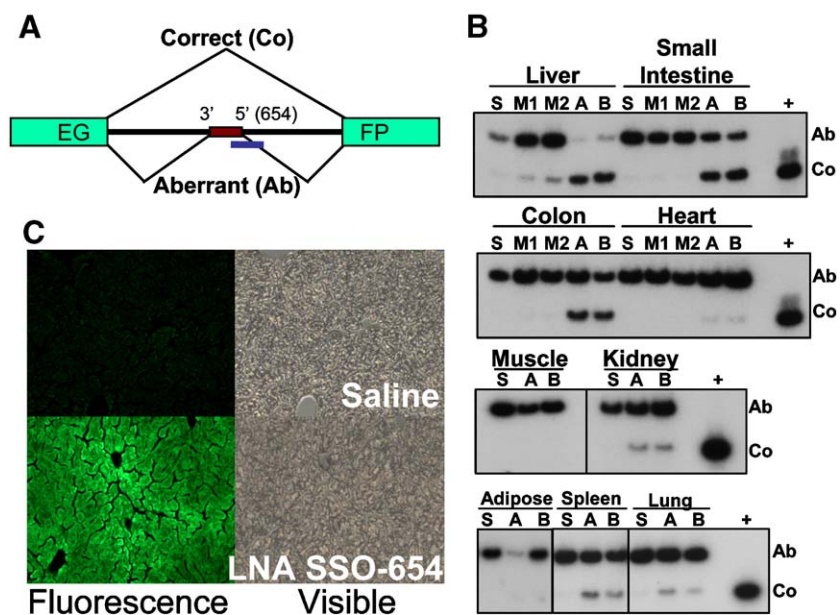
We assessed the splicing modulation ability of 16-mer LNA oligomers by administering (ip) 25 mg/kg LNA SSO-654 to our EGFP-654 transgenic mice (Fig. 1A) once a day for 4 days. We sacrificed the animals 1 day after treatment, isolated total RNA from various organs, and analyzed it by RT-PCR using primers that simultaneously detect correctly and incorrectly spliced EGFP-654 mRNA (see Materials and Methods). The results in Fig. 1B show that ip-injected LNA oligomers function as SSOs mainly in the liver, small intestine, and colon, where they induce

significant increases in correctly spliced EGFP. The effect is sequence specific; LNAs with mismatches to the target pre-mRNA were ineffective. At 25 mg/kg, splice switching in the liver was ~85% and induced clearly detectable fluorescence in the tissue (Fig. 1C); in small intestine and colon splice switching was at or above 50%, and in all other tissues the splice switching level was substantially less. In the liver and small intestine, LNA SSOs showed remarkable potency; at 6 mg/kg, 1/4 of the maximal tested dose, ~50% of the EGFP mRNA was still correctly spliced, with ~25% correctly spliced in colon (Fig. 2A). In liver, significant splice switching was still detectable at 0.75–1.5 mg/kg (see below). Quantification of these data indicates that in liver, the EC₅₀ of LNA SSO-654 was 3 mg/kg, with a maximum correction of 85%.

We assessed the persistence of splice switching after four daily injections of 25 mg/kg by analyzing treated animals 1, 8, 15, 22, or 29 days postinjection. The results in Fig. 2B clearly show that the LNA SSO effect can be seen to at least 22 days, at which splice switching remains up to 50%. At day 29, correctly spliced EGFP mRNA can still be detected above the saline-treated control. These functional data suggest that intact, functional LNA SSOs remain in the liver weeks after they are administered, since the EGFP mRNA half-life is approximately 24 h.

Mice treated with 25 mg/kg 2'OMe-modified SSO-654 showed a much lesser extent of splice switching in liver, small intestine, and colon than those treated with LNA. At 25 mg/kg 2'OMe in liver, we detected an approximate 25% shift in splicing (Fig. 3, liver, lanes A, B, and C), whereas LNA achieved the same level of splice switching at 1.5 mg/kg, a 17-fold lower dose (see Fig. 2A, liver, 1.5 mg/kg). Furthermore, the time course of 2'OMe-treated animals, compared with LNA, shows a much steeper

FIG. 1. Treatment of EGFP-654 transgenic mice with LNA SSO-654. (A) Transgenic mice express a modified EGFP gene driven by the β -actin promoter. The EGFP coding region (green) is interrupted by an aberrantly spliced intron from human β -globin in which a portion (red) is spliced into the mRNA, preventing EGFP expression. Blocking the aberrant 5' splice site within the intron with LNA oligonucleotide, SSO-654 (blue bar) prevents aberrant and restores correct splicing and proper translation of the EGFP coding regions. (B) Mice were injected ip with 25 mg/kg LNA SSO-654 once a day for 4 days. Total RNA was isolated from the indicated organs and analyzed by RT-PCR with EGFP-654-specific primers. Bands labeled "Co" (87 bp) and "Ab" (160 bp) represent correctly and aberrantly spliced EGFP mRNA, respectively. S, saline-treated mice. A, B and M1, M2, mice treated with SSO-654 and mismatch control oligonucleotide, respectively. For a given tissue, each lane represents a separate mouse experiment. Unless otherwise indicated this treatment and analytical procedures were used in the experiments illustrated in the subsequent figures. (C) Expression of EGFP protein in the liver of 25 mg/kg LNA SSO-654-treated animal.



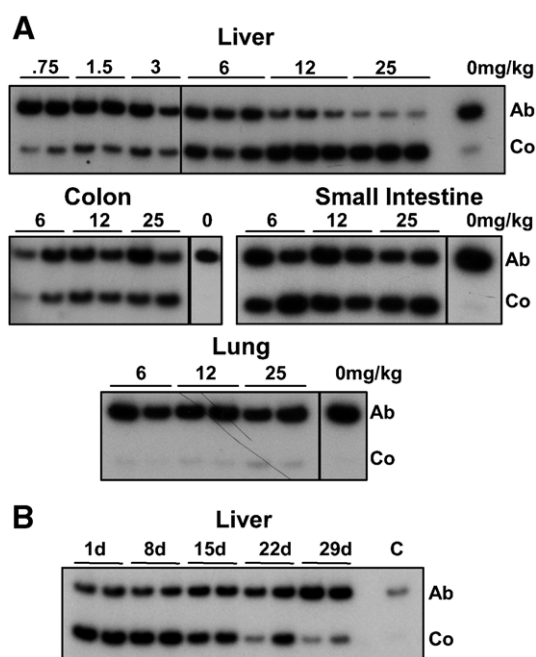


FIG. 2. Dose- and time-dependent splice switching by LNA SSO-654. (A) Analysis of total RNA from indicated organs by RT-PCR from mice injected with the indicated concentrations of LNA SSO-654. (B) Analysis of liver after mice were injected with 25 mg/kg LNA SSO-654 and sacrificed 1, 14, 21, and 29 days after the last injection.

extinction of the splice switching effect, indicating that it is cleared from the body faster and that it is less stable. By day 15, very little correctly spliced message was seen in the liver (data not shown). Interestingly, 2'OMe SSO-654-treated animals showed higher levels of splice switching in adipose tissue, approximately 20%, compared to animals treated with LNA SSO-654. We have seen such backbone-dependent difference in functional biodistribution of SSOs with other types of oligonucleotides [13].

We tested other routes of administration of LNA. Oral administration of these compounds was of particular interest, as previous work suggested that smaller sized nucleotide oligomers pass the intestinal barrier and gain entry into the bloodstream [14]. To test the oral bioavailability of the 16-mer LNA oligomers, we administered 50 mg/kg to mice by oral gavage and analyzed liver, small intestine, and colon for splice switching of EGFP pre-mRNA (Fig. 4). We found a small, but clearly above background (~5–10%), shift in splicing pattern in each of these tissues after treatment, suggesting that a modest fraction of the orally administered functional oligonucleotide was able to reach the bloodstream from the digestive tract. As we had taken no steps to buffer the dosing solution through the stomach, this suggests that a sufficient percentage of the acid-labile nucleosides remained intact upon entry into the small intestine. As expected, iv administration of LNA yielded functional

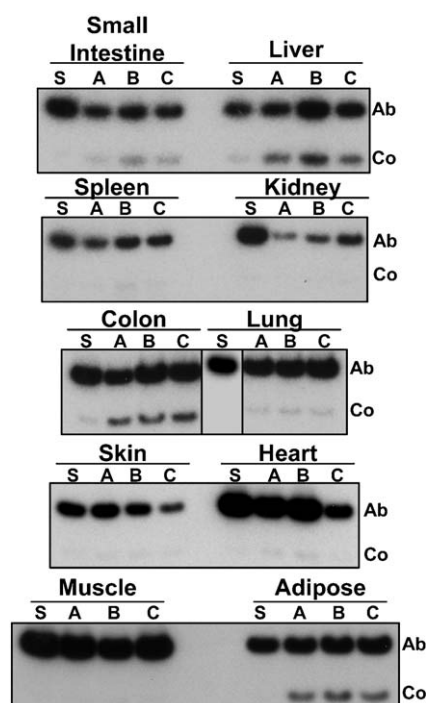


FIG. 3. Treatment of EGFP-654 transgenic mice with 2'OMe SSO-654. Mice were injected with 2'OMe SSO-654 oligonucleotide as for Fig. 1B.

distribution profiles similar to those seen after ip administration (data not shown).

Functional distribution is a subset of biodistribution in that it reflects not only where the oligonucleotide physically accumulates, but also where it exerts its effects. Interestingly, the functional distribution and biodistribution of the LNA SSOs were strikingly dissimilar. Our data show that after ip injection of LNA oligomers, most of the material is distributed to the kidney, followed by the liver, colon, and small intestine in that order (Fig. 5A). Previous biodistribution data for 2'OMe oligonucleotides show a similar profile [15]. However, neither SSO shows significant activity in the kidney, suggesting that although the compounds are concentrated there due to renal clearance, they do not enter the cells of this organ.



FIG. 4. Oral delivery of LNA SSO-654. 50 mg/kg SSO-654 was administered by oral gavage once a day for 4 days. Mice were sacrificed on the next day and analyzed for EGFP splicing patterns as for previous figures. +, 25 mg/kg ip treatment for comparison.

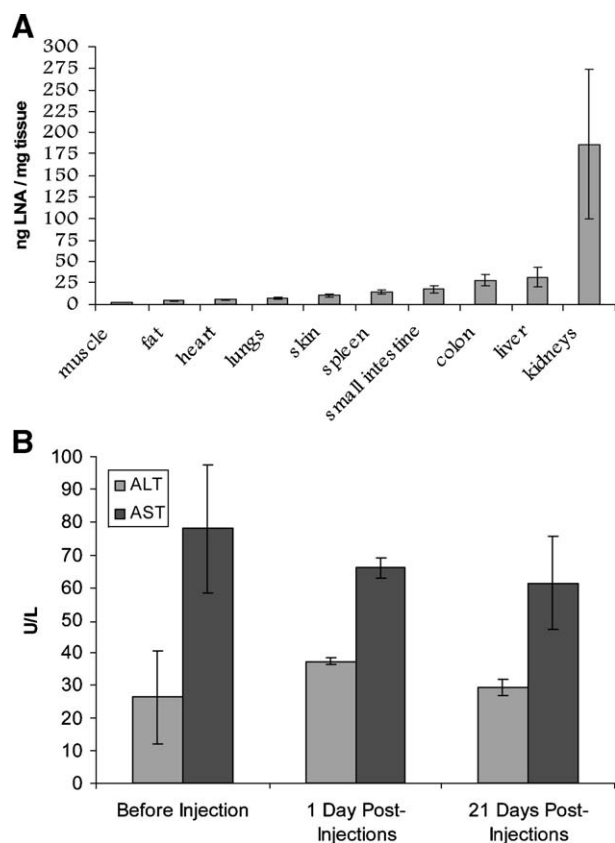


FIG. 5. Tissue distribution and toxicity of LNA oligomers. (A) Approximately 6 mg/kg tritium-labeled LNA ON-654 oligonucleotide was injected as for previous figures. The next day, animals were sacrificed and tritium levels in the indicated tissues were quantified per milligram of tissue. (B) Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were analyzed before and after mice were injected with LNA SSO-654 as for Fig. 1B.

Likewise, LNA SSO-654 shows slightly higher splice switching in the small intestine versus the colon, whereas more of this compound is accumulated in the colon per milligram of tissue. These data highlight the difference between biodistribution and functional distribution and the importance of a robust assay for the latter.

In our functional distribution assay, liver was the primary site of action of the LNA SSOs, suggesting intracellular accumulation was highest in that organ. However, we saw no signs of liver toxicity either 1 or 21 days after a 4-day, 25 mg/kg/day treatment with LNA SSO-654 as measured by serum levels of alanine and aspartate aminotransferase (Fig. 5B). These data suggest that LNA oligomers are both safe and effective *in vivo*.

DISCUSSION

We used the EGFP-654 mouse to study the potential of LNA oligonucleotides as SSOs. We found that systemically delivered LNAs with a phosphorothioate backbone show splice switching at doses as low as 1.5 mg/kg and

are thus significantly more potent per milligram than 2'OMe phosphorothioate oligonucleotides. In the context of these and other nucleic acid drugs, the potency of LNAs at doses less than 10 mg/kg is remarkable. For example, in recent studies, doses of 50–240 mg/kg siRNAs were required to demonstrate their efficacy [16,17]. There may be several reasons for the advantage of LNA oligomers. LNAs have superior serum stability compared to 2'OMe oligonucleotides [18], allowing more intact molecules to reach target tissues. LNA phosphorothioates may also have slower plasma and renal clearance, allowing greater blood concentrations to be achieved and therefore increasing the effective dose of the oligonucleotide. Slower plasma clearance also allows more time for target tissues to take up the drug. Finally, LNA oligonucleotides have a significantly higher melting temperature for target sequences [18,19] and therefore greater affinity for their target site of action. Note that to avoid the possibility of nonspecific binding, the LNA SSO-654 was synthesized as an alternating LNA/DNA 16-mer, while 2'OMe SSO-654 was a fully modified 18-mer.

We found that the antisense effect of LNA SSO-654 persisted for at least 3 weeks after the last injection, while 2'OMe oligonucleotides lost potency in less than 2 weeks. Since EGFP mRNA has a 24-h half-life, these data indicate that the oligonucleotides remained intact inside cells and were able to hybridize with target pre-mRNA for extended periods of time. Note also that the SSOs were targeted to introns, which during splicing are removed from mRNA and are rapidly degraded. Thus, it seems likely that LNAs, with their superior stability [20], are released from their duplexes and retargeted to newly synthesized pre-mRNA. This pseudo-catalytic effect may contribute to their effectiveness and persistence and shows one of the advantages of targeting splicing modulation. In addition, prolonged residence in the serum may allow LNAs to enter cells continuously at a controlled rate.

High potency, persistence, and tissue specificity make LNAs attractive candidates for drugs that act via modulation of aberrant and/or alternative splicing. Their low doses should result in low toxicity/side effects and their persistence in infrequent administration, a property desirable for an injectable. Further work may improve the oral bioavailability suggested in this work and could result in an oral LNA drug. The relatively restricted function of LNA in the liver, small intestine, and colon is advantageous as it provides additional specificity and reduces the possibility of deleterious splice switching in nontargeted tissues. Since the liver, small intestine, and colon are involved, for example, in diabetes, obesity, Crohn disease, irritable bowel syndrome, and some cancers, appropriately targeted LNA oligonucleotides may prove very useful as specific therapeutics for these serious disorders.

MATERIALS AND METHODS

Oligonucleotides. All 2'OMe oligonucleotides were purchased commercially (Trilink Biotechnologies, San Diego, CA, USA) as uniformly modified phosphorothioate 18-mers. The two LNA SSO-654 and LNA SSO-654M oligonucleotides were synthesized at 160 and 50 μmol scale, respectively, using the Äkta 100 platform and 2.5 eq of monomers of both DNA and LNA. Activation was achieved with dicyclohexylcarbodiimide and thioation with zanthane hydride as thiooxidizer. The oligonucleotides were purified on an El-column Source 30Q and desalted by ultrafiltration on a Millipore Pellicon 2 filter (MW 1000). The yields were 57 and 55%, respectively, with purity of >96%. The sequences of each oligonucleotide were as follows: LNA-654, 5'-GcTaTtAcCtTaAcCc-3'; LNA-654M, with 6 mismatches, 5'-GcAaAtTcCtAtTcCc-3'; where capital letters represent LNA monomers and lowercase letters represent deoxyribose monomers. Both molecules have phosphorothioate internucleotide linkages throughout.

Animal treatment. The EGFP-654 transgenic mice were injected ip once daily with 200 μl of the oligonucleotide saline solution at the concentrations indicated. Injections were performed at the same time each day, for the indicated number of days. Mice were sacrificed by CO₂ inhalation 1 day after the last injection and tissues harvested and analyzed as indicated. Tissues for RNA isolation were frozen on dry ice and treated as below. Tissues for EGFP imaging were fixed in 4% paraformaldehyde and processed as previously described [13].

RNA isolation and analysis. Total RNA from ~1–5 mg tissue was isolated using TriReagent (Molecular Research Center, Cincinnati, OH, USA) as per the manufacturer's directions and EGFP mRNA was amplified by RT-PCR using *rTth* polymerase and EGFP forward (5'-CGTAAACGGCCACAAGTTCAGCG-3') and reverse (5'-GTGGTGCAGATGAACCTCAGGGTC-3') primers. Cycles of PCR proceeded at 95°C for 60 s, 65°C for 60 s for 18 cycles total and contained ³²P-labeled dATP (GE Healthcare) for visualization (~2 μCi per 50- μl PCR). The PCR products were separated on a 10% nondenaturing polyacrylamide gel, and bands were visualized on photographic film (Kodak, Inc., Rochester, NY, USA).

Biodistribution analysis. Approximately 4 mg LNA was tritium-labeled following the procedure previously described [21]. Tritiated LNA (8.04 \times 10⁴ dpm/ μg) was brought to a final concentration of 0.95 mg/ml, and each EGFP-654 mouse (25 g) was injected ip once a day with 0.2 ml for 4 consecutive days. The next day, organs were collected and weighed. Tissues were homogenized in 2–3 ml cold homogenizing buffer (100 mM Tris-HCl, 10 mM EDTA, 1 mM β -mercaptoethanol, pH 7.5) supplemented with 1 ml of Solvable tissue solubilizer (Perkin-Elmer, Norwalk, CT, USA) per the manufacturer's instructions. Approximately 10 ml of scintillation cocktail fluid was added to each sample and tissue radioactivity was measured using a Packard Tri-Carb 2900TR liquid scintillation analyzer.

Liver toxicity analysis. Three mice were injected ip once daily for 4 days with 25 mg/kg LNA SSO-654. Tail bleeds were taken from mice before the injections and 1 day after the last injection. At day 21, mice were sacrificed and blood samples taken by cardiac puncture. All blood samples were treated with 8 μl anti-coagulate citrate-dextrose per 50 μl and centrifuged for 3 min at 2000 rpm, and the serum supernatant was collected. Samples were analyzed for alanine aminotransferase and

aspartate aminotransferase enzymes by the Animal Clinical Chemistry Laboratory (UNC, Chapel Hill, NC, USA) using a Vitros 250 instrument (Ortho-Clinical Diagnostics).

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