Vivo-Morpholinos: A non-peptide transporter delivers Morpholinos into a wide array of mouse tissues

Paul A. Morcos, Yongfu Li, and Shan Jiang
Gene Tools, LLC, Philomath, OR, USA

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We have developed a new transporter structure that provides effective delivery of Morpholino antisense oligomers into a wide variety of tissues in living mice. This transporter comprises a dendritic structure assembled around a triazine core which serves to position eight guanidinium head groups in a conformation effective to penetrate cell membranes. This transporter structure is conjugated to a Morpholino oligomer to form a delivery-enabled product referred to as a Vivo-Morpholino. Vivo-Morpholinos are shown to effectively enter and function within cultured cells in the presence of 100% serum using a rigorous positive test system based on correction of a defined splicing error in a pre-messenger RNA. In addition, Vivo-Morpholinos are demonstrated to enter into a wide variety of tissues in a similar positive test system in transgenic mice, as evidenced by correction of the targeted splicing error in all tissues assessed, including near-complete splice correction in the small intestine, colon, stomach, liver, kidney, and a number of muscles. Finally, Vivo-Morpholinos, which target the exon-skipping of exon 23 harboring a premature termination codon in the mdx *mouse model, effectively restore the reading frame of dystrophin and restore expression of a functional dystrophin protein.*

INTRODUCTION

Breaking the delivery barrier

Morpholino oligomers are a proven antisense reagent used to block translation or interfere with RNA processing, including splicing and miRNA maturation, with hundreds of successful applications primarily involving microinjection into embryos (zebrafish and frog). However, the introduction of unmodified Morpholino oligomers into in vivo systems has only been successful with leaky tissues such as damaged muscle. Systemic delivery of unmodified Morpholino oligomers into normal animals has not been successful, and delivery into tissue culture test systems requires the use of additional delivery reagents and low-serum conditions (1). We set out to develop a Morpholino oligomer transporter that could be coupled directly to Morpholino oligomers and facilitate system-wide delivery when introduced into the vascular system of animals.

Transporter design

The most developed in vivo delivery strategy employs peptides based on tat, penetratin, and, subsequently, polyarginine peptides to achieve delivery of oligomers. However, each of these has one or more serious drawbacks including poor efficacy, expensive synthetic routes, and cumbersome or inefficient coupling strategies.

The early touted natural transporter peptides tat and penetratin have been found inefficient at delivering oligomers (2,3). The inefficiency of tat delivery across the cell membrane was thought to be due to a requirement for folding and subsequent renaturation of the protein during membrane translocation (4,5). Penetratin coupled to steric-block peptide nucleic acid (PNA) oligomers (which, like Morpholinos, are uncharged and capable of interfering with RNA processing) has been shown to require 5–10 μM concentrations to achieve delivery into the cytosol but fails to deliver significantly to the nucleus (6,7). The inefficiency of tat and penetratin delivery to the nucleus is thought to be due to a majority of the materials entering cells via endocytosis and a subsequent inability to permeabilize the endocytic vesicles, as well as an inability to circumvent the electrostatic interactions with cellular heparin sulfates (8). In order to interfere with most RNA processing, and pre-messenger RNA (pre-mRNA) splicing in particular, an oligomer must be delivered to the nucleus.

A more promising delivery moiety evolved from these natural peptides, as the active components were defined as 6–9 arginine residues in a bio-available 6-aminohexanoic-spaced structure (9), with an optimal length defined as eight arginines (10). These arginine-based peptides with the 6-aminohexanoic–spaced oligoarginine (R-Ahx-R)×4 have successfully delivered Morpholinos to the nucleus and cytosol in vitro (11) and in vivo (12), including sustained induction of dystrophin expression in *mdx* mice (13). They have also been shown to actively or prophylactically knock down viral titers in various tissues of mice infected with corona virus (14), picornavirus (15), respiratory syncytial virus (16), and influenza A (17). However, it should be noted that arginine-based peptides are not generally available to the research community and that their greatest successes have been in delivering Morpholinos to the cytosol of what would be considered easily deliverable tissues like liver (18) or leaky muscle (19). The question of how well they deliver into a wide spectrum of tissues remains unanswered.

The active components of arginine peptides have been defined as the guanidinium head groups of the arginine residues; it has been further characterized that placing eight guanidinium head groups on a synthetic scaffold and coupling this scaffold to fluorescein leads to enhanced delivery as compared to arginine-based peptides (20,21). The guanidinium head groups have been predicted to interact with phosphates of phospholipids both by electrostatic attraction and multiple hydrogen bonds (22) and, as such, each guanidinium head group is capable of a charge-charge interaction plus hydrogen bonding.

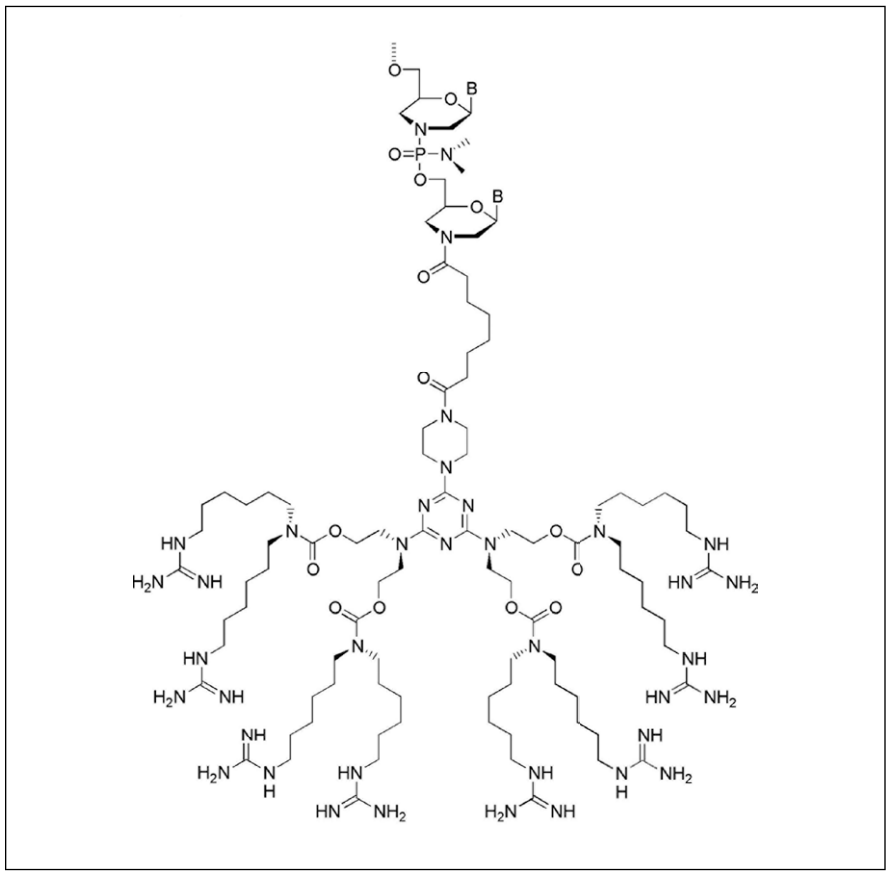

Figure 1. Structure of a Vivo-Morpholino. Shown are two 3′ end bases from a Morpholino oligomer (top portion) linked to the molecular transporter with eight guanidinium head groups (bottom portion).

We sought to exploit a nonlinear, non-peptidic and non-natural architecture for a transporter that provides cost-effective synthesis, convenient conjugation, and exhibits superior performance in transporting Morpholino oligomers across biological barriers. The design of our transporter is based on molecular transporters reported in the literature and the following factors. (*i*) Since guanidinium head groups of arginine-rich peptides are principally responsible for uptake into cells, we developed a synthesis scheme that makes use of a tri-functional triazine as a core scaffold to assemble and present guanidinium head groups in a nonlinear and economically feasible manner. (*ii*) Reports in the literature indicate that 7–15 guanidine head groups is optimal for efficient uptake (22) with 8 guanidine head groups exhibiting the most efficient internalization (10). We chose to install a total of eight guanidine head groups on two of the side chains of a triazine core, leaving the third for efficient on-column conjugation to the Morpholino oligomer with uncomplicated post-synthesis processing (Figure 1 and Reference 23). We call these novel, delivery-enabled Morpholinos Vivo-Morpholinos.

In this paper, we utilize three powerful test systems to assess delivery of Vivo-Morpholinos. Using a cell culture test system which involves splice correction and a subsequent up-regulation of luciferase expression (24), we demonstrate that Vivo-Morpholinos are able to overcome the inhibitory effects of serum in vitro, while cytosolic and nuclear delivery of Morpholinos with Endo-Porter, a proven cell culture delivery reagent, is abolished in full serum media. We further confirm the in vivo delivery efficiency in an equivalent transgenic mouse test system which involves splice correction and up-regulation of green fluorescent protein (GFP) expression in all mouse tissues into which splice-modifying oligomers are delivered to nuclei (25). Finally, we demonstrate that Vivo-Morpholinos can restore translation of the dystrophin protein by targeted deletion of exon 23 harboring a premature termination codon in the *mdx* mouse model. The data indicate that our molecular transporter delivers coupled Morpholino oligomers to the nucleus in a wide spectrum of mouse tissues.

MATERIALS AND METHODS

Making a Vivo-Morpholino

A key element in the synthesis of Vivo-Morpholinos is to use a triazine core to assemble a dendritic molecular transporter. Two sites of the tri-functional triazine are used for branching side arms, whereas its third functional site is used for linking covalently with a Morpholino oligomer. The dendritic moieties are conjugated with the Morpholino oligomer while the oligomer remains on the synthesis resin. After removal of all the protecting groups and detaching the oligomer from the synthesis resin, a subsequent perguanidinylation process results in a complete Vivo-Morpholino, a conjugate of Morpholino with a transporter moiety of a triazine core scaffold presenting eight guanidine head groups (Figure 1 and Reference 23). Morpholino oligomers were synthesized by Gene Tools, LLC (Philomath, OR, USA).

Functional quantitative assessment of delivery of a conjugate containing transporter and Morpholino composition in cultured cells

HeLa cells stably transfected with pLUC/705 (24), a plasmid comprising the firefly luciferase gene interrupted with human β-globin intron 2 containing an aberrant splice site mutation (IVS2–705) and referred to as the positive test system, were from Ryszard Kole (AVI BioPharma, Corvallis, OR, USA). The ON705 Morpholino oligomer targeting the β-globin test system was derived from pLUC/705 sequence and is as

follows: 5′-CCTCTTACCTCAGTTAC AATTTATA-3′. The single-stranded DNA primers used for RT-PCR analysis of the β-globin test system were purchased from Operon (Huntsville, AL, USA) and had the following sequences: 5′-CCATCACGGTTTTGGAATG-3′ and 5′-CCCTCGGGTGTAATCAGAA T-3′.

Cell culture and PCR reagents

Tissue culture cells were grown in D-MEM/F12 (Catalog no. 11330–032; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Catalog no. 16000–044; Invitrogen). Cells were trypsinized with 0.25% trypsin-EDTA (Catalog no. T-4049; Sigma, St. Louis, MO, USA). Dulbecco's Phosphate Buffered Saline (PBS) was purchased from Invitrogen (Catalog no. 14040–133). The firefly luciferase assay system (Catalog no. E1500) and passive cell lysis buffer (Catalog no. E1941) were purchased from Promega (Madison, WI, USA). The protein assay reagent was purchased from Bio-Rad (Catalog no. 500–0006; Hercules, CA, USA). The Access RT-PCR kits were purchased from Promega (Catalog no. A1250). Perfect RNA, eukaryotic mini prep kits were purchased from Eppendorf AG (Catalog no. 0032 006.108; Hamburg, Germany).

Oligomer delivery in cultured cells

Vivo-Morpholinos were added directly to 80% confluent HeLa cells to a final concentration of 2, 5, 10, or 50 µM. As a positive control, bare Morpholino oligomers at a final concentration of 5 µM were delivered using 6 µM Endo-Porter delivery reagent (Gene Tools, LLC), following the manufacturer's instructions (26).

RT-PCR analysis and luciferase assays

RT-PCR analysis was carried out as previously described (27). Firefly luciferase assays were performed as previously described (1).

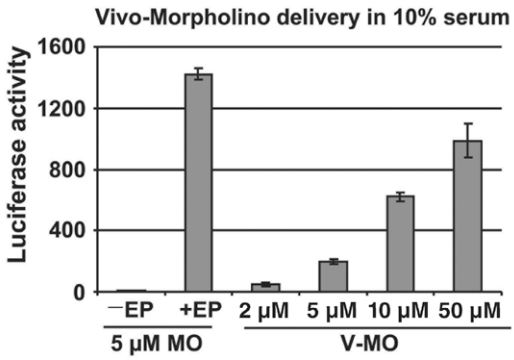

Figure 2. Assessment of Vivo-Morpholino delivery in cultured HeLa cells grown in 10% serum. The Vivo-Morpholino targeting the ON705 mutation (V-MO) was delivered at 2, 5, 10, or 50 µM final concentration and compared with 5 µM bare oligo (MO) against the same mutation delivered without (-EP) or with 6 µM Endo-Porter (+EP). Cells were harvested and luciferase activities were measured 24 h after delivery. The luciferase activities presented are an average of two samples for each condition, with error bars shown.

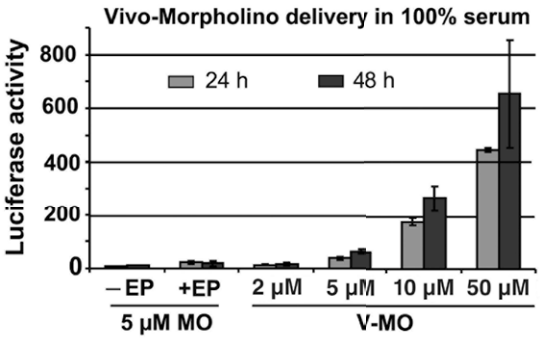

Figure 3. Assessment of Vivo-Morpholino delivery in cultured HeLa cells grown in 100% serum. The Vivo-Morpholino targeting the ON705 mutation (V-MO) was delivered at 2, 5, 10, or 50 µM final concentration and compared with 5 µM bare oligo (MO) against the same mutation delivered without (-EP) or with 6 µM Endo-Porter (+EP). Cells were harvested after incubation for 24 or 48 h. The luciferase activities presented are an average of two samples for each condition, with error bars shown.

Assessment of Vivo-Morpholino in vivo

Intravenous (i.v.) injections of a Vivo-Morpholino were carried out in Ryszard Kole's laboratory by Peter Sazani utilizing transgenic mice that ubiquitously express a modified enhanced green fluorescent protein (EGFP) pre-mRNA containing an aberrantly spliced β-globin intron (IVS2–654). The Vivo-Morpholino oligomer called 654–25 with the sequence 5′-TTGCTATTACCTTAA-CCCAGAAATT-3′ was dissolved in physiological saline and delivered to the transgenic mice by i.v. injection once daily at 12.5 mg/Kg for 4 consecutive days, sacrificed on day 5 and analyzed as described previously (28).

All animals were handled in accordance with the Institutional Animal Care and Use Committee (IACUC)–approved guidelines at Gene Tools, LLC. Nine-week-old *mdx* mice (C57BL/10ScSn-*Dmd*^{mdx}/J) and control normal mice (C57BL/10SnJ) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). The Vivo-Morpholino oligomer V-Mor23 with the sequence 5′-GGCCAAACCTCGGCTTAC-CTGAAAT-3′ was dissolved in physiological saline and delivered to *mdx* mice by i.v. injection once daily for 4 consecutive days at 0 mg/Kg (PBS), 12.5 mg/Kg, or 25 mg/Kg. The mice were sacrificed by isoflurane inhalation

12 days after the final injection. The muscle tissues were removed for immediate RNA isolation or snap-frozen in isopentane and stored at –80°C for immunofluorescence visualization. For immunfluorescence studies, tissues were mounted in Tissue-Tek optimal cutting temperature embedding medium (Sakura, Torrance, CA, USA).

RNA isolation and RT-PCR

Total RNA was isolated from ~10 mg tissue with the *MELT* Total Nucleic Acid Isolation System (Ambion, Inc., Austin, TX, USA), following the manufacturer's protocol. Total RNA (1 µg) was subjected to RT-PCR using the SuperScript One-Step RT-PCR Systems from Invitrogen and primers spanned dystrophin exon 21 (forward primer: 5′-TTCTGGATGCA GACTTTGTGGCCT-3′) and exon 24 (reverse primer: 5′-AGGGCAGGCCA TTCCTCTTTCA-3′). The predicted size of the amplified cDNA fragment containing dystrophin exon 23 is 517 bp, whereas a product generated from cDNA with exon 23 deletion would be 304 bp. The RT-PCR conditions were: 55°C for 30 min; 94°C for 2 min; 40 cycles of 94°C for 15 s, 57°C for 30 s, 68°C for 1 min; and ending with 68°C for 5 min. Sixty percent of the RT-PCR reactions were analyzed on a 2% agarose gel and visualized by ethidium bromide staining.

Dystrophin immunofluorescence

The 10-µm muscle cryostat sections were examined for dystrophin protein by staining with a monoclonal antibody NCL-DYS2 against C terminal dystrophin from Novocastra Laboratories (Newcastle, UK) using the Vector M.O.M. Immunodetection Kit from Vector Laboratories, Inc., (Burlingame, CA, USA), following the manufacturer's protocol. The sections were incubated with NCL-DYS2 antibody (1:20 dilution) for 2 h at room temperature, followed by incubation with the biotinylated anti-mouse IgG for 1 h at room temperature. Mounted sections were visualized and photographed using a Leica DMIL microscope (Wetzlar, Germany) and a Canon 20D camera (Tokyo, Japan) at 1280× magnification.

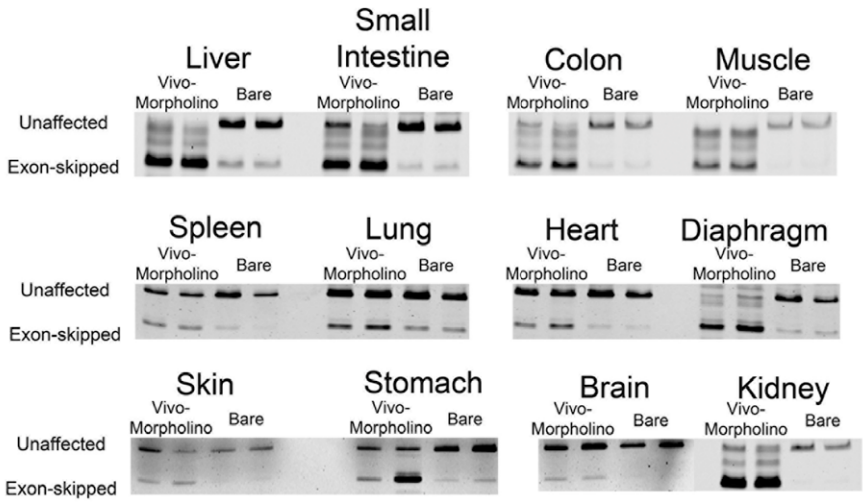

Figure 4. RT-PCR analysis of splice correction by Vivo-Morpholino in comparison with bare Morpholino. Transgenic mice which bear a human β-globin intron (with a splicing mutation) fused with the green fluorescent protein (GFP) gene were intravascularly injected with either the bare or Vivo-Morpholino form of the morpholino which corrects the splicing defect at 12.5 mg/Kg daily for 4 days followed by sacrifice and RT-PCR one day after the final injection. The upper band results from an unaltered transcript whereas the lower band is the RT-PCR product of the shorter corrected transcript. $n = 2$ per group.

RESULTS AND DISCUSSION

Cellular uptake in cultured cells

Cellular uptake of Vivo-Morpholinos was investigated by a functional and quantitative assessment in a well-characterized test system that incorporates a human β-globin intron fused with firefly luciferase stably integrated in HeLa cells (24). Blocking a dominant splice mutation within the globin intron with a Morpholino oligomer reverts normal splicing and brings luciferase in-frame, which results in up-regulation of luciferase activity. In this test system, splice modification can be characterized by luciferase activity or RT-PCR analysis and provides all the components necessary to assess cytosolic and, ultimately, nuclear delivery in one test system. Delivery is assessed by a quantifiable signal proportional to the amount of Morpholino oligomer delivered into the nucleus and a concomitant restoration of luciferase expression in the cytosol (29).

HeLa cells harboring the integrated test system and cultured in the presence of 10% or 100% serum were incubated with the Vivo-Morpholino targeting the splice mutation at 2, 5, 10, or 50 µM final concentration, or the bare Morpholino targeting the same splice mutation at 5 µM delivered with the proven and uncoupled cell culture delivery reagent Endo-Porter at 6 µM. For assessment of delivery in 10% serum, cells were lysed after 24 h and assayed for both luciferase activity and total cellular protein. The results of delivery in 10% serum are shown in Figure 2. Increasing concentrations of Vivo-Morpholino showed significant delivery compared with a 5 µM bare Morpholino control with greater delivery as concentration increased. However, a direct comparison between Vivo-Morpholino and Endo-Porter delivery at 5 µM Morpholino showed that in 10% serum, Endo-Porter achieves more than seven times the delivery at the same oligomer concentrations. At 100% serum concentrations, which better mimics conditions in vivo, Vivo-Morpholinos achieved nearly two times the delivery of Endo-Porter at 24 h and more than four times the delivery with Endo-Porter at 48 h (Figure 3). Interestingly, there

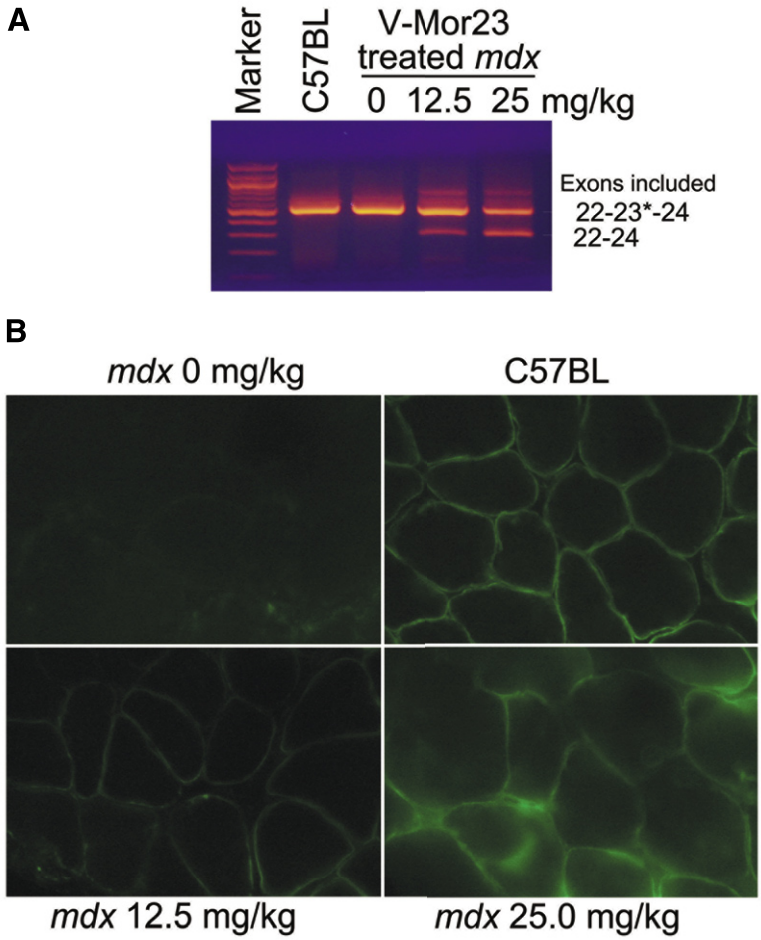

Figure 5. Restoration of dystrophin protein expression in *mdx* mice via Vivo-Morpholino-mediated dystrophin exon 23 skipping. *mdx* mice were injected with 0 (PBS), 12.5, or 25 mg/Kg daily of V-Mor23 for 4 consecutive days. Soleus muscles were examined for dystrophin expression 12 d after the final injections. (A) The effect of exon 23 skipping mediated by V-Mor23 was analyzed by RT-PCR. Full-length dystrophin mRNA harboring the exon 23 point mutation (22-23*-24) was detected in all muscles. In contrast, dystrophin mRNA with the exon 23 deletion (22-24) was detected only in *mdx* mice injected with V-Mor23. (B) The dystrophin protein level was detected by immunohistochemistry using a DYS2 dystrophin antibody. Muscles from normal mice C57BL/10SnJ (C57BL) were used as a positive control. $n = 2$ per group. Representative data are shown.

is a marked increase in delivery for Vivo-Morpholinos at 10 µM, with delivery >20 times background and overall activity doubling at 48 h. These results suggest that Vivo-Morpholinos can enter cells in a system mimicking in vivo conditions, whereas the free delivery peptide, Endo-Porter, is unable to deliver bare Morpholino in the same conditions.

Systemic delivery of Vivo-Morpholino in transgenic mice

To test the delivery efficiency of Vivo-Morpholino in animal models, we used a strain of transgenic mice developed by Ryszard Kole and co-workers which bear a human β-globin intron fused with the green fluorescent protein (GFP) gene (25). The GFP pre-mRNA is ubiquitously expressed and contains a globin intron mutation that causes a splicing error which prevents expression of the GFP protein. Association of a Morpholino antisense oligomer targeting the mutant splice site corrects the splicing error and results in the expression of green fluorescent protein. Thus, visualizing green fluorescence in a specific tissue or assessment of splice correction by RT-PCR can be used to assess in vivo cytosolic and nuclear delivery into cells of particular isolated tissues.

The ability of Vivo-Morpholinos to achieve cytosolic and nuclear delivery in vivo was assessed by administering a Vivo-Morpholino targeting the splice mutation intravenously at 12.5 mg/Kg for 4 consecutive days into two of the transgenic mice harboring the splice mutation followed by sacrifice on day 5. As a control, two mice were treated with bare Morpholino against the same target with the same injection regimen. Delivery was assessed by RT-PCR analysis of RNA transcripts isolated from various tissues (Figure 4). Near-complete splice correction was observed in tissues of the liver, small intestine, colon, skeletal muscle, and diaphragm. Delivery into the spleen and stomach was also high, although not sufficient for complete splice correction at the concentration tested. Modest delivery was achieved in lung, heart, skin, and brain tissues. The levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in the liver were also measured to assess toxicity, and the lack of significant change in either compared with untreated mice indicates that the Vivo-Morpholinos are not toxic to the liver in mice at 12.5 mg/Kg over 4 days (data not shown).

We have shown in mice that Vivo-Morpholinos are capable of altering mRNA splicing at a quantifiable level in all tissues tested by intravascular injections. Furthermore, at 12.5 mg/Kg, the final concentration of Vivo-Morpholino is <2 µM (as calculated by whole-animal volume) and at this concentration, near-complete splice correction is observed in the small intestine, colon, stomach, liver ,and all muscle types tested other than the heart. Together, these data suggest that Vivo-Morpholinos are both achieving a high level of access to the cytosol and nucleus of cells within tissues and are also able to avoid the inhibitory effects of serum and other system-wide cellular components. No other delivery-enabled oligomer has been shown to achieve this level of system-wide delivery at these concentrations. Vivo-Morpholinos exhibited little activity in the brain and skin tissues

which we attribute to poor direct access from the vascular system.

Vivo-Morpholino can induce dystrophin exon 23 skipping and restore dystrophin protein expression in dystrophic *mdx* mice

We have shown that Vivo-Morpholinos achieved systemic delivery in GFP transgenic mice and significantly altered mRNA splicing in most tissues tested. To test if Vivo-Morpholinos can alter gene expression at the protein level similar to the changes observed at the RNA level, we examined the efficiency of exon skipping mediated by a Vivo-Morpholino oligomer, V-Mor23, targeting dystrophin exon 23 in *mdx* mice. The *mdx* mouse bears a premature termination codon in dystrophin exon 23 which prevents translation of the dystrophin protein. Antisense oligonucleotide-mediated skipping of exon 23 during dystrophin mRNA splicing can restore its reading frame and produce a truncated dystrophin protein containing the carboxyl terminal domain (30). V-Mor23 was delivered to the *mdx* mice by daily i.v. injection over 4 consecutive days and the effect of V-Mor23 on dystrophin expression in skeletal muscle was assayed 12 days after the final injection. An mRNA transcript with an expected sized for deletion of exon 23 was detected by RT-PCR and this effect of V-Mor23 on splice alteration was dosage dependent (Figure 5A). Immunofluorescent staining with an antibody targeting the carboxyl terminal domain of dystrophin confirmed that dystrophin protein expression was restored when exon 23 was deleted (Figure 5B). Significantly, *mdx* mice injected with 25 mg/Kg of V-Mor23 daily restored dystrophin protein levels similar to that of normal mice, and that was with analysis 12 days after the final injection (Figure 5B). This data confirms that Vivo-Morpholinos can modulate gene expression at both the mRNA and protein level and also suggests that the response to Vivo-Morpholino delivery may be long-lived in terms of activity at the protein level.

An interesting finding from this work is that Vivo-Morpholinos achieve greater delivery in vivo than in vitro. Vivo-Morpholinos at a concentration <2 μM (by whole body volume) in vivo achieved near-complete splice correction in several tissues, whereas 10 μM was insufficient for complete correction in an equivalent in vitro test system. In 10 years of development of Morpholino delivery systems including EPEI (31) and Endo-Porter (26), only Vivo-Morpholinos have been capable of delivery in 100% serum in vitro. We speculate that the nonlinear guanidinium dendrimer that makes up the transporter component of Vivo-Morpholinos is capable of avoiding significant interaction with serum proteins and this ability translates well in vivo.

The high level of activity, the delivery into a wide-array of tissues, and the availability of these molecules to the research community bodes well for many research projects and ultimately shows great promise for antisense therapy using Vivo-Morpholinos.

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COMPETING INTEREST STATEMENT

The authors are employed by Gene Tools, LLC. Gene Tools, LLC is in the business of manufacturing the product described in this manuscript. All reagents, salaries, and space are provided by Gene Tools, LLC.

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Address correspondence to Paul A. Morcos, Gene Tools, LLC, 1 Summerton Way, Philomath, OR, 97370, USA. email: pmorcos@gene-tools.com