Saponins as Natural Adjuvant for Antisense Morpholino Oligonucleotides Delivery In Vitro and in *mdx* Mice

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Antisense oligonucleotide (AON) therapy for Duchenne muscular dystrophy has drawn great attention in preclinical and clinical trials, but its therapeutic applications are still limited due to inefficient delivery. In this study, we investigated a few saponins for their potential to improve delivery performance of an antisense phosphorodiamidate morpholino oligomer (PMO) both *in vitro* and *in vivo*. The results showed that these saponins, especially digitonin and tomatine, improve the delivery efficiency of PMO comparable to Endo-Porter-mediated PMO delivery *in vitro*. The significant enhancement of PMO targeting to dystrophin exon 23 delivery was further observed in *mdx* mice up to 7-fold with the digitonin as compared to PMO alone. Cytotoxicity of the digitonin and glycyrrhizin was lower than Endo-Porter *in vitro* and not clearly detected *in vivo* under the tested concentrations. These results demonstrate that optimization of saponins in molecular size and composition are key factors to achieve enhanced PMO exon-skipping efficiency. The higher efficiency and lower toxicity endow saponins as gene/AON delivery enhancing agents for treating muscular dystrophy or other diseases.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is an X-linked inherited muscle degenerative disorder caused by nonsense or frameshift mutations in the dystrophin gene affecting approximately 1 in 5,000 male births. Fundamental treatments of DMD requires either correction or replacement of the mutated gene to restore function. The large size of dystrophin protein and the requirement of lifetime administration to muscles throughout the body severely limit the progress in developing effective experimental therapies. Antisense oligonucleotide (AON)-mediated exon-skipping has recently demonstrated promising for the treatment of DMD by intentionally skipping one or multiple exons to restore the normal reading frame of the mutated transcripts, which results in the production of internally truncated but likely functional dystrophin proteins. AONs are short, single-stranded sequences of synthetic and chemically modified RNA or DNA, which are capable to hybridize to specific targets by the Watson–Crick base-pairing rules. They are easier to scale up for guanosine monophosphate (GMP) production than for viral vectors or cell therapies. Of the synthetic oligonucleotide chemistries, phosphorodiamidate morpholino oligomer (PMO) is the most commonly studied AON for exon-skipping in the dystrophin gene and has been recently approved by the US Food and Drug Administration (FDA) as the first drug specific to DMD (a 30-mer PMO marketed as EXONDys 51; [https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm521263.htm](https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm521263.htm)). PMOs have phosphorodiamidate linkages and morpholino rings instead of the deoxyribose rings, being neutral under physiological condition. These modifications confer more nuclease resistance, enhanced binding to mRNA, and preventing RNaseH activity, as well as lower toxicity compared with other counterparts. However, the uncharged nature of PMOs is associated with poor cellular uptake and fast clearance in bloodstream, which dramatically impedes pharmacological outcomes. Studies in animal models have demonstrated that a significant therapeutic effect on DMD can be achieved by the following: (1) increasing doses of PMOs, which could be cost inhibitive and increased risk of toxicity especially for long-term systemic administration in *mdx* mice and (2) chemical modification with cell-penetrating peptides (PPMO) or guanidine-rich dendrimer (Vivo-PMO), which have been reported with significant heightening in targeting dystrophin exons, leading to near normal levels of dystrophin expression in muscles throughout the body in *mdx* mice. However, the packed cationic charges are associated with higher toxicity, with LD50 only about 100 mg/kg and 10 mg/kg for PPMO and Vivo-PMO, respectively, preventing them from clinical applications. Furthermore, the complicated synthesis and purification procedures increase cost. In addition, potential peptide-related immune responses might prevent repeated administration: (3) small-molecule-aided, which have been demonstrated to promote exon-skipping of AONs in *mdx* mice. These include dantrolene-aided PMO delivery studied by Kendall et al. and monosaccharide-formulated AONs reported by Yin’s group; (4) the amphiphilic polymer-mediated delivery strategy has been recently studied by us and demonstrated promising...
in vitro and in vivo in mdx mice. The amphiphilic nature has been verified as key, especially for the delivery of uncharged PMO.27–30 Although some promising results have been demonstrated by the above mentioned, the development of an efficient and safe delivery system is still one of the most challenging hurdles to turn PMO into a significant therapeutic outcome for the treatment of DMD.

In this study, we tested our hypothesis that delivery efficiency of PMO could be improved by conjunction with saponins—a class of natural amphiphile composed of hydrophilic glucose and hydrophobic aglycone, commonly found in a set of plants and which are important nutrition for human and animals. The amphiphilic nature, immunological role, and divergent biological activities have made glycosidic saponins the best adjuvant for drug delivery.31,32 Although various saponin-rich extracts have demonstrated health beneficial effects on blood cholesterol levels, cancer, and bone health (http://www.phytochemicals.info/phytochemicals/saponins.php), there has been no report about the use of them as an oligonucleotide delivery vehicle.34,35 The third is tomatine—a glycoalkaloid found in the stems and leaves of tomato plants, and in the fruits at low concentrations—which has fungicidal, antimicrobial, and insecticidal properties, and the related aglycon derivative tomatidine has been shown to have multiple health benefits.38,39 The results of the above saponins for the delivery of PMO in cell culture and in vivo of the mdx mice were described herein.

RESULTS AND DISCUSSION

Cytotoxicity

Cytotoxicity of the saponins was determined using an MTS (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulphophenyl]-2H-tetrazolium)-based assay in C2C12E50 myoblast cell under different concentrations (from 2 \( \mu \)g/mL to 100 \( \mu \)g/mL) as shown in Figure 2. The glycyrrhizin (G) (molecular weight [MW]: 822.93; HLB: 8.97) showed less toxicity compared with digitonin (D) (MW: 1,229.32; HLB: 12.98) and tomatine (T) (MW: 1,034.19; HLB: 12.29), with about 90% cell remaining alive even at the highest dose of 100 \( \mu \)g/mL. This is likely due to its smaller molecule, even though it’s more hydrophobic compared with the other two counterparts. The highest toxicity was observed with T, even higher than that observed with Endo-Porter at the same concentration, probably resulting from the aglycon-alkaloid, different from the D and G
and more hydrophilic than D. The cell viability was 75.4%, 87.2%, 28.3%, and 37.5% at the dose of 20 μg/mL for D, G, T, and Endo-Porter, respectively. D and G maintained live-cell percentage of 24.7% and 84.6%, respectively, in contrast to 5.5% with Endo-Porter at the dose of 100 μg/mL. The toxicity study demonstrated the saponins, especially G and D, showed much less toxicity against Endo-Porter-current commercial standard vector for PMO delivery in vitro.

Delivery of PMO with Saponins In Vitro
The C2C12E50 myoblasts stably expressing a GFP reporter bifurcated by the insertion of the human dystrophin exon 50 (hDysE50) were used to evaluate the efficacy of saponins for the delivery of PMO. The expression of GFP in the reporter cells relies on the targeted skip of exon 50 by AONs. The PMO sequence PMOE50 (5’-AACTTCCTCTTTAAAACAGAAAAAGCATAC-3’) with previously confirmed efficacy of targeted removal of hDysE50 was used. C2C12E50 GFP reporter cells were treated with a fixed amount (5 μg) of PMOE50 in 500 μL 10% fetal bovine serum (FBS)-DMEM medium formulated with each of the saponins at six escalating doses (1, 2, 5, 10, 20, and 50 μg). Transduction efficiency was examined by fluorescence-activated cell sorting (FACS) analysis (Figure 3). The results showed that D and T at 2 μg improved GFP expression significantly compared with PMOE50 alone, a comparable outcome to Endo-Porter, and the celling doses were 10 μg and 2 μg for D and T, reaching GFP expression of 86.5% and 63.1%, respectively. The levels of GFP expression were dominantly saponin structure dependent: the larger molecule and/or more hydrophobic they are, the more effective as PMO delivery vector, which is in agreement with our previously reported. As the smaller molecule G (MW = 822.93 g/mol) showed much less efficiency (only 36.5% GFP expressed at the dose of 50 μg) compared with D (MW = 1,229.32 g/mol) and T (MW = 1,034.19 g/mol), both are larger and more hydrophilic; even the T gave the modest efficiency due to the toxicity when dosage increased. In contrast, around 5% of the cells were GFP positive when treated with PMOE50 only. The GFP expression produced from D- or T-mediated PMO delivery was about 17, 12-fold higher as compared with PMO alone at its optimum dosage.

D’s high efficiency also likely resulted from its permeabilizing cell membranes. The exonskipping efficiency is obviously dose dependent. Doses higher than those achieving the peak delivery rate inevitably resulted in lower viability and GFP expression. This is clearly indicated with D and T at the doses higher than 10 μg and 2 μg, respectively, showing a sharp decline in cell viability. Cell viability of saponin formulated with PMO was similar to that of saponin only.

Delivery potential of the saponins for PMO was further tested in C2C12E23 myoblasts expressing a mouse bifurcated dystrophin exon 23 (C2C12E23) and induced to differentiate toward myotubes. The C2C12E23 reporter construct uses a muscle creatine kinase (MCK) promoter, thus allowing us to evaluate more cell-type exon-skipping of AONs in differentiating or differentiated myotubes. Cells reaching around 70% confluence were incubated in the differentiation media for 2 days and then treated with saponin-formulated PMOE23. We examined the saponins at five doses (1, 2, 5, 10, and 20 μg formulated with 5 μg PMO in 0.5 mL medium) based on the results from PMOE50 delivery and Endo-Porter (5 μg) as control. Fluorescence images and RT-PCR detection of skipped exon 23 showed a similar trend, with D and T achieving higher GFP expression than PMO alone and comparable to Endo-Porter-mediated PMO delivery as illustrated in Figure 4. The levels of exon 23 skipping were 31.5% (D; 1 μg), 45.1% (D; 2 μg), 53.8% (D; 5 μg), 63.2% (D; 10 μg), 47.2% (D; 20 μg), 41.1% (G; 5 μg), 35.4% (G; 10 μg), 37.3% (G; 20 μg), 33.1% (T; 1 μg), 34.7% (T; 2 μg), 45.1% (T; 5 μg), 39.2% (Endo-Porter; 5 μg) formulated PMOs, and 27.5% (PMO only), respectively.

Delivery of PMO with Saponins In Vivo
Local Delivery
We next evaluated the effect of the saponins for PMO delivery in vivo by intramuscular (i.m.) injection in mdx mice. The mouse contains a nonsense mutation in the exon 23, preventing the production of the functional dystrophin protein. PMOE23-targeting dystrophin exon 23 was injected to tibialis anterior (TA) muscle, and removal of the mutated exon 23 could restore the reading frame of dystrophin transcripts and thus the expression of the dystrophin protein. Based on the delivery performance in vitro, we chose 10 μg saponins as an effective and safe dosage and premixed with 2 μg of PMOE23 in 40 μL saline for injection. The same amount of PMOE23 only was used as control. The treated TA muscles were harvested two weeks later.
Figure 3. Delivery Efficiency and Toxicity of PMOE50/Saponin Complexes in C2C12E50 Cell Line Determined by Fluorescence Microscope and FACS Analysis

(A) Representative fluorescence images of PMO-induced reporter GFP expression with exon 50 skipping in C2C12E50 cell line. The images were taken after three-day treatment (original magnification, 200x; scale bar, 500 μm). (B) Transduction efficiency of PMOs formulated with saponins is shown (Mann-Whitney U test; *p ≤ 0.05 compared with PMO only). (C) Cell viability is shown (Mann-Whitney U test; *p ≤ 0.05 compared with untreated cell as control). (D) Flow cytometry histogram of PMOE50/saponins complexes is shown. In this test, 5 μg PMOE50 was formulated with saponins (1, 2, 5, 10, 20, and 50 μg) and Endo-Porter (2, 5, and 10 μg) formulated as control in 0.5 mL 10% FBS-DMEM medium, respectively. The results are presented as the mean ± SEM in triplicate.
Immunohistochemistry showed that the mice treated with saponin-mediated PMOE23 dramatically increased the numbers of the dystrophin-positive fibers, reaching up to 72%, 53%, and 48% in one cross section of the TA muscle for D-, G-, and T-formulated PMOs, respectively, especially the digitonin, being over 7-fold as compared to control PMO with only 10% positive fibers. The levels of exon-skipping and corresponding dystrophin expression were also quantitatively determined by RT-PCR and western blot. D-, G-, and T-formulated PMO achieved the levels of exon-skipping at 41.3%, 31.2%, and 42.5%, respectively, and dystrophin protein expression at levels of 54.5%, 51.6%, and 56.7% (C57 normalized as 100%), respectively (Figure 5). These results correlated well with the data in muscle cell lines in vitro, supporting the notion that the composition within the vector molecule is crucial to improve the delivery efficiency of therapeutic agents in vivo also. Taken together, these results demonstrated that (1) the amphiphilic nature of delivery carrier is the key for PMO delivery, due to strong hydrophobicity of PMO compared with other chemistry of oligonucleotides. The hydrophobic interaction and potential H-bond between saponin and PMO is likely able to condense and stabilize the saponin/PMO complex, thus improving the delivery efficiency. (2) Positive charge of delivery vector is not a prerequisite for uncharged PMO delivery. This contrasts with the delivery of negatively charged oligonucleotides, which requires strong electrostatic interaction for stability of complex or polyplex in vivo microenvironment. These results further highlight the complexity of the interaction between delivery vector and their delivery cargos.

Systemic Delivery
DMD—a systemic disease—affects body-wide muscles, including cardiac muscle. Systemic treatment is therefore indispensable. Based on the results in vitro and in vivo locally, we evaluated their effects for PMO systemic delivery by intravenous (i.v.) injection at the dose of 0.2 mg formulated with 1 mg PMOE23 (Figure 6). The control PMOE23 alone induced dystrophin expression in less than 3% of muscle fibers in all skeletal muscles and no detectable dystrophin in cardiac muscle two weeks after injection. PMOE23 formulated with both D and G produced dystrophin-positive fibers over 15%–25% in skeletal muscles, with the highest levels (around 25%) with G in intercostal muscle and with D in biceps muscles, respectively. Induction of dystrophin expression was slightly lower with T (about 10%–17%). Importantly, immunohistochemistry demonstrated membrane-localized dystrophin in about 4%–5% and 2%–3% of...
cardiac muscle fibers in some areas of the heart treated with the single dose of D/G and T, respectively. In contrast, only occasional one or two positive fibers were observed in cardiac tissue in the mice treated with PMO alone. The low levels of dystrophin induction in cardiac muscle could still be beneficial to the patients.12,20

No signs of abnormal behavior or change in body weight and overall condition were observed during treatment with any saponin/PMO polyplexes in the mice receiving both local and systemic delivery. No pathologic changes of the liver, kidney, and lung of the treated mice were detected by H&E staining. These results suggest that saponins could be further explored for potential antisense PMO delivery to increase exon-skipping efficiency, especially for the treatment of muscular dystrophies.

Interaction between Saponin and PMO

The affinity between carrier and oligonucleotide is an important parameter for their efficient delivery into cells or tissues. To understand how the saponins improve the delivery performance of oligonucleotide PMO, we first examined the interactions between the carrier and PMO at the different weight by UV-Vis spectrum: we studied the G/PMO complex at the weight ratios from 1:5 to 50:5 (Figure 7A), because G molecule has absorbance at UV to be easily examined against D or T. The G/PMO complexes showed hyperchromic effect compared with PMO only at the ratio of 1:5 or 2:5, whereas the most hypochromic effect happened at the ratio of 5:5 at the 260 nm. In contrast, a little hypochromic shift compared with PMO only was observed at the ratios of 20:5 or 50:5, even at 10:5, which stands for the G-covered PMO completely and shields the absorbance from...
PMO. In addition, we investigated the D/G/T-complexed PMO at same ratio of 5:5 (Figure 7B). The results showed that all three D/PMO, G/PMO, and T/PMO had hypochromic effect against PMO only, with G/PMO giving higher absorbance intensity compared with the other two saponin complexes probably resulted from G’s UV absorbance, whereas the D/T has no absorbance over 220 nm. The results indicated that the D/G/T, although different in structure, have similar affinity with PMO, due to their shared amphiphilic nature and similar composition. Second, we further examined saponin/PMO polyplex at the weight ratio of 5:5 in 0.9% saline solution under transmission electron microscopy (TEM), and G/PMO at the ratio of 1:5 and 20:5 also were assessed. As illustrated in Figure 7C, the PMO oligonucleotides alone formed particles with the size below 50 nm, which is most likely a result of hydrophobic interaction and hydrogen bond among PMO molecules; the molecule D or T only sized below 30 nm in particles, whereas the G only formed uniform particles below 20 nm, probably due to its smaller and relatively rigid structure as well as its being more hydrophobic compared with D or T molecules. When the complex of saponin/PMO at the ratio of 5:5, the D/PMO complex aggregated to larger particles, probably resulting from strong hydrophilic interaction; T/PMO complex produced separated particles below 40 nm, likely due to its more lipophilic compared with D, whereas the G/PMO shaped smaller particles compared with D or T/PMO complexes at the ratio of 5:5 and more condensed particles were observed at the ratio 20:5 due to G’s more hydrophobic nature. However, G/PMO particles at the ratio 1:5 were of similar size as PMO only, suggesting that G could not cover the PMO completely at the low ratio and the particles were dominantly controlled by PMO. Clearly, mechanisms of interaction between PMO and the saponins remain to be further clarified for improving delivery efficiency.

Conclusions
In this study, selected saponins have been evaluated for the first time as delivery vector for antisense PMO in vitro and in dystrophic mdx mice. The results show that saponins, especially D, improve the delivery and achieve efficiency of PMO comparable to Endo-Porter-mediated PMO delivery in vitro. The significant enhancement of PMO delivery is also demonstrated in vivo, up to 7-fold with the D when compared with PMO only. No obvious toxicity was observed in local and systemic delivery at the tested dosage. These data suggest that optimization of saponins in molecular size and component has potential for enhanced delivery of PMO oligonucleotide in vivo.

MATERIALS AND METHODS
Materials
DMEM, penicillin-streptomycin, FBS, L-glutamine, and HEPES (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid) buffer solution
(1 M) were purchased from Gibco, Invitrogen Corp (Carlsbad, CA, USA). 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was ordered from BioVision Technologies (San Francisco, CA, USA). Phosphorodiamidate morpholino oligomer PMOE50 (5′-AACTTCCTCTTTAACAGAA AAGCATAC-3′), PMOE23 (5′-GGCCAAACCTCGTACCTG AAAT-3′), and Endo-Porter were purchased from Gene Tools (Philomath, OR, USA). Saponins and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated. Investigated saponins’ structures were illustrated in Figure 1.

**Cell Viability Assay**

Cytotoxicity was evaluated in C2C12E50 cell line using the MTS (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium)-based assay. Cells were seeded in a 96-well tissue culture plate at $1 \times 10^4$ cell per well in 200 µL 10% FBS-DMEM. Cells achieving 70%–80% confluence were exposed to saponin at different doses for 24 hr followed by addition of 20 µL of Cell Titer 96 Aqueous One Solution Proliferation Kit (Promega, Madison, WI, USA). After further incubation for 4 hr, the absorbance was measured at 570 nm using a Tecan 500 Plate Reader (Tecan, Morrisville, NC, USA) to obtain the metabolic activity of the cell. Untreated cells were taken as controls with 100% viability and wells without cells as blanks; the relative cell viability was calculated by $\left( \frac{A_{\text{treated}} - A_{\text{background}}}{A_{\text{control}} - A_{\text{background}}} \right) \times 100$. All viability assays were carried out in triplicate.

**In Vitro Transfection**

The C2C12E50 myoblast and C2C12E23 differentiated cell lines expressing the reporter GFP were used in this study. The expression
of GFP was controlled by the effective skipping of the inserted human dystrophin exon 50 sequence (hDysE50) and mouse dystrophin exon 23 sequence (mDysE23), respectively.40

C2C12E50
The C2C12E50 cell line was maintained in 10% FBS-DMEM in a humidified 10% CO2 incubator at 37°C. About 5 × 105 cells/well in 500 μL 10% FBS-DMEM medium were seeded and allowed to grow until a confluence of 70%. Cell culture medium was replaced before addition of saponin/PMOE50 (fixed at 5 μg) formulation with varying ratio. Endo-Porter was used as comparison. Transfection efficiencies indicated by GFP production were recorded after three-day incubation with the Olympus IX71 fluorescent microscope (Olympus America, Melville, NY, USA) and digital images taken with the DP Controller and DP Manager software (Olympus America, Melville, NY, USA). Transfection efficiency was also examined quantitatively using flow cytometry. Cells were washed twice with PBS (1×; pH 7.4), treated with 0.2 mL 0.05% trypsin-EDTA, followed by incubation for 3 min at 37°C. The cells were then treated with cooled growth medium (1 mL), collected by centrifugation, and then resuspended in 0.5 mL of ice-cold PBS (1×; pH 7.4). Samples were run on a FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). At least 1 × 105 cells were counted and analyzed with CellQuest Pro (BD, Franklin Lakes, NJ, USA) software package.

C2C12E23
The cell culture and delivery protocol are the same as in C2C12E50, the images taken and cell collected after six-day treatment. Collected cells were initially washed twice with PBS, and RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) per manufacturer’s instructions. RNA was stored at −80°C for later use. RT-PCR was performed with RT-PCR Master Mix (USB, Cleveland, OH, USA) to amplify the sequence of interest. 100 ng of template RNA was used for each 25 μL RT-PCR reaction. The primer sequences for the RT-PCR were eGFP5 (5′-TTGCTGAG-3′) and eGFP3 (5′-TTCTTCAGCTTGTGTCATCC-3′). The cycle conditions for reverse transcription were 43°C for 15 min and 94°C for 2 min. The reaction was then cycled 30 times at 94°C for 30 s, 65°C for 30 s, and 68°C for 1 min. The products were examined by electrophoresis on a 1.5% agarose gel. The intensity of the bands was measured with the NIH ImageJ 1.42 (NIH, Bethesda, MD, USA) and percentage of exon-skipping was calculated with the NIH ImageJ software 1.42 (NIH, Bethesda, MD, USA). The bound primary antibody was detected by horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (IgG) Alexa Fluor 594 (Invitrogen, Carlsbad, CA, USA). As for dystrophin-positive fiber counting, the number of dystrophin-positive fibers in one section was addressed using the Olympus BX51 fluorescent microscope (Olympus America, Melville, NY, USA).

In Vivo Delivery
This study was carried out in strict accordance with the recommendations in Guide for the Care and Use of Laboratory Animals of the NIH. The protocols were approved by the Institutional Animal Care and Use Committee (IACUC), Carolinas Medical Center (breeding protocol: 10-13-07A; experimental protocol: 10-13-08A). Mice were housed with 12-hr light-dark cycles in individually ventilated cages and had access to standard chow and water ad libitum.

Mice of mixed gender were used in all studies. All injection was performed under isoflurane anesthesia, and all efforts were made to minimize suffering.14–16,40

Animals and Injections
Dystrophic mdx mice (C57BL/10 as genetic background) aged 4 or 5 weeks were used for in vivo testing (5 mice per group, mixed male/female [m/f], 3 m + 2 f or 2 m + 3 f in the test and control groups) unless otherwise stated. The PMOE23 targeting the boundary sequences of exon and intron 23 of mouse dystrophin gene (Gene Tools, Philomath, OR, USA) was used. For i.m. injections, 2 μg PMOE23 with or without saponin was formulated in 40 μL saline for each TA muscle. For i.v. injection, 1 mg PMO with or without saponin (0.2 mg) in 100 μL saline was used. The muscles were examined 2 weeks later, snap frozen in liquid-nitrogen-cooled isopentane, and stored at −80°C.

Immunohistochemistry and Histology
Serial sections of 6 μm were cut from the treated mice muscles. The sections were stained with a rabbit polyclonal antibody P7 for the detection of dystrophin protein as described previously.16,17 Polyclonal antibodies were detected by goat anti-rabbit immunoglobulin G (IgG) Alexa Fluor 594 (Invitrogen, Carlsbad, CA, USA). As for dystrophin-positive fiber counting, the number of dystrophin-positive fibers in one section was addressed using the Olympus BX51 fluorescent microscope (Olympus America, Melville, NY, USA).

Western Blot and RT-PCR for In Vivo Samples
Protein extraction and western blot were done as described previously.14,16,17 The collected sections were ground into powder and lysed with 200 μL protein extraction buffer (1% Triton X-100, 50 mM Tris [pH 8.0], 150 mM NaCl, and 0.1% SDS), boiled at 100°C water for 1 min, and then centrifuged at 18,000 × g at 4°C for 15 min. The supernatants were quantified for the protein concentration with a protein assay kit (Bio-Rad, Hercules, CA, USA). Proteins were loaded onto a 4%–15% Tris–HCl gradient gel. Samples were electrophoresed 4 hr at 120 V at room temperature (RT). Then, the gel was blotted onto nitrocellulose membrane for 4 hr at 150 V at 4°C. The membrane was probed with NCL–DYS1 monoclonal antibody against dystrophin rod domain (1:200 dilutions; Vector Laboratories, Burlingame, CA, USA). The bound primary antibody was detected by horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:3,000 dilutions; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and the ECL Western Blotting Analysis System (Perkin-Elmer, Waltham, MA, USA). The density of the bands obtained from the treated mdx mice muscles was measured with ImageJ software 1.42 (NIH, Bethesda, MD, USA) and compared with that of normal muscles of C57BL/6 mice. α-actin was detected by rabbit anti-actin antibody (Sigma, St. Louis, MO, USA) as a sample loading control.

Total RNA was extracted from the muscle after dissection; 100 ng of RNA template was used for a 25 μL RT-PCR with the Fidelitaq RT-MasterMix (USB, Cleveland, OH, USA). The primer sequences for
the RT-PCR were Ex20Fo 5'-AGAATTCTGGAATTGAG-3' and Ex26Ro 5'-CTCTACGCTTTGCTCATCC-3' for amplification of mRNA from exons 20 to 26. Unskipped band, including exon 23, is 1,093 bp, and skipped band without exon 23 is 880 bp.

**UV-Vis Study**

The Thermo Scientific NanoDrop 2000 spectrophotometer was used to determine the absorbance of saponin and PMO in both simple and chemical conjugation. 1.5 μL of each sample was measured at desired concentration under room temperature.

**TEM**

The saponin/PMO polyplex solution containing 1 μg of PMO was prepared at different weight ratios of saponin/PMO in 100 μL 0.9% saline and analyzed using TEM (JEM-1400Plus Transmission Electron Microscope by JEOL USA) with AMT-XR80S-B Wide-Angle Side-Mount 8 M Pixel, CCD Camera. The samples were prepared using negative staining with 1% phosphotungstic acid. Briefly, one drop of sample solution was placed on a formvar and carbon-coated carbon grid (Electron Microscopy Sciences, Hatfield, PA, USA) for 1 hr and blotted dry, followed by staining for 3 min. Samples were analyzed at 60 kV. Digital images were captured with a digital camera system from 4 pi Analysis (Durham, NC, USA).

**Statistical Analysis**

All the results were expressed as mean ± SEM, and the data were analyzed using Mann-Whitney U test and two-tailed Student t test with a value of *p ≤ 0.05 being considered statistically significant.

**AUTHOR CONTRIBUTIONS**

M.W. and Q.L. conceived and designed the experiments; M.W. wrote the paper and performed the whole experiments except for in vivo study; B.W. supervised and performed the in vivo experiments with S.N.S.; P.L. contributed in cell culture; and Q.L. and B.W. reviewed the manuscript.

**CONFLICTS OF INTEREST**

The authors declare no conflicts of interest in relation to this paper.

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