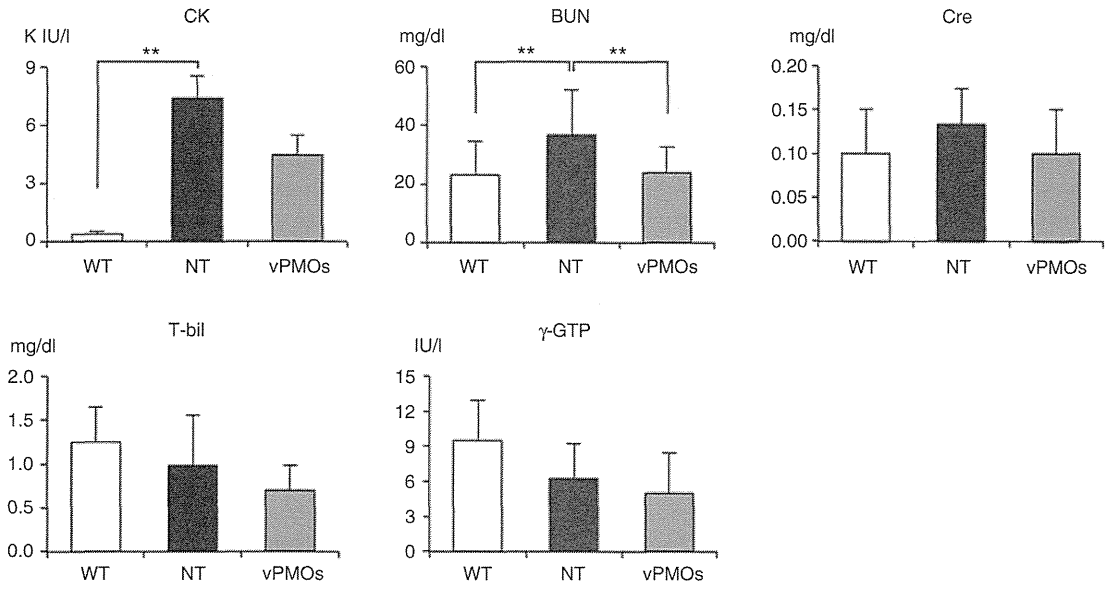

Figure 5 Evaluation of serum creatine kinase level and renal and hepatic toxicity in the blood test at the end-point of 2 weeks after the last of nine systemic injections of 6 mg/kg 10-vPMO cocktail at 2-week intervals. Blood biochemical tests were performed for serum creatine kinase (CK), blood urea nitrogen (BUN), creatinine (Cre), total bilirubin (T-bil), and γ-glutamyl transpeptidase (γ-GTP) in wild-type (WT), nontreated (NT), and treated *mdx52* mice ($n = 4$ in WT, $n = 6$ in NT and $n = 4$ in treated group). Data are presented as mean ± SE.

no overt toxicity due to the chemical components of vPMO was recorded at up to 15–20 mg/kg in mice.[25,44] However, the presence of dose-dependent and delayed side effects by long-term treatment must be determined to use this chemistry as a clinical grade. Neither the current 18-week regimen of 6 mg/kg vPMOs (0.6 mg/kg each vPMO) every 2 weeks, nor the previous study using the vPMO cocktail at a higher dose of 12 mg/kg, lead to any deterioration of the serum parameters that were tested.[26] Notably, we found a significant reduction in BUN, an indicator of progression of protein catabolism, to the normal range observed in WT mice. Mussini *et al.* also have shown that catabolism in DMD muscles is increased.[45] A relatively higher level of BUN is reported in *mdx* mice compared with WT C57BL/6 mice.[46] The reduced BUN level with no significant change in the creatinine level may result from amelioration of muscle degeneration by the treatment. In addition, we confirmed that our vPMO cocktail does not induce a detectable immune response of T cells. Although pathological and immunological side effects for long-term treatment with vPMOs need to be more closely examined in various tissue types of animal models, these results indicate that this regimen may have clinical potential for DMD patients who require repeated administration over the course of their lifetime.

In summary, we demonstrated that long-term repeated intravenous injections of the 10-vPMO cocktail at a lower dose than described previously was both effective and safe in the DMD animal model. However, further investigations of vPMO cocktails will need to be conducted at the preclinical stage, such as dose-escalation and -reduction studies, as well as acute and chronic toxicity assessments, since multiexon skipping has more potential for off-target effects due to the repeated administration of many different AOs. Intermediate products induced by AO cocktails also should be considered a potential side effect, as shown in this study. Multiexon skipping with a number of AOs is likely to generate a variety of dystrophin mRNA/protein types including in-frame and out-of-frame transcripts due to the unequal skipping efficiency of individual AOs.[26] Reducing the number of AOs used in a cocktail—depending on mutation patterns in the patients—or developing a new strategy with fewer AOs to induce skipping of the entire exons 45–55 region, regardless of the mutation patterns, may prevent unexpected effects and lead to formation of intended dystrophin proteins more effectively. Such advancements would aid in forwarding this strategy and other multiple exon skipping strategies into clinical application.[33] In addition, sequence-specific AO cocktails optimized for the human *DMD* gene will need to be tested with human DMD patient skeletal muscle cell lines. Currently, the drug regulatory authorities consider individual AOs targeting different sequences to be separate drugs. This stance may need to be changed for developing cocktail drugs. Nevertheless, the results of this study should contribute not only to the clinical development of an exon 45–55 skipping therapy for DMD, but should also open up the possibility of using antisense drug cocktails for other genetic diseases in which long-term administration is required.

Material and methods

Animals. Eight-week-old male exon 52–deficient *mdx52* mice were used in this study. As a control in the systemic treatment, male WT C57BL/6J mice at 6 months old were used for comparing to treated *mdx52* at the end-point of the treatment. All mice were housed in an individually ventilated cage system with a 12-hour light–dark cycle; they received standard mouse chow (Harlan Teklad, Madison, WI) and water *ad libitum*. All mice were allowed to rest for at least 7 days in the facility before acclimatizing them on the instruments and taking baseline readings for behavioral assays. All mice were handled

according to local Institutional Animal Care and Use Committee (IACUC) guidelines (University of Alberta, Edmonton, Canada and Children's National Medical Center, Worthington, DC).

Antisense oligonucleotides. Ten AOs targeted to exons 45–51 and 53–55 in mouse *dystrophin* gene were designed using ESEfinder software to anneal to the exon-splicing enhancer sites of each exon or exon/intron boundary, as previously described (Table 1).[26,27] Specificity of the designed AOs was confirmed by NCBI blast software (https://www.ncbi.nlm.nih.gov/), which shows that our AO sequences theoretically do not bind any untargeted RNA sequences in 100% identity. All sequences were synthesized using vPMO backbones (Gene-Tools, LLC, Philomath, OR).[21]

Vivo-Morpholino injections. Animals were anesthetized by inhalation of isoflurane (Baxter, Deerfield, IL) for injections. A total of 0.3 µg of vPMOs targeting exons 45–55 in a total volume of 36 µl of saline were injected into the TA muscle of *mdx52* mice. Muscle samples were obtained 2 weeks after the intramuscular injection. For long-term systemic treatment, a total of 6 mg/kg per injection of 10-vPMOs (0.6 mg/kg for each) in 150 µl of saline was injected into the tail vein of *mdx52* mice, nine times at 2-week intervals. The mice were examined 2 weeks after the final injection. Muscle samples were obtained immediately after the mice were killed. The samples were snap frozen in liquid nitrogen–cooled isopentane and stored at –80 °C before use.

RT-PCR. Total RNA from muscle sections of WT, nontreated *mdx52*, and treated *mdx52* mice were extracted as previously described.[47] Total RNA template (200 ng) was used for a 25 µl RT-PCR with the SuperScript III One-Step RT-PCR System (Invitrogen, Carlsbad, CA) and 0.2 µmol/l of each primer, in accordance with the manufacturer's instructions. Primer sequences for the PCR were designed with Primer3Plus software: Exon 44_F, CAGTTGAAAAATGGCGACAC and Exon 56_R, GTAACA GGGGTGCTTCATCC. The cycling conditions were as follows: 55 °C for 30 minutes; 94 °C for 2 minutes; 35 cycles at 94 °C for 15 seconds, 60 °C for 30 seconds, and 68 °C for 1.2 minutes; and 68 °C for 5 minutes. PCR products were separated on a 2% agarose gel and then visualized by SYBR Safe DNA Gel Stain (Invitrogen). Skipping percentage was calculated as $\frac{\text{Exons } 45-55 \text{ skipped transcript}}{\text{Native} + \text{skipped transcripts}} \times 100$ using ImageJ software (NIH). Bands of the expected size for the transcript were extracted with a gel extraction kit (Promega, Madison, WI). The sequencing reactions were performed with Big Dye Terminator v3.1 (Applied Biosystems, Foster City, CA).

Immunohistochemistry. Sections (7 µm thickness) of the TA muscle after single intramuscular injection were incubated with two anti-dystrophin antibodies: mouse monoclonal DYS1 against peptides encoded by exons 26–30 (1:200; Novocastra Laboratories, Newcastle upon Tyne, UK) and rabbit polyclonal P7 against peptides encoded by exon 57 (1:200; Fairway BioTech, Shrewsbury, UK). For quantification of the number of dystrophin-positive fibers, we made several tissue sections and selected representative sections of TA muscles, then stained these sections with DYS1 antibody (n = 3). The number of dystrophin-positive fibers were counted in sections having at least 200 total muscle fibers using a BZ-9000 fluorescence microscope (Keyence, Osaka, Japan), as previously described.[48] In systemic treatment with the 10-vPMO cocktail, the diaphragm, biceps femoris, quadriceps, gastrocnemius, tibialis anterior, biceps brachii, triceps brachii, and heart muscles were examined 2 weeks after the final injection using anti-dystrophin (P7) antibody and antibodies against dystrophin-associated proteins: anti-α1-syntrophin rabbit polyclonal antibody (1:200, Abcam, Cambridge, UK), anti-nNOS rabbit polyclonal antibody (1:100, Invitrogen), anti-α-sarcoglycan mouse monoclonal antibody (1:10, Novocastra Laboratories), and anti-β-dystroglycan mouse monoclonal antibody (1:5, Novocastra Laboratories). To detect the primary antibodies, Alexa Fluor 594–conjugated goat antimouse or rabbit IgG (1:2,000; Invitrogen) were used as secondary antibodies. To examine IgG accumulation in muscle fibers and immune response to the vPMO cocktail, anti-mouse IgG F(ab')2 (1:750, Invitrogen) and anti-CD3 rabbit monoclonal (1:25, Abcam) antibodies were used on nonfixed and 4% PFA-fixed sections (7 µm thickness), respectively. The number of IgG-positive fibers and sporadic CD3-positive cells as pan T cells were counted in 10 section areas randomly selected through a 20× objective lens. In immunostaining against CD3 antigen, cells were regarded as positive when more than half the membrane circumference was stained on cross-sections.

Hematoxylin and eosin staining. For counting CNFs, muscle sections (7 µm thickness) were stained with Mayer's hematoxylin and eosin (H&E) solutions and images were taken with a DMR microscope (Leica Micro-systems, Newcastle upon Tyne, UK) with a 20× objective lens, as previously described.[49] The percentage of CNFs was calculated in 400–1,100 fibers in DIA, BF, QUA, GC, and TA muscles of nontreated (n = 4) and treated *mdx52* mice (n = 6–7).

Western blotting. Protein extraction from frozen muscle sections and Western blot analysis were performed as previously described.[47] In brief, 10 and 1% (4 and 0.4 µg of protein, respectively) of the TA muscle from WT mice were used as a positive control, 40 µg of protein of the TA muscle from nontreated *mdx52* mice were used as a negative control, and 40 µg of protein from the indicated muscles of treated *mdx52* mice were loaded onto a NuPAGE Novex 3–8% Tris-Acetate Midi Gel (Invitrogen) and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis at 150 V for 75 minutes. The proteins were transferred onto an Immobilon PVDF membrane (Millipore, Billerica, MA) by semidry blotting at 20 V of constant voltage for 70 minutes. The membrane was blocked with phosphate-buffered saline containing 0.05% Tween 20, 0.1% casein, and 0.1% gelatin, then incubated with the DYS1 monoclonal antibody (1:400 in blocking solution) at 4 °C overnight. Using ImageJ software, the intensities of the bands were compared with those from WT muscles, as previously described.[48] Myosin heavy chain (MyHC) stained by Coomassie Brilliant Blue was used as a loading control.

Muscle function test. The grip strength test for hind and forelimbs of the mice was performed 2 weeks after the eighth of the every 2 weeks intravenous injections, as previously described.[50]

Biochemical blood test. Serum samples were collected from WT mice, nontreated and treated *mdx52* mice 2 weeks after the final injection of vPMO cocktail. Serum biochemical parameters of creatine kinase, blood urea nitrogen, creatinine, total bilirubin, and γ-GTP were assayed with the Fuji Drychem system (Fuji Film Medical, Tokyo, Japan).

Statistical analysis. For analysis of dystrophin-positive fibers in the locally injected TA muscle and CNFs in the systemic treatment, statistical differences were assessed by *F* test and Student's *t*-test. Mann–Whitney *U*-test was performed to analyze the number of IgG-positive fibers and CD3-positive cells. One-way analysis of variance with a Tukey–Kramer multiple-comparison test was performed for statistical analysis of the serum biochemical test. Data are reported as mean values ± SD or ± SE. The level of significance was set at $P < 0.05$.

Supplementary material

Figure S1. Intramuscular (i.m.) injection of the 10-vPMO cocktail at 0.3 μg (0.03 μg of each vPMO).

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Supplementary Information accompanies this paper on the Molecular Therapy–Nucleic Acids website (http://www.nature.com/mtna)