Citation: Molecular Therapy—Nucleic Acids (2016) 5, e329; doi:10.1038/mtna.2016.46
Official journal of the American Society of Gene & Cell Therapy
www.nature.com/mtna

Fructose Promotes Uptake and Activity of Oligonucleotides With Different Chemistries in a Context-dependent Manner in *mdx* Mice

Limin Cao[1,2], Gang Han[1], Caorui Lin[1], Ben Gu[1], Xianjun Gao[1], Hong M Moulton[3], Yiqi Seow[4] and HaiFang Yin[1]

Antisense oligonucleotide (AO)-mediated exon-skipping therapeutics shows great promise in correcting frame-disrupting mutations in the *DMD* gene for Duchenne muscular dystrophy. However, insufficient systemic delivery limits clinical adoption. Previously, we showed that a glucose/fructose mixture augmented AO delivery to muscle in *mdx* mice. Here, we evaluated if fructose alone could enhance the activities of AOs with different chemistries in *mdx* mice. The results demonstrated that fructose improved the potency of AOs tested with the greatest effect on phosphorodiamidate morpholino oligomer (PMO), resulted in a 4.25-fold increase in the number of dystrophin-positive fibres, compared to PMO in saline in *mdx* mice. Systemic injection of lissamine-labeled PMO with fructose at 25 mg/kg led to increased uptake and elevated dystrophin expression in peripheral muscles, compared to PMO in saline, suggesting that fructose potentiates PMO by enhancing uptake. Repeated intravenous administration of PMO in fructose at 50 mg/kg/week for 3 weeks and 50 mg/kg/month for 5 months restored up to 20% of wild-type dystrophin levels in skeletal muscles with improved functions without detectable toxicity, compared to untreated *mdx* controls. Collectively, we show that fructose can potentiate AOs of different chemistries *in vivo* although the effect diminished over repeated administration.

Molecular Therapy—Nucleic Acids (2016) **5**, e329; doi:10.1038/mtna.2016.46; published online 28 June 2016
Subject Category: Nucleic acid chemistries

Introduction

Duchenne muscular dystrophy (DMD) is a devastating monogenic disease, caused by frame-disrupting mutations in the *DMD* gene,[1,2] which result in the lack of functional dystrophin protein. Currently there is no effective treatment available in the clinic apart from palliative care. Antisense oligonucleotide (AO)-mediated exon-skipping therapeutics is promising for DMD patients based on encouraging preclinical and clinical outcomes.[3–6] Two particular AO compounds targeting at human *DMD* exon 51 (drisapersen and eteplirsen) showed promising results and herald new hope for DMD patients. Despite the promise, low systemic delivery efficiency remains a critical challenge for therapeutic use of AOs, such as phosphorodiamidate morpholino oligomer (PMO), peptide nucleic acid (PNA), 2'O-methyl phosphorothioate RNA (2'OMe), and tricycle-DNA (tcDNA)[3,4,7–9] among other AOs. Therefore, development of safe and compatible delivery technologies is crucial for the clinical adoption of AOs.

Different strategies are under investigation, *e.g.*, the use of polymers and cell-penetrating peptides (CPPs) for enhancing the delivery of AOs.[4,10–12] However, only marginal effect was achieved with polymers for delivering AOs to muscle with limited applicability to negatively charged AOs.[4] Although CPPs can significantly increase the uptake of AOs to muscle and restore the expression of dystrophin in *mdx* mice, its toxicity is of concern.[13,14] Chimeric or tissue-targeting peptides can improve the exon-skipping efficiency of PMO in *mdx* mice, but the issues with safety and cost need to be tackled prior to its clinical use.[15]

Recently, we discovered that carbohydrates can potentiate the uptake of nucleic acids in muscle in *mdx* mice. Notably, a glucose/fructose mixture (GF) and 5% fructose outperformed other analogues in promoting PMO-mediated exon-skipping and dystrophin restoration in *mdx* mice intramuscularly.[16] Here, we further explore the effect of fructose alone on potentiating other AO chemistries and its systemic potential in *mdx* mice. Investigation of four different AO chemistries with four top hexose candidates from previous screening in *mdx* mice indicated that hexose can increase the activities of different AOs. In particular, fructose showed preferable augmenting effect on PMO, suggesting fructose functions in a backbone-dependent manner. Short-term repeated systemic administration of PMO in fructose (PMO-F) elicited a systemic enhancement in dystrophin restoration in *mdx* mice compared to PMO in saline (PMO-S) under identical dosing conditions. Consistently, tissue distribution data revealed higher uptake of lissamine-labeled PMO in peripheral muscles of *mdx* mice treated with PMO-F compared with PMO-S. Long-term repeated intravenous administration of PMO-F at 50 mg/kg/week for 3 weeks followed by 50 mg/kg/month for 5 months induced effective exon-skipping and dystrophin

The first two authors contributed equally to this work and should be recognized as co–first authors.
[1]Department of Cell Biology and Research Center of Basic Medical Science, Tianjin Medical University, Tianjin, China; [2]Tianjin Key Laboratory of Lung Cancer Metastasis and Tumor Microenvironment, Tianjin Medical University General Hospital, Tianjin, China; [3]Biomedical Sciences, College of Veterinary Medicine, Oregon State University, Corvallis, Oregon, USA; [4]Molecular Engineering Laboratory, Biomedical Sciences Institutes, Agency for Science Technology and Research, Singapore, Singapore. Correspondence: HaiFang Yin, Department of Cell Biology and Research Center of Basic Medical Science, Tianjin Medical University, Qixiangtai Road, Heping District, Tianjin, 300070, China. E-mail: haifangyin@tmu.edu.cn
Keywords: antisense oligonucleotide; Duchenne muscular dystrophy; exon skipping; fructose
Received 7 March 2016; accepted 22 May 2016; published online 28 June 2016. doi:10.1038/mtna.2016.46

restoration in multiple peripheral muscles in *mdx* mice with modest functional improvement in the absence of detectable toxicity when compared to untreated *mdx* controls; however no enhancement in PMO activities was observed with PMO-F compared with PMO-S over repeated systemic administration. In summary, our studies demonstrate that fructose promotes the activities of oligonucleotides with different chemistries in a context-dependent manner and can be used as a delivery tool for assessing the efficacy of various AO sequences and backbones in *mdx* mice.

Results

Hexose augments different AO activities in *mdx* mice intramuscularly

Previously, we evaluated hexoses and mixtures for their ability to improve the exon-skipping activity of PMO after intramuscular injections into tibialis anterior (TA) muscles of *mdx* mice.[16] A mixture of glucose/fructose (GF), fructose, galactose, and mannose achieved comparable improvements of dystrophin restoration over saline. Therefore, we further characterized the effect of these four candidates on the exon-skipping activity of other AO chemistries in *mdx* mice (**Table 1**).

As PNA AOs were shown previously to induce limited dystrophin expression in *mdx* mice at repeated doses,[4,7] we first investigated the effect of these four candidates on the activity of PNA AOs in *mdx* mice. Coadministration of PNA AOs (5 µg) in galactose, GF, mannose, or fructose into TA muscles of *mdx* mice resulted in comparable improvements of dystrophin expression over saline shown by the increased number of dystrophin-positive fibres (**Figure 1a**,**b**), exon skipping (**Figure 1c**) and dystrophin expression (**Figure 1d**). Although fructose and galactose displayed similar levels of enhancement, fructose demonstrated a more uniform enhancement effect on PNA AOs than galactose. Further examination on 2'OMe AOs (5 µg) in galactose, GF, mannose, or fructose in *mdx* mice intramuscularly indicated similar levels of enhancement over saline with more uniform enhancement effect achieved with fructose as shown by the increased number of dystrophin-positive fibres (**Figure 1a**,**b**) and exon skipping (**Figure 1c**), suggesting fructose is capable of enhancing the activities of AOs with different chemistries. We then investigated if peptide-conjugated PMO (PPMO) including M12-PMO, B-MSP-PMO and R-PMO.[15,17,18] M12-PMO (2 µg), B-MSP-PMO (1 µg), or R-PMO (1 µg) were similarly potentiated in fructose after administration into TA muscles of *mdx* mice. As expected, significant increases in the number of dystrophin-positive fibres were achieved in samples treated with B-MSP-PMO (2.27±0.5-fold), M12-PMO (2.05±0.4-fold), or R-PMO (1.18±0.54-fold) in fructose, respectively, compared to the corresponding saline groups (**Figure 1e**,**f**). Consistently, RT-PCR and western blot showed that higher levels of exon-skipping and dystrophin restoration were also detected in TA muscles of *mdx* mice treated with all three PPMOs in fructose than the corresponding PPMOs in saline (**Figure 1g**,**h**). The results demonstrated that fructose can enhance AO uptake independent on uptake enhancing modifications such as targeting or cell-penetrating peptides.

Table 1 Oligonucleotide and peptide sequences and nomenclature

Name	Sequence
PMO	5′-GGCCAAACCTCGGCTTACCTGAAAT-3′
Peptide nucleic acid	5′-GGCCAAACCTCGGCTTACCTGAAAT-3′
2'OMe	5′-GGCCAAACCTCGGCTTACCT-3′
B-MSP-PMO	5′-RXRRBRRXRRBRB-ASSLNIAX-ggccaaacctcggcttacctgaaat-3′
M12-PMO	5′-RRQPPRSISSHP-ggccaaacctcggcttacctgaaat-3′
R-PMO	5′-RXRRXRRXRRXRXB-ggccaaacctcggcttacctgaaat-3′

B, β-alanine; R, L-arginine; X, 6-aminohexanoic.

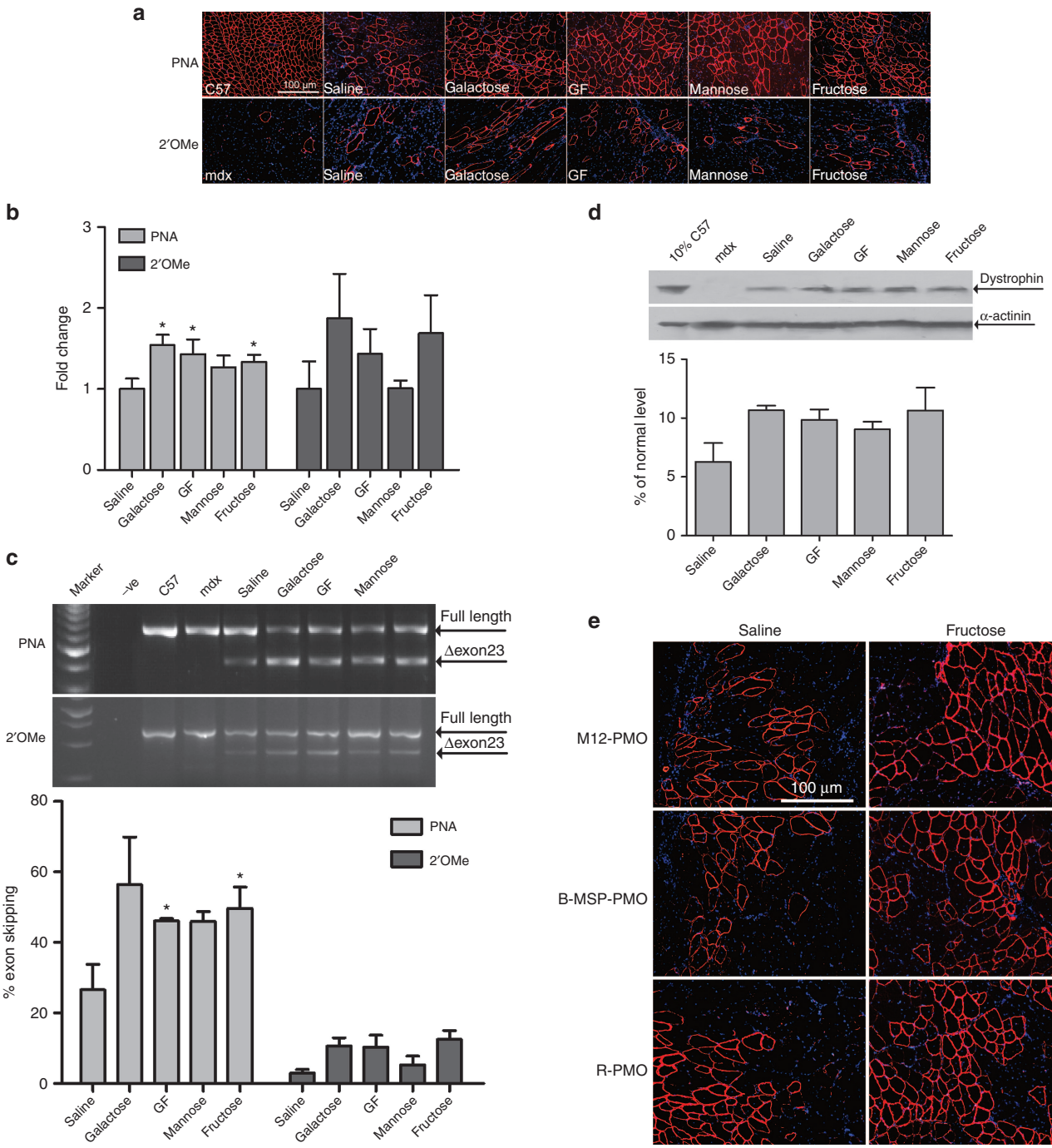
Fructose increases activities of oligonucleotides in a backbone-dependent manner

Since fructose demonstrated different magnitudes of enhancement on different AOs, we then assessed if fructose shows any preference in AO backbones. 2'OMe, PNA, and PMO were tested in *mdx* mice intramuscularly. The results demonstrated that fructose had the greatest improvement for PMO among the three AO types. PMO-F treatment resulted in 4.25±0.83-fold increase in the number of dystrophin-positive fibres compared to PMO-S; to a lesser extent with 2' OMe (1.7±0.81-fold) or PNA AOs (1.33±0.08-fold), respectively (**Figure 2a**,**b**). Consistently, RT-PCR analysis indicated that higher levels of exon-skipping were detected in samples treated with AOs in fructose than corresponding samples treated with AOs in saline, with PMO-F showing the highest level of exon-skipping (69.6±7.9%) compared with PNA or 2'OMe in fructose (**Figure 2c**). Western blot results also demonstrated the significantly enhanced level of dystrophin expression in TA muscles from *mdx* mice treated with PMO-F compared to PMO-S (**Figure 2d**). Thus, fructose appears to function in a backbone-dependent manner and be more potent at enhancing PMO activity than other AO chemistries.

Next, we investigated if fructose concentration affects the magnitude of enhancement, thus 2.5, 5, or 7.5% fructose with PMO were administrated into TA muscles of *mdx* mice. Uniform distribution of dystrophin-positive fibres was found throughout TA sections from *mdx* mice treated with PMO in 5% fructose and to a lesser extent with PMO in 2.5 or 7.5% fructose (**Supplementary Figure S1a**). Quantitative analysis of dystrophin-positive fibres revealed that a 4.25±0.83-fold increase was achieved with PMO in 5% fructose compared to PMO-S, whereas 2.12±0.23 or 2.68±0.63-fold improvement was obtained with 2.5 or 7.5% fructose compared to the corresponding saline groups, respectively (**Supplementary Figure S1b**). Corroborating with immunostaining results, RT-PCR and western blot data demonstrated that slightly higher levels of exon-skipping and dystrophin restoration were yielded with PMO in 5% fructose than 2.5 or 7.5% fructose treatments, respectively, though the difference was not significant. These data suggest that 5% fructose is likely the optimal and saturated concentration (**Supplementary Figure S1c–e**). Despite up to 7.5% fructose was tested in *mdx* mice intramuscularly, no muscle damage was found in treated TA muscles demonstrated by H&E staining (**Supplementary Figure S1f**), further supporting that fructose is safe to use in *mdx* mice.

Fructose augments PMO exon-skipping activity by enhancing its cellular uptake in muscles of *mdx* mice

As we had shown that GF augmented PMO's exon-skipping activity by enhancing its cellular uptake, we wanted to ascertain that fructose itself also worked via the same way.[16] Lissamine-labeled PMO was injected intravenously at 25 mg/kg/day for 3 days in *mdx* mice and body-wide muscles were harvested 4 days after last injection. Quantitative analysis of red fluorescence signals showed significantly enhanced PMO uptake in multiple peripheral muscles from *mdx* mice treated with PMO-F compared to PMO-S (**Figure 3a**,**b**), consistent with observations with GF[16]. Further RT-PCR analysis revealed significantly higher level of exon-skipping in TA, quadriceps, gastrocnemius and abdominal muscles from *mdx* mice treated with PMO-F over PMO-S (**Figure 3c**,**d**). Corroborating with RT-PCR results, approximately 2% of wild-type levels of dystrophin protein were restored in TA, quadriceps and abdominal muscle from *mdx* mice treated with PMO-F, whereas only trace amount of dystrophin was detected in counterparts treated with PMO-S (**Figure 3e**), suggesting that the augmented PMO activity is attributed to enhanced cellular uptake. The systemic potentiating effect



of fructose on PMO was also verified with another different dosing regimen (intravenous administration of PMO at 25 mg/kg/week for 3 weeks) in *mdx* mice (**Supplementary Figure S2a,b**), further strengthening the conclusion that fructose can potentiate PMO activity in *mdx* mice systemically.

Fructose promotes sustained molecular correction and phenotypic rescue in *mdx* mice

To examine the long-term effect of fructose, we employed a protocol consisting of repeated intravenous injections of PMO at 50 mg/kg/week for 3 weeks followed by 50 mg/kg/month for 5 months in *mdx* mice, an identical dosing condition to GF[16]. Substantial numbers of dystrophin-positive fibres were found in the TA, quadriceps, gastrocnemius and abdominal muscles, fewer in other peripheral muscles and no dystrophin expression in heart from *mdx* mice treated with PMO-F (**Figure 4a**). Compared with PMO-S, there was no evident difference in the number and distribution of dystrophin-positive fibres in body-wide muscles from *mdx* mice treated with PMO-F except for

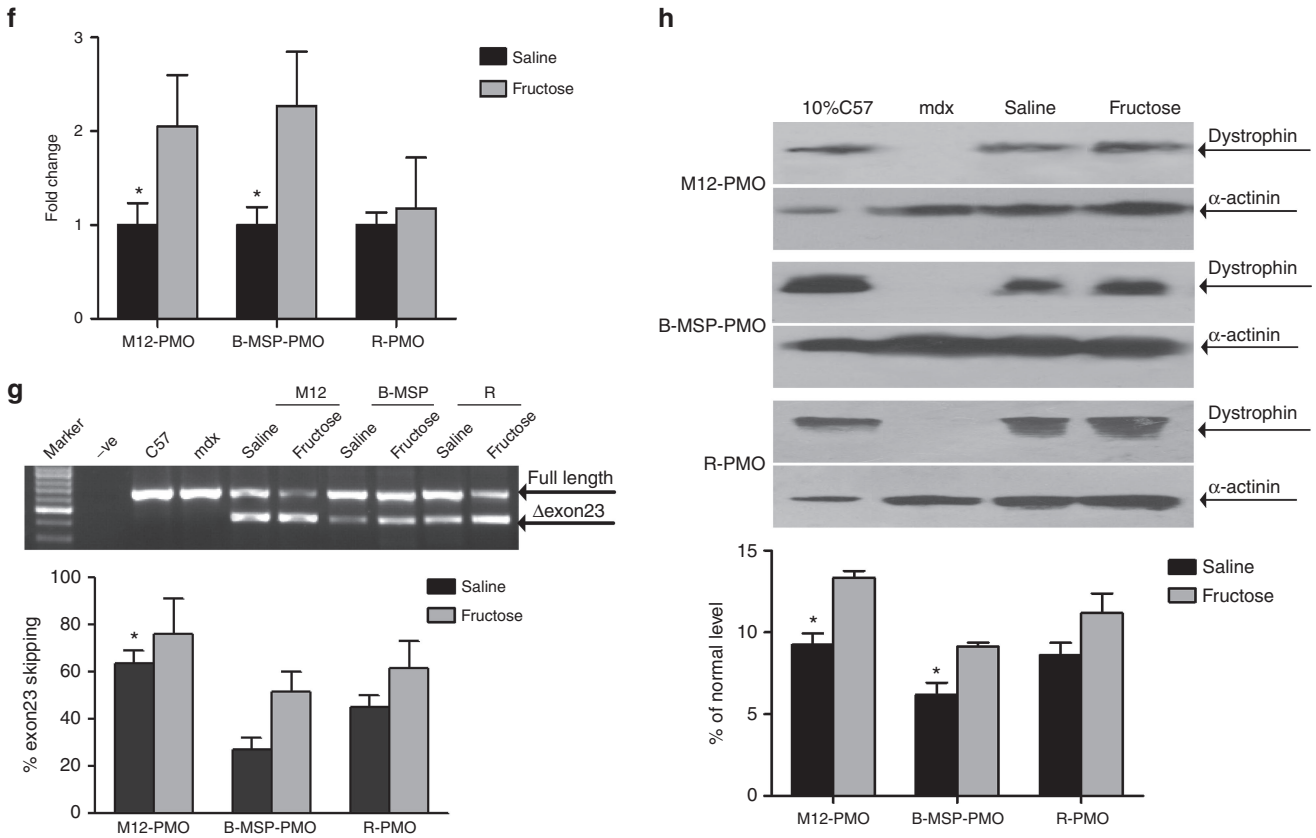


Figure 1 Evaluation of different AOs in hexose solutions in *mdx* mice intramuscularly. Dystrophin expression following one single intramuscular injection of 5 μg peptide nucleic acid (PNA), 2'OMe AOs or 2 μg M12-PMO, 1 μg B-MSP-PMO, or 1 μg R-PMO in hexose solutions in adult *mdx* mice. (**a**) Immunohistochemistry for dystrophin protein expression in *mdx* mice treated with PNA or 2'OMe AOs in different solutions, respectively. Data from control normal *C57BL6* and untreated *mdx* mice were shown (scale bar = 100 μm). (**b**) Quantitative analysis of dystrophin-positive fibres in TA muscles from *mdx* mice treated with different combinations. The comparison was normalized to the corresponding saline group and presented as fold change relative to saline. Significant improvement was detected in TA muscles treated with PNA in galactose, GF, or fructose compared with PNA in saline ($n = 3$, two-tailed *t*-test, $^*P < 0.05$). (**c**) RT-PCR analysis to detect dystrophin exon-skipping transcripts in the treated tissues with PNA AOs in different solutions, respectively. Δexon 23 is for exon 23 skipped bands. Significant increases were observed in the levels of exon skipping in TA muscles treated with PNA in GF or fructose compared with PNA in saline ($n = 3$, two-tailed *t*-test, $^*P < 0.05$). (**d**) Western blot to detect dystrophin protein expression in the indicated muscle groups from treated *mdx* mice compared with *C57BL6* and untreated *mdx* mice. Total protein (5 μg) from tibialis anterior of *C57BL6* and treated and untreated *mdx* mice (50 μg) were loaded with α-actinin used as a loading control ($n = 3$). (**e**) Immunohistochemistry for dystrophin protein expression in *mdx* mice treated with M12-PMO, B-MSP-PMO, or R-PMO in 5% fructose (scale bar=100 μm). (**f**) Quantitative analysis of dystrophin-positive fibres in TA muscles from *mdx* mice treated with different peptide-PMO conjugates in fructose. The comparison was normalized to the saline treatment group and presented as fold change relative to saline. Significant improvement was detected in TA muscles treated with M12-PMO or B-MSP-PMO in fructose compared with the corresponding saline groups ($n = 3$, two-tailed *t*-test, $^*P < 0.05$). (**g**) RT-PCR analysis to detect dystrophin exon-skipping transcripts in the treated tissues with different peptide-PMO conjugates in fructose. Δexon 23 is for exon 23 skipped bands. A significant increase was observed in the level of exon skipping in TA muscles treated with M12-PMO in fructose compared with the saline group ($n = 3$, two-tailed *t*-test, $^*P < 0.05$). (**h**) Western blot to detect dystrophin protein expression in the indicated muscle groups from treated *mdx* mice compared with *C57BL6* and untreated *mdx* mice. Different amounts of total protein (5, 10, 20 μg) from tibialis anterior of *C57BL6* and treated and untreated *mdx* mice (50 μg) were loaded with α-actinin used as a loading control. Significant increases were detected in the levels of dystrophin expression in TA muscles treated with M12-PMO or B-MSP-PMO in fructose compared with the corresponding saline groups ($n = 3$, two-tailed *t*-test, $^*P < 0.05$).

gastrocnemius, which showed slightly more dystrophin-positive fibres in PMO-F than PMO-S (**Figure 4a**). Consistent with immunostaining, RT-PCR analysis indicated that significantly higher level of exon-skipping was achieved in gastrocnemius treated with PMO-F compared with PMO-S (**Figure 4b**). Corroborating with immunostaining and RT-PCR results, up to 21.3% of wild-type level of dystrophin protein was restored in gastrocnemius treated with

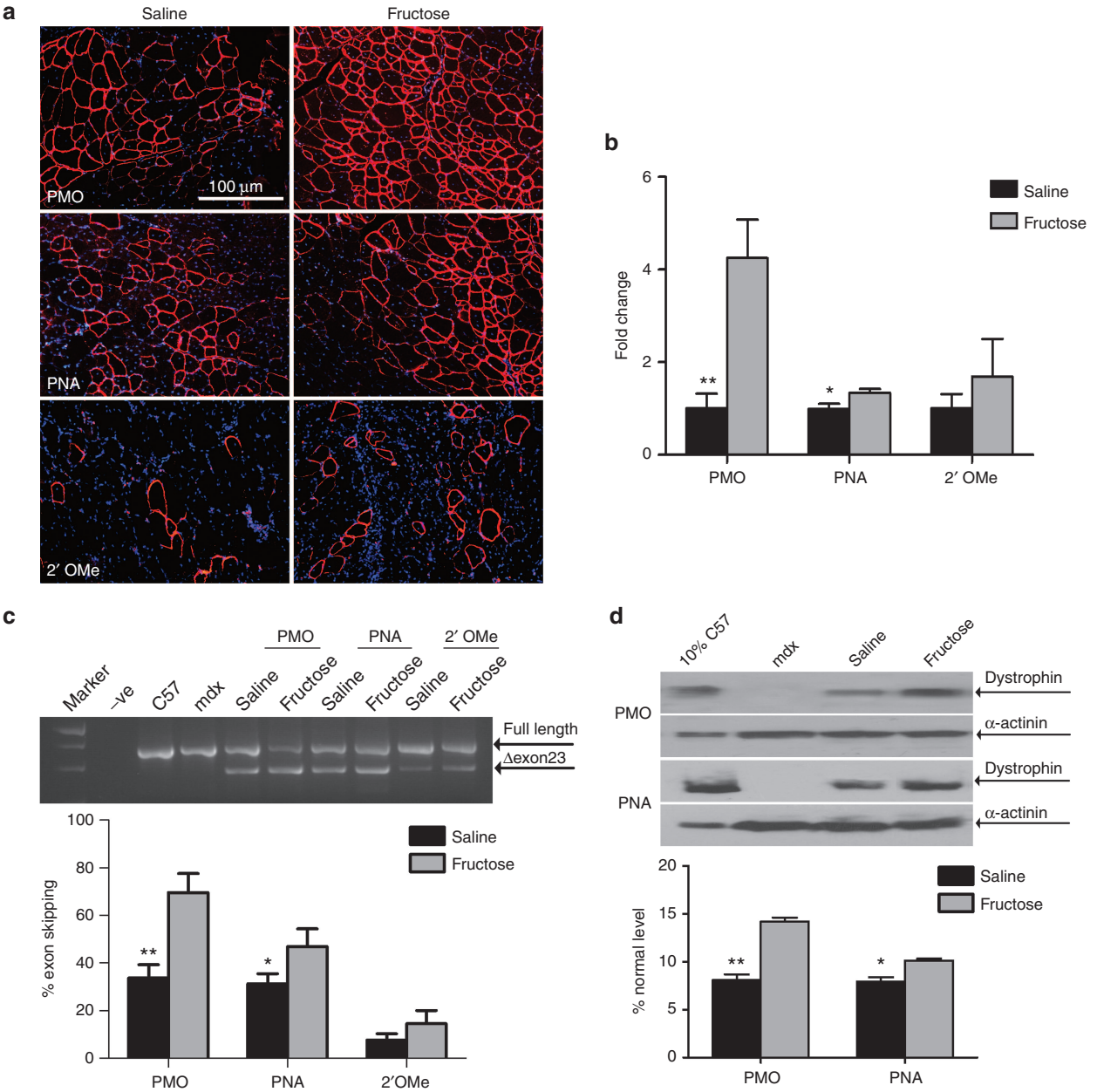


Figure 2. Investigation of the potentiating effect of fructose on PMO, peptide nucleic acid (PNA), and 2'OMe AOs in *mdx* mice intramuscularly. (**a**) Immunohistochemistry for dystrophin protein expression in TA muscles from *mdx* mice treated with 2 µg PMO, 5 µg PNA, and 5 µg 2'OMe AOs in 5% fructose (scale bar = 100 µm). (**b**) Quantitative analysis of dystrophin-positive fibres in TA muscles from *mdx* mice treated with PMO, PNA and 2'OMe in fructose, respectively. The comparison was normalized to the saline treatment group and presented as fold change relative to saline. Significant increases were detected in TA muscles treated with PMO or PNA in fructose compared with the corresponding saline groups ($n = 3$, two-tailed *t*-test, $^{*}P < 0.05$, $^{**}P < 0.01$). (**c**) RT-PCR analysis to detect dystrophin exon-skipping transcripts in treated TA muscles. Δexon 23 represents exon 23 skipped bands. Significant increases were detected in the levels of exon skipping in TA muscles treated with PMO or PNA in fructose compared with the corresponding saline groups ($n = 3$, two-tailed *t*-test, $^{*}P < 0.05$, $^{**}P < 0.01$). Also, there was a significant increase in the level of exon skipping in TA muscles treated with PMO-F compared with PNA in fructose ($n = 3$, two-tailed *t*-test, $P = 0.023$). (**d**) Western blot to detect dystrophin protein expression in TA muscles from treated *mdx* mice compared with *C57BL6* and untreated *mdx* mice. Total protein (5 µg) from tibialis anterior of *C57BL6* and treated and untreated *mdx* mice (50 µg) were loaded with α-actinin used as a loading control. Significant increases were detected in TA muscles treated with PMO or PNA in fructose compared with the corresponding saline groups ($n = 3$, two-tailed *t*-test, $^{*}P < 0.05$, $^{**}P < 0.01$).

PMO-F, whereas only 3.8% was detected in PMO-S. Notably, a significantly higher level of dystrophin expression was achieved in diaphragm treated with PMO-F compared with PMO-S (**Figure 4c**). But overall, comparable levels of dystrophin protein were achieved and no evident difference was observed between PMO-F and PMO-S treatments under this dosing condition (**Figure 4c**).

Further examination of the dystrophin-associated protein complex (DAPC), an indicator for functional improvement,[19] including α-sarcoglycan, β-dystroglycan and neuronal nitric oxide synthase (nNOS), demonstrated that DAPC components correctly relocalized to the muscle membrane of PMO-F-treated *mdx* mice (**Figure 4d**). A significant decline in serum creatine kinase (CK) levels was observed in mice

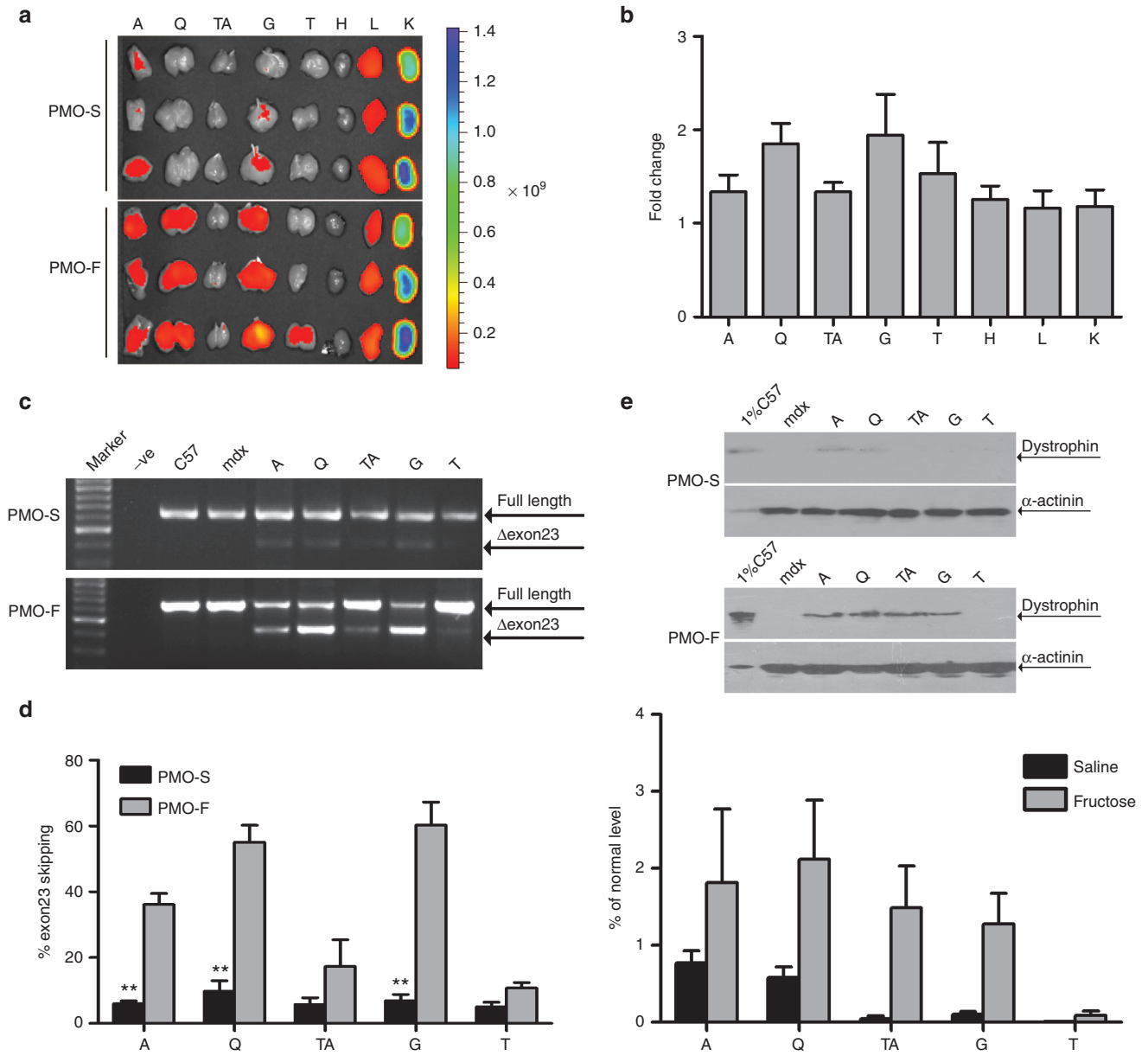
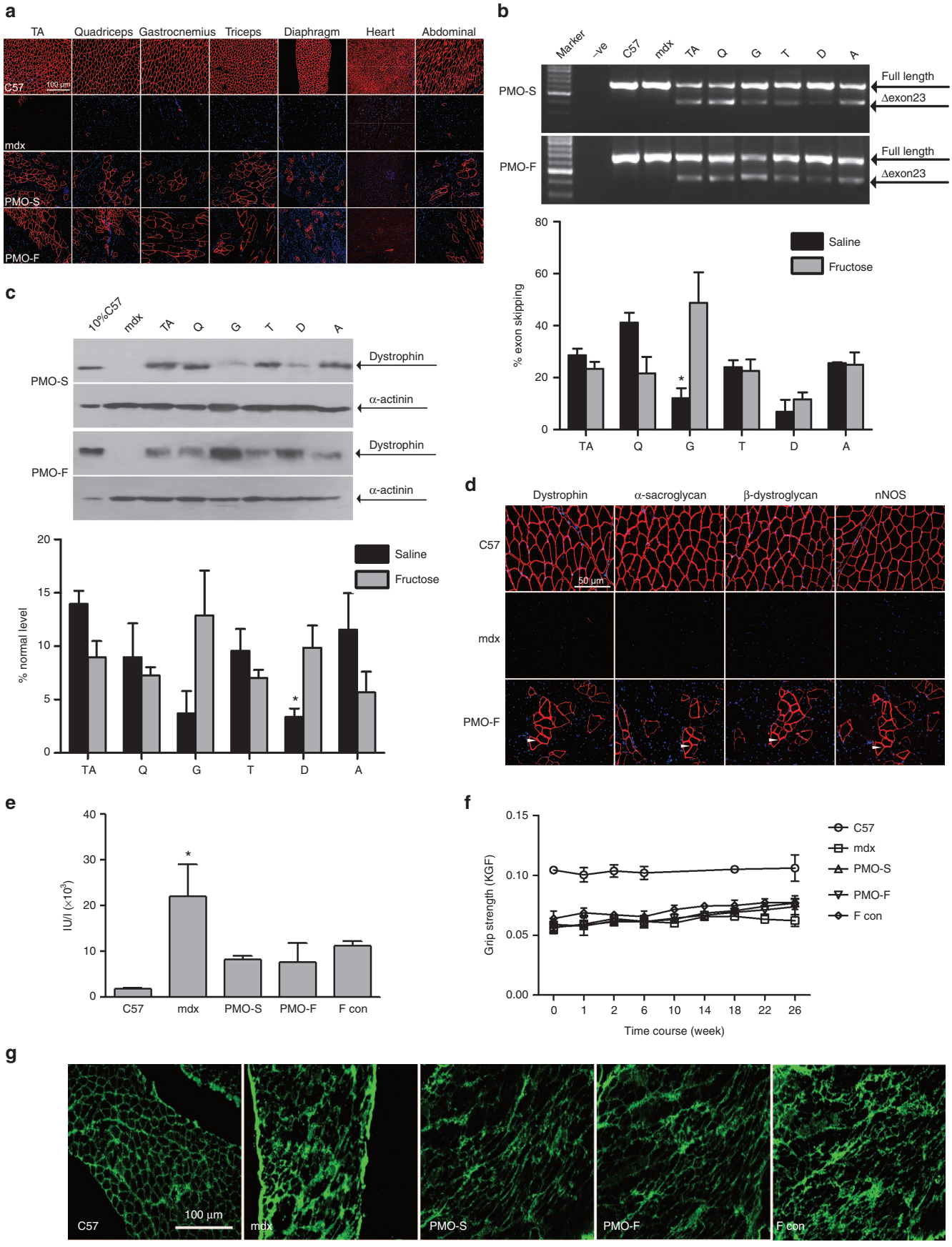


Figure 3 Evaluation of tissue distribution and exon-skipping activity of lissamine-labeled PMO-F in *mdx* mice. Lissamine-labeled PMO-F was injected intravenously into *mdx* mice at 25 mg/kg/day for 3 days. (**a**) Tissue distribution of lissamine-labeled PMO in *mdx* mice 4 days after three daily intravenous injections of either PMO-F or PMO-S at the 25 mg/kg/day doses. NC represents untreated *mdx* controls. A, abdominal muscle; Q, quadriceps; TA, tibialis anterior; G, gastrocnemius; T, triceps; H, heart; L, liver, and K, kidney ($n = 4$). (**b**) Quantitative evaluation of fluorescence intensity in body-wide tissues with IVIS spectrum series. The comparison was normalized to the corresponding saline group and presented as fold change relative to saline. (**c**) RT-PCR analysis to detect dystrophin exon skipping transcripts in the treated TA muscles. Δexon 23 represents exon 23 skipped bands. (**d**) Quantitative analysis of exon 23 skipping efficiency in muscles from *mdx* mice treated with PMO-F or PMO-S. Significant increases were detected in abdominal, quadriceps or gastrocnemius muscles treated with PMO-F compared with counterparts treated with PMO-S ($n = 4$, two-tailed *t*-test, $^{**}P < 0.01$). (**e**) Western blot to detect dystrophin protein expression in muscles from treated *mdx* mice compared with *C57BL6* and untreated *mdx* mice. Total protein (0.5 μg) from tibialis anterior of *C57BL6* and treated and untreated *mdx* mice (50 μg) were loaded with α-actinin used as a loading control ($n = 4$).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treated with PMO-F or PMO-S compared with untreated *mdx* mice, respectively (**Figure 4e**).[20] Although therapeutic level of dystrophin protein was restored in PMO-F and PMO-S treatment groups (**Figure 4c**), marginal force recovery was achieved only at later time-points compared to untreated *mdx* controls (**Figure 4f**), which is likely attributed to the accumulation of dystrophin protein in triceps. Examination on the muscle membrane integrity with a fluorescently-conjugated goat-anti-mouse IgG showed substantially less IgG-positive staining in diaphragm from *mdx* mice treated with PMO-F compared to untreated *mdx* controls and fructose alone (**Figure 4g**), suggesting that PMO-F treatment improved the muscle membrane conditions. Overall, it appears that the potentiating effect of fructose diminishes with long-term repeated administration unlike GF, which appears to enhance activity over multiple injections. Thus, fructose functions in a context-dependent manner and may be considered to be suitable for enhancing AO delivery in the short term.

Long-term use of fructose does not elicit any overt toxicity in *mdx* mice

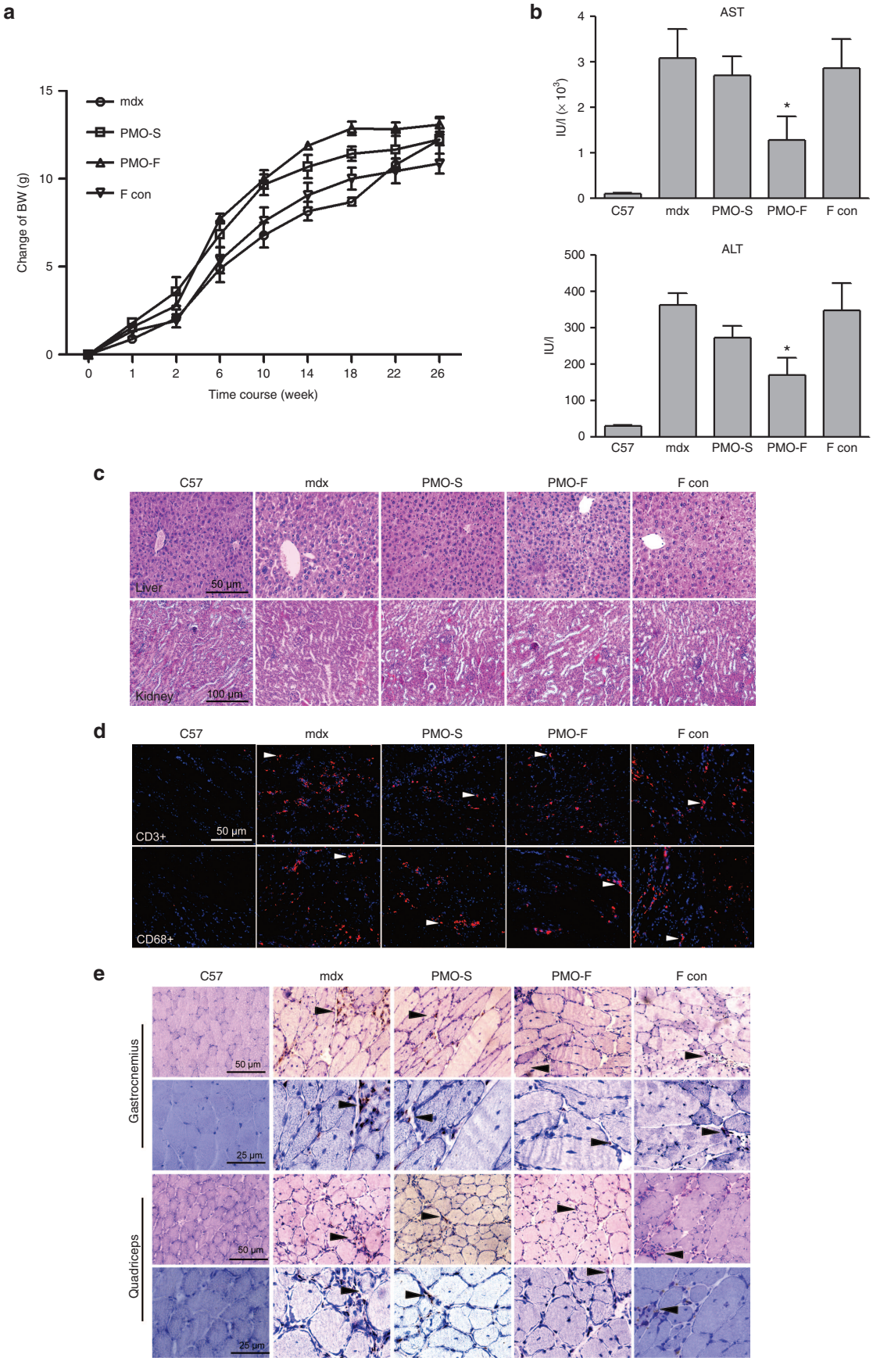
To examine whether long-term repeated intravenous injections of fructose or PMO-F could elicit any detectable toxicity, we monitored treated animals closely during the course of experiments and no behavioral abnormality was found. We measured the body-weight change of treated *mdx* mice periodically and the results showed that no difference was observed between mice treated with fructose alone or PMO-F compared to PMO-S or untreated *mdx* controls (**Figure 5a**), indicating that no abnormal body-weight gain occurred during the experimental period of 6 months. Strikingly, significant decreases in levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were found in serum from *mdx* mice treated with PMO-F compared with untreated *mdx* controls, suggesting that the combination of fructose with PMO generated more beneficial effects synergistically (**Figure 5b**). Histological assessment of liver and kidney evidenced no morphological alteration in *mdx* mice treated with PMO-F, PMO-S or fructose alone compared with untreated *mdx* and normal *C57BL6* controls (**Figure 5c**). Importantly, neither inflammation nor activation of immune response was triggered by long-term use of fructose alone or PMO-F shown by staining of CD68+ macrophages and CD3+ T lymphocytes in diaphragms (**Figure 5d**). Further, morphological and immunohistochemical examination of quadriceps and gastrocnemius showed more uniform myofibre sizes and fewer CD68+ macrophages present in *mdx* mice treated with PMO-F compared to PMO-S and age-matched untreated *mdx* controls (**Figure 5e**), strengthening the conclusion that the use of PMO-F is safe, well-tolerated, and beneficial.

Discussion

Low systemic delivery efficiency is a hurdle for therapeutic use of AOs in DMD. Development of safe and efficient delivery technologies will be immensely useful for the clinical translation of various AOs. Here, we demonstrate that fructose can augment the activity of a variety of AO chemistries in *mdx* mice. A strong potentiating effect on PMO exon-skipping activity was yielded with fructose in *mdx* mice with intramuscular and short-term repeated systemic use of fructose. Further tissue distribution studies revealed that the augmented PMO activity is attributed to the enhanced cellular uptake of PMO in muscles in *mdx* mice, consistent with previous observations with GF[16]. Long-term repeated administration of PMO-F showed limited enhancement in dystrophin expression in *mdx* mice compared to PMO-S contrary to the effect shown by GF, though the functional benefits were evident with significantly decreased levels of serum CK, AST, and ALT compared to age-matched *mdx* controls. Overall, our study provide evidences for the first time that fructose can be a safe and efficient delivery formulation for enhancing the uptake of AOs to muscle in a context-dependent manner and shed light on the new role of fructose in drug delivery.

It was reported that high amount of fructose intake likely contribute to obesity,[21] though the amount of fructose we used was much lower than dietary carbohydrate intake. Importantly, we did not observe any abnormal animal behavior or body-weight gain in *mdx* mice treated with PMO-F or fructose alone during the experimental period. Although it was documented that long-term consumption of high concentrations of fructose results in the elevated level of AST in liver,[22] our study demonstrated that long-term PMO-F treatments contributed to significantly decreased levels of AST and ALT compared to age-matched *mdx* controls, suggesting the combination of fructose and PMO is beneficial. Since the studies on low doses of fructose are limited, it is likely that other roles of fructose remained to be discovered.

Figure 4 Sustained dystrophin expression and functional improvement in *mdx* mice following repeated administration of PMO-F at 50 mg/kg/week for 3 weeks and 50 mg/kg/month for 5 months. (**a**) Immunohistochemistry for dystrophin protein expression in indicated muscles from *mdx* mice treated with repeated intravenous injections of PMO-F at 50 mg/kg/week for 3 weeks and 50 mg/kg/month for 5 months (scale bar = 100 µm). (**b**) RT-PCR analysis to detect dystrophin exon skipping transcripts in the treated muscles from *mdx* mice treated with PMO-F or PMO-S. Δexon 23 represents exon 23 skipped bands. A, abdominal muscle; Q, quadriceps; TA, tibialis anterior; G, gastrocnemius; T, triceps, and D, diaphragm. A significant increase was detected in gastrocnemius treated with PMO-F compared with PMO-S ($n = 4$, two-tailed *t*-test, $^*P = 0.041$). (**c**) Western blot to detect dystrophin protein expression in treated muscles from *mdx* mice treated with either PMO-F or PMO-S compared with *C57BL6* and untreated *mdx* mice. Total protein (5 µg) from tibialis anterior of *C57BL6* and treated and untreated *mdx* mice (50 µg) were loaded with α-actinin used as a loading control. A significant increase was detected in diaphragm treated with PMO-F compared with PMO-S ($n = 4$, two-tailed *t*-test, $^*P = 0.043$). (**d**) Restoration of the dystrophin-associated protein complex (DAPC) was studied to assess dystrophin function and recovery of normal myoarchitecture. DAPC protein components β-dystroglycan, α- and β-sarcoglycan and nNOS were detected by immunostaining in serial tissue cross-sections of quadriceps (scale bar = 50 µm). (**e**) Measurement of serum creatine kinase (CK) levels as an index of ongoing muscle membrane instability in treated *mdx* mice compared with untreated control group. Significant falls in serum CK levels were detected in mice treated with PMO-F, PMO-S, and fructose alone (F con) compared to age-matched untreated *mdx* controls ($n = 4$, two-tailed *t*-test, $^*P < 0.05$). (**f**) Muscle function was assessed using a functional grip strength test to determine the physical improvement of *mdx* mice treated with PMO-F. There was no statistical difference detected between PMO-F, PMO-S, F con and untreated *mdx* controls ($n = 4$, two-tailed *t*-test, $P > 0.05$). (**g**) Analysis of muscle membrane integrity in diaphragm from *mdx* mice treated with PMO-F, PMO-S, or F con with IgG staining.


a
Change of BW (g)
Time course (week)
mdx
PMO-S
PMO-F
F con
b
AST
ALT
IU/l (× 10³)
IU/l
C57
mdx
PMO-S
PMO-F
F con
c
Liver
Kidney
50 μm
100 μm
d
CD3+
CD68+
50 μm
e
Gastrocnemius
Quadriceps
50 μm
25 μm

Surprisingly, unlike GF[16], we failed to achieve significant potentiaing effect on PMO exon-skipping activity with long-term use of fructose under an identical dosing condition in *mdx* mice, though a functionally beneficial effect was observed. We speculated two reasons likely responsible for the unexpected results, though other possibilities cannot be excluded. It was documented that co-administration of glucose and fructose at equal quantities could facilitate cotransport of fructose even beyond the saturation concentration, so the presence of glucose is somehow important for the uptake of fructose.[23,24] An alternative possibility is that the dosing regimen we applied might, for unknown reasons, be the suboptimal choice for fructose. Therefore further studies are warranted to fine-tune the fructose concentration and the dosing regimen for PMO-F, based on our current findings. Nevertheless, coadministration of fructose with PMO mitigated the pathologies in *mdx* mice. As expected, no dystrophin restoration was detected in heart with PMO-F, suggesting different mechanisms might exist in heart as reported previously.[16] However, our recent preliminary data showed that the combination of carbohydrates with B-MSP-PMO, a chimeric peptide-PMO conjugate reported earlier,[15] can enhance dystrophin restoration in heart (Han *et al*., unpublished data).

In summary, our results demonstrate that fructose is a safe and efficient delivery formulation for enhancing the uptake of different AOs to muscle and can be used as a delivery tool for optimization of AO sequences and backbones. Our findings unveil a new role of fructose in drug delivery and might have implications for current exon-skipping clinical trials for DMD.

Materials and methods

Oligonucleotides. Four different AO chemistries were evaluated in this study. PMO and lissamine-labeled PMO were purchased from GeneTools (OR), and peptide-PMO conjugates were kindly synthesized by Dr Hong M Moulton (Oregon State University, Corvallis, OR). PNA AOs were synthesized by Panagene (Daejeon, Korea) with 90% purity. And 2'O-methyl phosphorothioate RNA (2'OMe) AOs were purchased from TriLink BioTechnologies (San Diego, CA). Details of all tested AOs were shown in **Table 1**.

Animal experiments. Six- to eight-week old *mdx* mice were used in all experiments (three mice in each group unless otherwise specified). The experiments were carried out in the animal unit (Tianjin Medical University, Tianjin, China), according to procedures authorized by the institutional ethical committee. For intramuscular studies, tibialis anterior (TA) muscles of 6–8-week old *mdx* mouse were injected with various amounts of AOs in 40 µl saline or hexose solutions including 5% fructose, 5% galactose, 5% mannose, and GF (2.5% glucose: 2.5% fructose). For systemic intravenous injections, various amounts of PMO were dissolved in 100 µl saline or 5% fructose solutions at the final dose of 25 or 50 mg/kg, respectively. Mice were sacrificed by terminal anesthesia followed by cervical dislocation at desired timepoints, and muscles and other tissues were snap-frozen in liquid nitrogen-cooled isopentane and stored at –80 °C.

RNA extraction and nested RT–PCR. Total RNA was extracted with Trizol (Invitrogen, Paisley, UK) and 400 ng of RNA template was used for 10 µl RT–PCR with OneStep RT–PCR kit (Qiagen, Manchester, UK) . The primer sequences were used as Exon20Fo: 5′-CAGAATTCTGCCAATTGCTGAG-3′ and Exon26Ro: 5′-TTCTTCAGCTTGTGTCATCC-3′. The RT-PCR product was then used for a nested PCR performed in 20 µl with 0.5 U Taq DNA polymerase (Invitrogen, UK). The primer sequences for the second round were Exon20F1: 5′-CCCAGTCTACCACCCTATCAGAGC-3′ and Exon24R1: 5′-CCTGCCTTTAAGGCTTCCTT-3′. The products were examined by electrophoresis on a 2% agarose gel.

Immunohistochemistry and histology. Series of 8 µm sections were examined from TA, quadriceps, gastrocnemius, triceps, abdominal and diaphragm, and cardiac muscles. Sections were then examined for dystrophin expression with a rabbit polyclonal antibody P7 against the dystrophin carboxyl-terminal region (the antibody was kindly provided by Dr Qilong Lu, North Carolina University, Chapel Hill, USA). Inflammation and immune response was detected by CD68+ macrophages (rabbit polyclonal, Abcam, Cambridge, UK) and CD3+ T lymphocytes (rat polyclonal Ab).[25] Polyclonal antibodies were detected by goat anti-rabbit or -rat IgG Alexa Fluor 594 (Invitrogen, New York, NY). Routine H&E (hematoxylin and eosin) staining was used to examine the overall muscle, liver and kidney morphology. For examination of CD68+ monocytes in quadriceps and gastrocnemius, the same rabbit polyclonal Ab (Abcam, Cambridge, UK) was used, followed by detection with peroxidase-conjugated goat anti-rabbit IgG (Sigma, Beijing, China). The serial sections were also stained with a panel of polyclonal and monoclonal antibodies for the detection of DAPC protein components. Rabbit polyclonal antibody to neuronal nitric oxide synthase and mouse monoclonal antibodies to β-dystroglycan, and α-sarcoglycan were used according to the manufacturer's instructions (Novocastra, Newcastle upon Tyne, UK). Polyclonal antibodies were detected by goat anti-rabbit IgGs Alexa 594 and the monoclonal antibodies were detected by goat anti-mouse IgGs Alexa 594 (Invitrogen, New York, NY). The M.O.M. blocking kit (Vector Laboratories, Burlingame, CA) was applied for the immunostaining of the DAPC.

Figure 5 Investigation of potential toxicity and immune activation of repeated administration of PMO-F at 50 mg/kg doses in *mdx* mice. (**a**) Body-weight measurements of *mdx* mice treated with PMO-F or PMO-S over 26 weeks. Data shows a steady body-weight increase and the same pattern of growth with both treatments as untreated *mdx* controls ($n = 4$). BW, body weight; F con, fructose alone. (**b**) Measurement of serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) enzymes in *mdx* mice treated with PMO-F compared with untreated *mdx* mice (two-tailed *t*-test, $P = 0.036$, $n = 4$). (**c**) H&E staining of liver (upper panel) and kidney (lower panel) tissues sections from *mdx* mice treated with PMO-F, PMO-S, F con, untreated *mdx* mice and *C57BL6* normal controls. (**d**) Detection of CD3+ T lymphocytes and CD68+ macrophage in the diaphragms of treated and untreated *mdx* mice (scale bar = 50 µm). Arrows indicate T lymphocytes detected by CD3+ and CD68+ mouse monoclonal antibodies. (**e**) Histological and immunohistological examination of quadriceps and gastrocnemius from *mdx* mice treated with PMO-F, PMO-S, F con, untreated and *C57BL6* normal controls. Data shows decrease in the number of CD68+ macrophages in skeletal muscles from *mdx* mice treated with PMO-F compared with untreated *mdx* controls.

Protein extraction and western blot. Protein extraction and western blot were carried out as previously described.[16] Various amounts of protein from wild-type *C57BL6* mice were used as positive controls and corresponding amounts of protein from muscles of treated or untreated *mdx* mice were loaded onto sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels (4% stacking, 6% resolving). The membrane was then washed and blocked with 5% skimmed milk and probed overnight with DYS1 (Abcam) for the detection of dystrophin protein and α-actinin as a loading control. The bound primary antibody was detected by peroxidase-conjugated goat anti-mouse IgG (Sigma, Beijing, China) and the ECL western blot analysis system (Millipore, Billerica, MA). The quantification is based on band intensity and area with Image J software, and compared with that from *C57BL6* TA muscles. Briefly, the densitometric intensity of each band, including dystrophin and α-actinin, was measured; next, the dystrophin values were divided by their respective α-actinin values. The dystrophin/ α-actinin ratios of treated samples were normalized to the average *C57BL6* dystrophin/α-actinin ratios. Each experiment was performed at least three times (at least three animals).

In vivo distribution test. Lissamine-labeled PMO were diluted in 100 µl of saline or 5% fructose solutions, respectively, and administered into 6–8-week-old *mdx* mice intravenously at a dose of 25 mg/kg/day for 3 days. Treated mice were perfused with 50 ml of cold phosphate-buffered saline to wash out unbound compounds. Heart, quadriceps, TA, gastrocnemius, triceps, liver, and kidney were harvested for imaging with IVIS spectrum (PE, Waltham, MA).

Grip strength test. Grip strength was assessed using grip strength meter consisting of horizontal forelimb mesh (BIOSEB, GT-31003004, Vitrolles, France). Each mouse was held 2 cm from the base of the tail, allowed to grip the metal mesh attached to the apparatus with their forepaws, and pulled gently until they released their grip. The force exerted was recorded and five sequential tests were carried out for each mouse, averaged at 1 minute apart. Five successful forelimb strength measurements were recorded, and data were normalized to body weight and expressed as kilogram force.

Serum enzyme measurements. Mouse blood was taken immediately after cervical dislocation and centrifuged at 1,500 rpm for 10 minutes. Serum was separated and stored at –80 °C. Analysis of levels of serum creatinine kinase, aspartime transaminase (AST), and alanine transaminase (ALT) was performed by the clinical laboratory (Tianjin Metabolic Diseases Hospital, Tianjin, China).

Statistical analysis. All data are reported as mean+SEM. Statistical differences between treatment and control groups were evaluated by SigmaStat (Systat Software, London, UK). Both parametric and nonparametric analyses were applied, in which the Mann-Whitney rank sum test (Mann-Whitney *U*-test) was used for samples on a non-normal distribution, whereas a two-tailed *t*-test was performed for samples with a normal distribution, respectively.

Supplementary material

Figure S1. Optimization of different concentrations of fructose with PMO in *mdx* mice intramuscularly.
Figure S2. Evaluation of PMO-F at 25 mg/kg/week dose for 3 weeks in *mdx* mice intravenously.

Acknowledgments The authors acknowledge Professor Wenyan Niu (Tianjin Metabolic Diseases Hospital, Tianjin, China) for assistance with the clinical biochemistry assays. This work was supported by National Key Basic Research Program of China (973) (No. 2012CBA01305, 2012CB932503); National Natural Science Foundation of China (No. 81361128013, 81273420 and 81301526) and Key Program of National Natural Science Foundation of Tianjin (No. 14JCZDJC36000). The authors declare no conflict of interest.

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