Morpholino antisense oligonucleotide induced dystrophin exon 23 skipping in *mdx* mouse muscle

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The *mdx* mouse model of muscular dystrophy arose due to a nonsense mutation in exon 23 of the dystrophin gene. We have previously demonstrated that 2′-O-methyl phosphorothioate antisense oligonucleotides (AOs) can induce removal of exon 23 during processing of the primary transcript. This results in an in-frame mRNA transcript and subsequent expression of a slightly shorter dystrophin protein in *mdx* muscle. Refinement of AO design has allowed efficient exon skipping to be induced in *mdx* mouse muscle cultures at nanomolar concentrations. In contrast, splicing intervention by morpholino AOs has been applied to the β-globin gene pre-mRNA in cultured cells to correct aberrant splicing when delivered in the micromolar range. The morpholino chemistry produces a neutral molecule that has exceptional biological stability but poor cellular delivery. We present data showing that exon skipping in *mdx* cells may be induced by morpholino AOs at nanomolar concentrations when annealed to a sense oligonucleotide or ‘leash’, and delivered as a cationic lipoplex. We have investigated a number of leash designs and chemistries, including mixed backbone oligonucleotides, and their ability to influence delivery and efficacy of the morpholino AO. Significantly, we detected dystrophin protein synthesis and correct sarcolemmal localisation after intramuscular injection of morpholino AO:leash lipoplexes in *mdx* muscle in vivo. We show enhanced delivery of a morpholino AO, enabling the advantageous properties to be exploited for potentially therapeutic outcomes.

INTRODUCTION

The degenerative muscle diseases, Duchenne and Becker muscular dystrophy (DMD/BMD) are allelic X-linked recessive disorders, arising from mutations within the dystrophin gene (1). DMD is characterized by the progressive wasting of skeletal and cardiac muscle and presents between the ages of 3 and 5 years (2). Affected boys are restricted to a wheelchair by the age of 12 years and 90% die in their late teens or early twenties due to cardiac or respiratory failure (2). In the absence of prenatal screening and genetic counselling, DMD occurs at a frequency of ~1 in 3500 live male births (3). DMD typically arises from nonsense or frame-shift mutations within the gene, which cause premature termination of translation and synthesis of a non-functional dystrophin protein. BMD, a milder, allelic form of DMD, manifests as a wide spectrum of phenotypes, from mild to borderline DMD (4). BMD mutations are generally such that the reading-frame is maintained, enabling a shortened, but still functional, protein to be produced.

Current clinical treatments of DMD/BMD generally involve the use of palliative agents, steroid treatment and intermittent positive pressure ventilation later in life (5,6). However these treatments do not address the primary genetic defect. Several potential therapies have been proposed, including viral-mediated gene transfer, myoblast transplantation, homologous gene up-regulation and targeted gene correction amongst others (7). Although promising, clinical trials have been limited and many hurdles need to be overcome to improve specificity, safety and efficiency.

As an alternative to these potential therapies, we and others have investigated the possibility of using antisense oligonucleotides (AOs) to redirect gene transcript processing and restore dystrophin protein synthesis (8–11). We have previously reported the use of 2′-O-methyl (2OMe) phosphorothioate (PS) AOs to block motifs involved in normal dystrophin pre-mRNA transcription and splicing, leading to an in-frame transcript and subsequent expression of a dystrophin protein that can correct the muscle phenotype of the *mdx* mouse model of DMD. We have also identified a number of leash chemistries that can enhance delivery and efficacy of morpholino AOs.

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splicing in order to induce excision of exon 23, the exon containing the mdx nonsense mutation, from the processed mRNA transcript (8–10).

One chemistry that is gaining wide recognition for use in antisense applications is the morpholino oligonucleotide developed by Summerton and Weller (12). These authors developed the morpholino structural type with the intention that this chemistry could provide several advantages in the clinical application(s) of antisense therapeutics, such as strong nucleic acid binding, resistance to nuclease, minimal non-antisense effects, high aqueous solubility and relatively low synthesis costs (12). However, transfection with uncomplexed morpholino AOs (naked transfection) has been shown to require relatively high concentrations (13,14). Because morpholino AOs are neutral molecules, they do not readily diffuse across the cell membrane and cannot be transfected using standard cationic transfection reagents. To overcome this limitation, a morpholino AO directed at the mouse dystrophin exon 23 donor splice site was annealed to various complementary DNA/RNA molecules, or ‘leashes’ (15).

Varying the length, chemistry and structure of the leash was shown to influence the ability of the morpholino AO to induce exon 23 skipping, presumably by affecting the delivery of the morpholino AO:leash lipoplex to the nucleus. This is the first reported use of a morpholino AO able to efficiently induce the removal of exon 23 from mdx dystrophin pre-mRNA as determined by both RT–PCR and western blotting. When delivered as a leash duplex and complexed with Lipofectin, the morpholino AO was effective at concentrations several orders of magnitude lower than previously reported for other applications of this chemistry (13,14). Finally, we also demonstrated dystrophin synthesis and correct sarcolemmal localization in vivo in mdx mice treated by intramuscular injection of morpholino AO:leash lipoplexes. Our results suggest that the morpholino structural type may be the chemistry of choice for inducing targeted removal of dystrophin exons.

RESULTS

Leash design

We wished to investigate the potential of a morpholino AO to induce dystrophin exon 23 skipping when targeted to the donor splice site of exon 23 (Fig. 1A). Exon skipping is dependent upon the ability of the AO to reach the nucleus and then interfere with spliceosome assembly. To facilitate delivery and nuclear uptake of the morpholino AO, it was annealed to a range of complementary DNA/RNA oligonucleotides, or leashes. It was anticipated from the work of others (15,16), that parameters such as leash annealing length, extent and position of non-complementary overhangs and chemistry could alter the uptake and subsequent biological activity of the morpholino AO. To ensure the leash annealed to the morpholino AO at 37°C, the complementary region consisted of at least 17 bases (15). Various chemistries were also evaluated in leash design to achieve a balance between resistance and susceptibility to degradation (Fig. 1B). It was considered necessary to create a leash sufficiently stable to ensure delivery to the nucleus while retaining some lability to nucleases to facilitate liberation of the morpholino AO from the AO:leash duplex, and thus enable subsequent annealing to the target sequence. Three chemistries were used with the following hierarchy of nuclease resistance: phosphodiester (PO) < phosphorothioate (PS) < 2OMe PS. Leash arrangements with respect to chemistry, annealing domain and non-complementary overhangs are detailed in Figure 1B. It has been shown that the base composition of the non-complementary overhangs had no significant impact whereas the position of the overhang was more important (15).

Delivery of morpholino:leash duplexes

Prior to transfection with the various morpholino:leash complexes, it was necessary to confirm that all leashes had correctly annealed to the morpholino AO. Morpholino:leash duplexes were electrophoresed through non-denaturing 20% polyacrylamide gels alongside the leash alone to demonstrate a mobility shift, where duplexes were retarded compared to the single-stranded leashes. All leashes were found to have annealed to the morpholino AO (data not shown).

Delivery of a morpholino:leash duplex into the nucleus of the cells was monitored using the morpholino AO annealed to leash 4, which was labelled with FITC-at the 3' end. Untreated cells displayed no fluorescence, whereas cells transfected with ψM23D(+07–18):leash 4 lipoplexes showed efficient uptake of the complex into the nucleus after 24 h (Fig. 2). While it was possible that intracellular fluorescence was due to transfection of leash 4 only and not the morpholino:leash duplex, several experiments were included to confirm morpholino AO delivery to the nucleus. Firstly, the mobility shift studies indicated that the ψM23D(+07–18) effectively formed a duplex with leash 4. Cells transfected with the ψM23D(+07–18):leash 4 lipoplex produced dystrophin gene transcripts missing exon 23 (Fig. 3). Although H-2K mdx cells transfected with lipoplexes of leash 4 alone demonstrated identical intracellular fluorescence to cells transfected with ψM23D(+07–18):leash 4 lipoplex, no transcripts excluding exon 23 were ever observed in cells transfected with individual leashes alone, including leash 4 (data not shown). No intracellular fluorescence was observed when the ψM23D(+07–18):leash 4 duplexes were transfected in the absence of Lipofectin (uncomplexed), or when the cells were exposed to the supplied FITC-labelled control morpholino, even at high doses of 1 and 10 μM, in the presence or absence of Lipofectin. Similarly, no exon 23 skipping was observed after transfection of uncomplexed ψM23D(+07–18):leash duplexes or when ψM23D(+07–18) was transfected without a leash (Fig. 3). In summary, these results suggest that leashes and Lipofectin were essential for efficient morpholino AO delivery and activity, but did not induce exon 23 skipping on their own.

Assessment of exon skipping induced by morpholino:leash duplexes

Initial experiments designed to assess the ability of morpholino:leash duplexes to induce exon 23 skipping involved transfecting H-2K mdx cells, 24 h after seeding, with ψM23D(+07–18) at 300 nM in combination with each of 12
different leashes. The nature of the leash was found to influence the ability of the morpholino AO to induce exon skipping, as determined by the presence and relative intensity of the shortened 688 bp RT–PCR product, representing mRNA transcripts missing exon 23, compared with the intact full-length product of 901 bp (Fig. 3).

When transfected as lipoplexes, all 12 $\psi$M23D(+07–18) : leash duplexes demonstrated some capacity to induce exon 23 skipping (Fig. 3). When the morpholino AO was annealed to leash 1, a 17mer complementary PO oligonucleotide, exon 23 skipping was routinely weaker when the morpholino was annealed to a 21mer all PS oligonucleotide (leash 2) with a 20-base complementary core and a single base overhang. Efficient removal of exon 23 from the dystrophin transcript was also observed when the morpholino was annealed to leashes of mixed backbone chemistries (leashes 3–9; Fig. 3). The

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**Figure 1.** (A) Annealing of the morpholino AO, $\psi$M23D(+07–18), to the 5′ donor splice site of intron 23 in mouse dystrophin pre-mRNA. Exonic region is shown in upper case whereas the intron is shown in lower case. (B) Leashes are shown 5′→3′ with annealing positions relative to the morpholino AO $\psi$M23D(+07–18). Only complementary bases of the leash to the morpholino are underlined. Chemistries are indicated according to the following key: morpholino = bold; phosphodiester = lower case; phosphorothioate = upper case; 2′-O-methyl phosphorothioate = lower case and italics.
annealed to progressively shorter leashes without overhangs (Fig. 1B), as judged by the relative intensity of the 688 bp product to the full length fragment (Fig. 3). No exon 23 or exon 22+23 skipping was ever observed in untreated cells, or those treated with a 300 nM dose of ψM23D(+07–18) transfected without leash or Lipofectin, or when ψM23D(+07–18) was annealed to a leash and delivered without Lipofectin.

**Titration of ψM23D(+07–18): leash hybrids**

Since all morpholino:leash lipoplexes consistently induced the removal of exon 23 in cultured H-2K mdx cells at a transfection dose of 300 nM, titration studies were then performed to determine the minimum effective doses and thus identify the more effective leash designs. To encompass the spectrum of leash designs, six leashes were chosen for titration studies on the basis of their design and chemistry and to refine observed trends. Leashes 1, 2 and 10 were chosen to represent pure PO, PS and 2OMe PS chemistries, respectively, whereas, leashes 3, 6 and 8 represented different facets of the mixed backbone design. The morpholino AO annealed to leashes 3, 6 and 8 by 17–20 complementary PO core bases (Fig. 1B). Leashes 3, 6 and 8 also differed in the number of non-complementary PS bases with 5’ and 3’ overhangs of 4–10 bases. The six ψM23D(+07–18):leash duplexes were transfected at doses between 5 and 300 nM with a 2:1 Lipofectin:leash ratio. Morpholino:leash duplexes were also transfected at 300 nM in the absence of Lipofectin.

When the morpholino AO was annealed to leash 1, weak exon 23 skipping was only induced at doses above 100 nM (Fig. 4). Lipoplexes of ψM23D(+07–18):leash 2 consistently induced strong exon 23 skipping at a transfection dose as low as 30 nM, with weaker, inconsistent skipping evident at 5 nM. Consistent exon skipping was observed when the morpholino AO was annealed to leash 6, compared with the stronger skipping induced by the morpholino AO when it was annealed to leashes 3 or 8 (Figs 3 and 4). The removal of exon 23 from dystrophin mRNA was more prominent at 10 nM when the morpholino was annealed to leash 8, than when it was annealed to leash 3 (Fig. 4). When annealed to leash 10, ψM23D(+07–18) consistently induced strong skipping at a dose of 30 nM, with weak and inconsistent skipping observed at 10 nM. The induction of exon 22+23 skipping was generally only observed at the higher doses as previously reported for 2OMe PS AOs (10). No exon 23 skipping was observed when duplexes were exposed to cells without prior complexing with Lipofectin, as described above. Furthermore, the minimum effective doses, varying from 5 to 30 nM for the more efficient morpholino:leash lipoplexes, were several orders of magnitude less than the uncomplexed morpholino AO was annealed to a leash and delivered without Lipofectin.

Simultaneous skipping of exons 22+23 was often observed to varying degrees (Fig. 3). When annealed to leashes of pure 2OMe PS chemistry (leashes 10–12), the induced exon 23 skipping in cells was not as efficient when the shorter leashes (11 and 12) were used. The efficiency of exon skipping was generally found to decrease when the morpholino AO was annealed to progressively shorter leashes without overhangs (Fig. 1B), as judged by the relative intensity of the 688 bp product to the full length fragment (Fig. 3). No exon 23 or exon 22+23 skipping was ever observed in untreated cells, or those treated with a 300 nM dose of ψM23D(+07–18) transfected without leash or Lipofectin, or when ψM23D(+07–18) was annealed to a leash and delivered without Lipofectin.
Time course analysis of induced exon skipping

The results of the titration studies yielded several leash designs that were effective at delivering the morpholino AO at low doses. Persistence studies were performed with three leashes, 3, 8 and 10, over a 10 day period to further assess their influence on morpholino AO delivery and the subsequent duration of \( \psi M23D(+07–18) \)-induced exon 23 skipping. Cells were transfected with duplexes of \( \psi M23D(\text{þ}07–18) \) and leashes 3, 8 or 10 at 300 and 100 nM doses, 24 h after seeding. Total RNA was extracted 1, 4, 7 and 10 days after transfection and RT–PCR undertaken between exons 20 and 26.

All three morpholino:leash complexes included in this study demonstrated persistent exon 23 skipping for up to 10 days after a single transfection at a dose of 300 nM (Fig. 5). The influence of the leashes on morpholino AO activity was more evident when the morpholino:leash complexes were transfected at 100 nM. At this dose, the morpholino AO induced the most efficient and persistent removal of exon 23 when annealed to leash 3. Exon 23 skipping was consistently shown to be induced efficiently for up to 7 days post transfection, with weaker skipping evident until day 10, the latest time point tested (Fig. 5). The intensity of the 688 bp RT–PCR product, missing exon 23, was strong only at day 1 post-transfection when cells were treated with \( \psi M23D(\text{þ}07–18) \) annealed to leashes 8 or 10. For these treatments, the intensity of the 688 bp band declined after the day 1 time point when compared with the intensity of exon 23 skipping induced by \( \psi M23D(\text{þ}07–18) \):leash 3 duplexes. The persistence of the out-of-frame product missing exons 22+23 was profoundly weaker at the 100 nM dose in all treated samples (Fig. 5). No exon 23 or 22+23 skipping was detected in untreated cells or cells exposed to naked morpholino:leash duplexes (data not shown).

Western blot analysis of treated cells

We wished to determine if the exon 23 skipping induced by the morpholino AO : leash lipoplexes correlated with the restoration of protein synthesis using the three leashes and a range of control conditions, including transfection with the 2OMe PS AO M23D(\text{þ}02–18) as a positive control (10). Total protein extracted from treated and control cultures was loaded onto denaturing SDS gradient gels after normalization for the myosin heavy chain, fractionated, transferred to nitrocellulose and detected with the Dys2 monoclonal antibody to the C-terminal of dystrophin (10). Cells were transfected at day 4 in the absence of serum and harvested at day 8. We detected near-full-length dystrophin in extracts of cells treated with the morpholino AO annealed to leashes 3, 8 and 10 when complexed with Lipofectin (Fig. 6A). Other leashes were not examined, although it was anticipated from RT–PCR studies that variable levels of dystrophin protein would have been produced. Levels of protein induced by two lipoplexes were higher than those achieved after transfection with an equivalent dose of M23D(\text{þ}02–18), previously the most effective 2OMeAO (10). Levels of dystrophin induced by \( \psi M23D(\text{þ}07–18) \):leash 10 lipoplex were similar to those obtained by treatment with M23D(\text{þ}02–18) (Fig. 6A), consistent with the slightly weaker influence of leash 10 as determined by RT–PCR analysis (Fig. 5). No protein was observed when \( H-2K \) mdx cells were exposed to unannealed \( \psi M23D(+07–18) \), with or without Lipofectin at 300 nM, or...
when morpholino : leash duplexes were exposed to cells without prior complexing with Lipofectin (Fig. 6A).

However, when cells were exposed to a 10 μM dose of uncomplexed cM23D(+07–18) in the absence of serum, low levels of dystrophin were detected upon over-exposure of the membrane (Fig. 6B). In contrast, a single 1 μM dose administered under identical conditions failed to produce any detectable dystrophin, even after overexposure of the membrane (Fig. 6B). No dystrophin was detected in extracts of cells left untreated or exposed to Lipofectin only [Lipofectin-only dose was equivalent to that administered with cM23D(+07–18):leash 3 duplex].

Restoration of dystrophin synthesis in vivo

Based on the strength of the in vitro results, we injected 1 μg of ψM23D(+07–18):leash 3 duplex complexed to Lipofectin at a 2:1 ratio into the left tibialis anterior (TA) muscle of 3-week-old mdx mice. The contralateral leg was injected with phosphate buffered saline as an untreated control. Mice were sacrificed 2 days or 2 weeks after injection. Dystrophin was detected and found to be localized to the sarcolemma of muscle fibres in the left TA 2 weeks after injection (Fig. 7C and D). The dystrophin signal was strong and continuous, reminiscent of the C57Bl/10ScSn normal control (Fig. 7A). The induced protein was determined to be near full-length in size by western blotting (not shown). Dystrophin was not detected in the contralateral, untreated limb (Fig. 7B), examined after sham injection. Sections of treated muscle examined 2 days after injection only showed low levels of diffuse staining for dystrophin (data not shown). A 688 bp product representing the dystrophin transcript missing exon 23 was detected in the treated left TAs 2 days and 2 weeks after injection, but not in

**Figure 4.** Titration of morpholino : leash duplexes to determine the minimum effective lipoplex dose in tissue culture. ψM23D(+07–18):leash hybrids were transfected either naked or as lipoplexes at a range of doses. RT–PCR across exons 20–26 was used to ascertain the minimum dose at which each morpholino : leash lipoplex could induce exon 23 skipping (688 bp product). Exon 23 skipping was routinely detected at a minimum dose of 5 nM with leash 2, 10 nM with leashes 3, 6 and 8, 30 nM with leash 10 and 100 nM with leash 1. No exon 23 skipping was detected after naked transfections or in untreated cells. Some sporadic exon skipping was seen in some samples.

**Figure 5.** Time course analysis of exon 23 skipping induced by morpholino : leash complexes. H-2K mdx cells were transfected 24 h after seeding with either 100 or 300 nM doses of ψM23D(+07–18):leash lipoplex. Total RNA was extracted 1, 4, 7 or 10 days after transfection for RT–PCR across exons 20–26. The 688 bp product representing exon 23 skipping was detected for up to 10 days with all leashes and doses although at different intensities relative to the intact product. As expected, skipping was more pronounced with all morpholino : leash lipoplexes at 300 nM than at 100 nM. The most efficient skipping at 100 nM was observed with leash 3. At 100 nM, all other morpholino AO : leash lipoplexes produced comparable levels of exon 23 skipping that were sustained for 10 days, but at lower levels than those produced by the ψM23D(+07–18):leash 3 complex.
the untreated contralateral TAs (Fig. 7E). The persistence of the induced dystrophin mRNA transcript missing exon 23 for at least 2 weeks is of longer duration than we have observed when using the 2OMe PS AO M23D(+12–13) in vivo (9) (unpublished observations). This is likely to be the result of the more stable chemistry and/or perhaps the slow release of the morpholino AO due to the presence of the leash. The identity of the induced product was confirmed by direct DNA sequencing, which also showed that the fidelity of the exon boundaries had been maintained (data not shown).

**DISCUSSION**

AOs have been successfully used to produce shortened mRNAs by inducing specific exon skipping of dystrophin transcripts in mdx muscle in vitro and in vivo, and in cultured human cells (8,9,11,17,18). Previously, our laboratory has reported that the efficiency of exon 23 skipping induced by 2OMe PS AOs in mdx muscle cells was dependent on the target site of the AO rather than the length of the AO (9). Recent improvements to the target design of the 2OMe PS AOs identified a 20mer M23D(+02–18) and a 17mer M23D(+02–18), which induced enhanced levels of exon skipping and protein synthesis (10). When targeting exon 23 for omission from the pre-mRNA, out-of-frame transcripts missing both exons 22 and 23 were frequently detected and reflect the intimate processing of these 2 exons (10). The generation of dystrophin transcripts skipping exons 22 and 23 does not reflect a lack of specificity in the AO target, that is the donor splice site, but rather the processing of the targeted exon (18). With these principles and improvements further established, investigating alternative oligonucleotide structural types is an important consideration, as it could ultimately yield a molecule with enhanced safety, efficiency, specificity and affordability. The feasibility of using alternative oligonucleotide chemistries to modulate the splicing process has only been explored for the dystrophin pre-mRNA using the nuclease-sensitive PO chemistry (19).

The morpholino structural type was developed in response to the limitations and non-specific effects observed with some early AO molecules (12). The unnatural morpholino chemistry renders the molecule highly resistant to nucleases. An essential property for our application is that the morpholino AOs will not induce RNase H-mediated down-regulation of the target pre-mRNA (20). However, the uncharged backbone compromises delivery, for non-ionic AOs cannot easily be delivered into cultured cells using delivery agents such as cationic liposomes. To circumvent this difficulty, we investigated the use of single stranded (anionic) nucleic acid ‘leashes’ which were annealed...
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to the morpholino AO, allowing the AO:leash duplex to be complexed with Lipofectin.

In this study, a single morpholino AO was used, whilst the annealed leashes varied, with several parameters considered significant. Firstly, the length of complementary core sequence defines the melting temperature \( T_m \) and has been shown to greatly influence the activity of peptide nucleic acid AOs to down-regulate luciferase expression in co-transfected COS-7 cells (16). Secondly, another study used a morpholino AO in an inducible luciferase system (21) and proposed that the presence of non-complementary overhangs, particularly a 5’ overhang, could increase luciferase expression by increasing accessibility of nucleases which liberated the morpholino AO from the leash (15). We also investigated the use of mixed chemistry backbones which have previously been used for therapeutic AOs designed to down-regulate gene expression (22), but to our knowledge have never been employed in leash-like molecules. Finally, a cholesterol moiety was included at the 3’ end of leash 5 in an attempt to enhance uptake of the morpholino:leash lipoplex.

The pure PO chemistry leash, leash 1, was consistently less effective than the other leashes at delivering the morpholino AO to promote dystrophin exon 23 skipping. This may have been due to the labile PO backbone being too rapidly degraded, or the absence of any non-complementary overhang. Duplexes comprising of morpholino AO:leash 2, the pure PS chemistry leash, were able to induce exon skipping at much lower doses after transfection \( \text{in vitro} \). This could be due to either the increased leash stability and/or the leash length, which subsequently improved delivery of the morpholino AO to the nucleus.

The proposed requirement for nucleases to degrade the leash and liberate the morpholino AO is not supported by the observation that the more stable 2OMe PS leashes 10, 11 and 12 facilitated exon skipping with a hierarchy of efficiency 10 > 11 > 12, inversely proportional to their length. The 2OMe PS leashes are several-fold more nuclease resistant than the PO or PS oligonucleotides (23,24). Leash 10 contained a 5 base overhang at the 5’ end of the leash and was consistently found to be more effective in delivery of the morpholino AO as determined by relative amounts of induced exon skipping. Indeed, we observed that the morpholino AO was less effective when annealed to pure 2OMe PS leashes without overhangs, or the absence of any non-complementary overhang (leashes 11 and 12; Fig. 3). It must be considered that the leash length and/or the overhang may mediate enhanced lipoplex formation, potentially through the formation of higher order structures that affect complexing with Lipofectin.

Both leash 2 and leash 10 were complementary to \( \psi M23D(07–18) \) over a 20 nucleotide region, although at different annealing sites, suggesting that the \( T_m \)s of the two morpholino:leash duplexes should be similar. However, the two leash chemistries have contrasting effects on the \( T_m \) where PS modifications decrease the \( T_m \) while 2OMe modifications enhance binding to the target (25). Thus, the \( T_m \) of leash 10 should be slightly higher than that of leash 2. The use of mixed PO/PS structural designs proved effective in delivering the morpholino AO to the cell nucleus. When the morpholino was annealed to mixed backbone leashes (leashes 3, 6 and 8), exon 23 skipping was consistently shown to be induced at doses as low as \( 10 \text{ nM} \). In assessing the mixed backbone leashes, the presence of the non-complementary overhangs also appeared to be an important factor. It is interesting to note a report that leash 5’ overhangs increase susceptibility to nucleases (15), despite the accepted convention that most exonuclease degradation of AOs occurs in the 3’–5’ direction (26,27). Leashes 4 and 5 were of identical nucleotide composition to leash 3 and differed only by either an FITC or cholesterol group at the 3’ terminus, respectively. The addition of either FITC or cholesterol moiety appeared to lower the efficiency of exon skipping after transfection of the morpholino:leash lipoplex when compared with leash 3.

The detection of dystrophin protein induced by the \( \psi M23D(07–18) : \) leash 3 lipoplex is one of only a few reports to date describing \( \text{in vivo} \) results achieved with AO molecules designed to induce dystrophin exon skipping and the first using the morpholino chemistry. The TA muscle fibres positively stained by the dystrophin monoclonal antibody are not likely to be revertant fibres, since the latter usually only occur as single fibres or small clusters (28). Furthermore, the protein detected by western blotting was near full-length, and presumed to be missing only the 71 amino acids encoded by exon 23 by extrapolation from the \( \text{in vivo} \) RT–PCR assay. Studies suggest that revertant fibres skip multiple exons at a time (29) and they occur at levels generally too low to be detectable by western blotting (30,31). The weaker diffuse dystrophin immunostaining on sections of muscle treated with the morpholino:leash lipoplex after only 2 days most likely reflects the time required to transcribe and translate the induced exon 23 skipped dystrophin mRNA and the subsequent localization of the dystrophin. The nuclease resistance associated with the morpholino chemistry (20) and the stability of the induced dystrophin mRNA transcript are likely to be a major factors contributing to the continued presence of the induced transcript 2 weeks after treatment \( \text{in vivo} \).

In some cases, restoration of the dystrophin-associated protein complex has been shown to be insufficient to reverse or prevent the pathological process in muscular dystrophy (32). Therefore, further tests in addition to immunohistochemical localization are needed to demonstrate restored function, although no convention currently exists for either defining the minimum number of tests to prove functionality or to provide a relative measure between groups (33,34). Our purpose was to demonstrate the potential and feasibility of using morpholino AO:leash lipoplexes to induce exon 23 skipping and protein restoration, thus experiments to ascertain function are beyond the scope of this report.

For clinical applications of AOs as a potential therapy for DMD, AO delivery needs to be efficient, tissue specific (if possible) and have negligible side-effects. Our results, coupled with the chemical properties of the morpholino structural type (12), suggest that morpholino AOs may be well suited for therapeutic induction of exon skipping. Furthermore, when annealed to leash 3 or 8, the levels of induced dystrophin protein were higher than those observed after transfection of an equivalent dose of 2OMe PS M23D(02–18). However, as the target sites of the two AOs differ by five nucleotides, in addition to the chemistries, generalizations regarding the inferiority of the morpholino over the 2OMe PS chemistry are purely speculative at this stage. We are expanding this work by directly comparing a range of chemistries at the target sites.
to further explore the relationship of chemistry and target site sensitivity (B.L. Gebski, S. Fletcher and S.D. Wilton, manuscript in preparation).

Morpholino AOs have been previously reported to be able to restore correct β-globin splicing in erythroid cells from thalassemic patients (35). However, concentrations as high as 45 μM were required when the morpholino was delivered by a syringe-loading method to suspension cultures of mononuclear cells (equivalent to scrape loading) (35). Similarly, high doses of morpholino were required over longer periods in order to produce the same effect after free uptake of morpholino AO in a related cell line (14). Delivery of morpholino AOs into cultured HeLa cells, when annealed to a DNA leash and complexed with the weakly basic molecule ethoxylated polyethylenimine (EPEI), resulted in improved activity (15). When comparing this delivery system to the conventional scrape loading method at the same dose, 1 μM of the morpholino : DNA hybrid complexed with EPEI yielded a 10-fold increase in luciferase activity (15). These reports serve to emphasize the benefits of using a leash to enhance delivery of a morpholino AO. Although the use of greatly reduced AO doses may be preferable in a clinical setting, the requirement for delivery reagents contribute their own disadvantages, such as potential toxicity (36,37) and/or modified tissue distribution and potential serum and cell interactions (36). Exploring the use of various chemical structural types and/or modifications to AO design should ultimately lead to compromises where optimal efficiency, stability and safety of AOs and delivery agents can be achieved.

This is the first study in which morpholino AOs have been used to restore gene function in the mdx mouse model of DMD, both in vitro and in vivo. Data presented here has demonstrated that efficient exon 23 skipping and restoration of protein synthesis can be induced by a morpholino AO, ψM23D(−07−18), when annealed to a range of leashes and delivered as lipoplexes. Exploration of leash design resulted in improved delivery of a morpholino AO, such that effective doses were several orders of magnitude lower than those reported by others (14,15,35).

While leash design will obviously be restricted to complementary regions of the morpholino AO, more important factors appear to be the length of the leash and non-complementary overhangs at one or both termini. It has been reported that the activity associated with the non-complementary overhangs was sequence-independent (15). Since the sequence of the overhangs in this study were different from those used by others, it is most likely that this approach can be readily applied to a wide range of morpholino AOs directed at other gene transcripts. Morpholino AOs thus appear favourable for future in vivo studies as they have also been reported to be stable in serum and plasma.

**MATERIALS AND METHODS**

**Morpholino AO design and synthesis**

Morpholino AOs were purchased from Gene Tools, USA and were HPLC-purified. Previous studies indicated that the 2OMe AO M23D(−02−18) directed against the 5′ donor splice site of intron 23 was the most efficient AO tested for inducing exon 23 skipping (10). The sequence of this AO was supplied to Gene Tools, who recommended that the morpholino AO be at least 25 bases in length. The morpholino AO was redesigned such that it extended five bases further into exon 23 (Fig. 1A) and had the coordinates M23D(+07−18) according to previously described nomenclature (10). We have used the prefix ‘ψ’ throughout to indicate the morpholino chemistry. For fluorescence studies, we also employed the Gene Tools supplied FITC-labelled control morpholino AO (5′-CCTTTACCTCA-GTTACAATTATA-3′; see www.gene-tools.com/Antisense/body_products.HTML#StandardControl). The morpholino AO was resuspended in 600 μl of distilled water under sterile conditions to produce a stock concentration of 500 μM and stored at −20°C. Stock solutions were warmed to room temperature, and then pre-heated to 65°C for 5 min before being added to the annealing mixture.

**Design and synthesis of oligonucleotide leashes**

All leash oligonucleotides were synthesized on an Expedite 8909 (Applied Biosystems) using a modified 1 μmol thiocarbamate synthesis protocol. Leashes 10, 11 and 12 were synthesized as 20Me PS and HPLC-purified by Geneworks (Adelaide, Australia). All leash sequences, chemistries and annealing positions relative to ψM23D(+07−18) are indicated in Figure 1B.

**Annealing of the morpholino AO to the leashes**

The morpholino AO was annealed to the leashes according to the method of Braasch and Corey described for preparing PNA : DNA complexes (38). Briefly, all morpholino : leash working solutions were prepared at a final concentration of 60 μM. Each solution contained the morpholino and respective leash at a 1 : 1 (mol : mol) ratio using 6 μl of 500 μM morpholino stock solution. The volume of leash added to each mix depended on the individual leash concentration. For all annealing reactions, 12.5 μl of 10×PBS (pH 7.4) was added to a microfuge tube resulting in a final concentration of 2.5×PBS in 50 μl. Tubes were incubated in an MJ research thermal cycler equipped with a hot bonnet according to the following temperature profile: 95°C for 5 min, 85°C for 1 min, 75°C for 1 min, 65°C for 5 min, 55°C for 1 min, 45°C for 1 min, 35°C for 5 min, 25°C for 1 min and 15°C for 1 min. Working stocks were stored at 4°C.

**Confirmation of successful annealing of morpholino AO to leashes**

To confirm that the leash oligonucleotides had annealed to the morpholino AO, complexed samples were assessed for a mobility shift after non-denaturing polyacrylamide gel electrophoresis. Samples were prepared by mixing 1 μl of the morpholino : leash complex, 2 μl of PBS and 1 μl of water. Samples were then incubated at 37°C for 30 min. Following incubation, 1 μl of 5× glycerol loading buffer was added and samples were electrophoresed through a 19 : 1 20% polyacrylamide non-denaturing gel in TBE running buffer at 175 V for 100 min. Gels were stained with Sybr Gold (Molecular Probes,
OR, USA) and photographed under UV light trans-illumination using a Kodak ID 2.0 gel documentation system.

**Cell culture and transfection**

H-2K<sup>+</sup>-tsA58 (H-2K) mdx cells were cultured as described previously (9,10). Morpholino : leash duplexes were delivered to the nucleus of the cells with Lipofectin (Invitrogen, Melbourne, Australia) at the ratio of 2 : 1 Lipofectin : AO (9,10). The amount of Lipofectin used to deliver each morpholino : leash complex was calculated on the w : w ratio of Lipofectin : leash, as it is the charged leash that interacts with Lipofectin. When delivering AOs of the 20Me PS chemistry using Lipofectin, lipoplexes were formed on the basis of 2 : 1 (w : w) ratio of Lipofectin : AO as previously described. All lipoplexes were prepared in serum-free OptiMEM (Invitrogen) according to the manufacturer’s instructions, to a final transfection volume of 500 μl/well of a 24-well plate. Since morpholino : leash complexes were always formed at a 1 : 1 mol : mol ratio, the concentration indicated is a measure of each component.

**RNA extraction, RT–PCR analysis**

RNA was extracted using RNA Bee (Tel-Test, Friendswood, TX, USA) 24 h after transfection as described previously (9). For persistence studies, RNA was extracted 24 h after transfection, then on days 4, 7 and 10. RT–PCR analysis was performed as described (9).

**Protein extraction and western blots**

Protein extraction and western blots were performed as described previously (9,10). All in vitro samples were loaded on a denaturing 4–8% gradient gel after normalization for myosin heavy chain as described.

**In vivo treatment and immunohistochemistry**

Twenty-one-day-old C57Bl/10ScSn mdx mice were given a single 1 μg dose of 9'M23D(±07–18) : leash 3 duplex complexed with Lipofectin at a 2 : 1 (w : w) Lipofectin : leash ratio in a 10 μl volume of phosphate buffered saline. The left tibialis anterior (TA) muscle was injected with the morpholino : leash lipoplex while the right was injected with phosphate buffered saline. Lipoplexes were prepared in saline as previously described (9). Mice were sacrificed either 2 days or 2 weeks after treatment. Injected and contralateral muscles were dissected, embedded in OCT compound (Tissue-Tek, Sakura Finechemicals, Tokyo, Japan) and snap frozen in liquid nitrogen-cooled isopentane and stored at −80°C. Serial 10 μm frozen sections were adhered to silanated slides for immunohistochemistry or collected in tubes for either protein or total RNA studies as described above. Immunohistochemistry was performed using the Mouse-on-Mouse kit with Texas Red substrate according to the manufacturer’s instructions (Vector Laboratories, Burlingame, USA) with the monoclonal antibody DYS2 (1:30; Novoceastra, Newcastle-upon-Tyne, UK). Fluorescence was visualized with an Olympus IX70 microscope and images recorded with an Olympus DP11 digital camera.

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**REFERENCES**


