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Review Article

Antisense oligonucleotides for the treatment of cardiomyopathy in Duchenne muscular dystrophy

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Abstract: Duchenne muscular dystrophy (DMD) is an X-linked recessive fatal neuromuscular disorder characterized by progressive muscle degeneration which affects one in 3500-5000 males born worldwide. DMD is caused by loss-of-function mutations in the *dystrophin* (*DMD*) gene encoding for dystrophin, a cytoskeletal protein that supports the structural integrity of myofibers during cycles of muscle contraction and relaxation. DMD patients do not only experience skeletal muscle deterioration but also severe cardiomyopathy, which is recognized as the current leading cause of death for the disease. Among the therapies being developed, exon skipping using antisense oligonucleotides (AOs) is one of the most promising approaches. AOs effectively restore dystrophin expression in skeletal muscles; however, they are highly inefficient in the heart due to endosomal entrapment. Improving skeletal muscle function without restoring dystrophin expression in cardiac tissue may exacerbate cardiomyopathy due to increased voluntary activity. This review consolidates the preclinical antisense approaches to improve dystrophin restoration, with a special focus on the heart.

Keywords: Duchenne muscular dystrophy, dystrophin, cardiomyopathy, antisense therapy, morpholinos, drug delivery

Introduction

Duchenne muscular dystrophy (DMD) is an X-linked recessive disorder characterized by progressive muscle degeneration [1]. The prevalence of DMD is estimated to be 1 in 3500-5000 live male births, making it the most common lethal neuromuscular disorder [2, 3]. Patients with DMD generally stay asymptomatic at birth although they could show signs of delayed gross motor development compared to their peers [4-6]. The disease progresses rapidly, with muscle weakness and wasting observed first in the proximal muscles then extending to the more distal muscles [7]. Affected boys often lose ambulation by the age of 12 and experience multiple organ system dysfunction such as neuromuscular scoliosis, joint contracture, osteoporosis, restrictive lung disease, obstructive sleep apnea, cardiomyopathy, and psychological problems [8-10]. The age of onset and the rate of decline are highly variable among patients. Due to continuous muscle damage, patients with DMD have markedly elevated serum levels of creatine kinase (CK), even at birth [11]. Death usually occurs in their 20s due to respiratory or cardiac complications [11-14].

DMD is caused by mutations in the *DMD* gene, which encodes a membrane-associated protein called dystrophin. Dystrophin has 4 domains: an actin-binding N-terminal domain, a rod domain consisting of 24 spectrin-like repeat motifs for structural flexibility, a cysteine-rich domain for facilitating protein-protein interaction, and a C-terminal domain for binding sarcolemmal proteins [15, 16]. As a member of the dystrophin-glycoprotein complex (DGC), dystrophin functions to link the actin cytoskeleton of muscle cells to the extracellular matrix, providing mechanical support and membrane stabilization during muscle contraction and relaxation cycles [15, 17-20]. In the absence of dystrophin, muscle fibers experience mechanical stress and become susceptible to tearing and frag-

mentation, resulting in degeneration [21, 22]. Other studies have suggested that dystrophin also serves non-mechanical roles [23].

The *DMD* gene is located on the short arm of the X chromosome (Xp21.3-p21.2) spanning 2.4 Mb with 79 exons which produces a 14 kb transcript [24-26]. The *DMD* gene has 3 main promoters that produce full-length dystrophin (M, B, and P), with other promoters responsible for the transcription of different, smaller isoforms [27, 28]. The M promoter produces the Dp427m isoform which is expressed in cardiac and skeletal muscle, the B promoter produces the Dp427c isoform which is expressed in neurons of the cortex and the Cornu Ammonis (CA) region of the hippocampus, and the P promoter produces the Dp427p isoform which is expressed in the Purkinje cells of the brain. Other shorter isoforms include Dp260 expressed in the retina, Dp140 expressed in the kidney and brain, Dp116 expressed in the peripheral nerves and Dp71 which is ubiquitously expressed [29-33].

DMD is considered one of the largest genes in the human genome. Furthermore, it is located in a genomic region with high rates of recombination [34-36]. Due to its length and location, *DMD* is highly susceptible to mutation. Approximately 60% of DMD cases are due to deletions of one or more exons, ~6% due to duplications, and the rest due to small insertions/deletions or point mutations [11, 16, 37, 38]. These mutations usually disrupt the reading frame or introduce a premature stop codon, both of which lead to the absence of dystrophin. There is no significant correlation between mutation size and disease severity [39-41]. Most mutations that maintain the *DMD* reading frame produce truncated yet partly functional dystrophin, and often result in a milder form of the disease known as Becker muscular dystrophy (BMD) [42-44]. Although the skeletal muscle symptoms are less severe, the majority of BMD patients also develop cardiomyopathy, and it is the leading cause of death in patients with BMD [45].

Cardiomyopathy in DMD patients

The life expectancy for patients with DMD used to be less than 20 years of age just two decades ago. Due to advancements in ventilator support and spinal surgery, the average age of mortality has been pushed into the late 20s [12, 13]. Since respiratory dysfunction is now better managed, cardiomyopathy is presently the leading cause of death among DMD patients. Cardiomyopathy is estimated to present in 25% of patients at the age of 6, and 59% of patients at the age of 10 [27, 46]. The majority of patients will have developed cardiomyopathy by 18 years of age. Recognition of signs and symptoms of cardiac dysfunction can be challenging since DMD patients are usually wheelchair-bound and do not perform increased cardiac workload [47]. The correlation between a patient's genotype and the severity of their cardiac phenotype remains uncertain [48-52].

The earliest clinical signs of cardiomyopathy are either decreased systolic function or sinus tachycardia, with the latter commonly seen in teenage DMD patients [53, 54]. Sinus tachycardia results in elevated heart rate, which increases the workload of the already deteriorating dystrophic myocardium [55-57]. Arrhythmias are a common cardiac involvement in patients with DMD and are significantly correlated with decreased heart function. Approximately 44% of DMD patients show signs of arrhythmias [58]. Supraventricular tachycardia (SVT) and ventricular tachycardia (VT) are observed in 10% of individuals with DMD [58]. Although DMD cardiomyopathy is routinely described as dilated cardiomyopathy (DCM), patients with decreased systolic or diastolic function do not necessarily show ventricular dilation [49, 59, 60]. Additionally, myocardial fibrosis usually precedes left ventricular dysfunction (LVD) [61, 62]. The age of onset and the rate of progression are variable for LVD. However, patients with onset of LVD before 18 years of age were found to have a significantly shorter lifespan compared to those who had LVD after 18 years old [48]. Other features of DMD cardiomyopathy include abnormal cardiac conduction (as caused by, for example, vacuole degeneration in the Purkinje fibers), congestive heart failure and sudden cardiac arrest [27, 28, 47, 63-66].

Myocardial issues in DMD patients are unavoidable since dystrophin serves the same functions in cardiomyocytes as in skeletal muscle cells [67]. In the absence of dystrophin, cardiomyocytes experience increased structural vulnerability and are susceptible to membra-

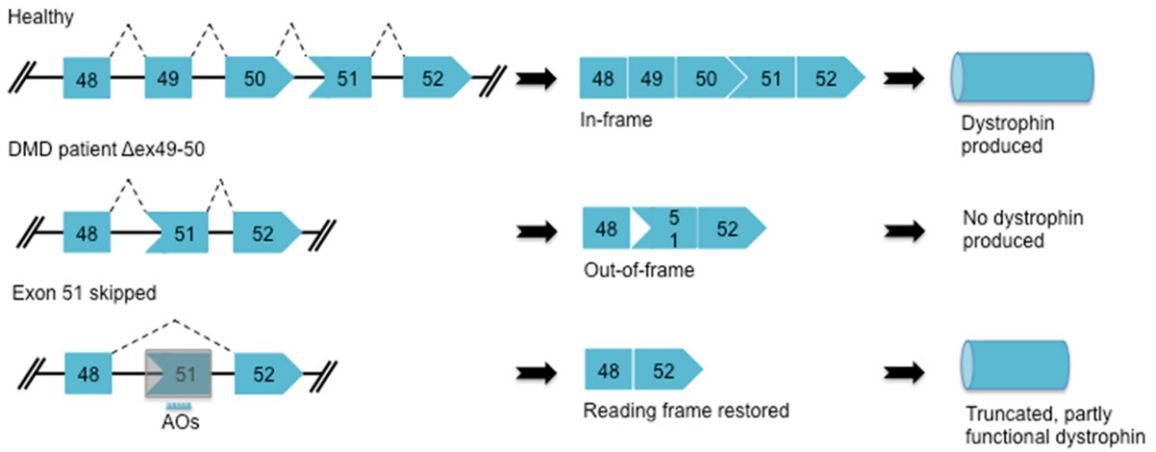

Figure 1. Antisense-induced exon skipping in DMD. Deletion of exon 49 and 50 in the DMD gene creates a frame-shift mutation which results in a premature stop codon and no dystrophin produced. In the case shown, AOs can specifically bind to exon 51 and interfere with the splicing machinery to exclude exon 51 from the mature mRNA, thereby restoring the reading frame and producing truncated but partly functional dystrophin.

ne instability and tearing [22]. Cardiomyocyte remodeling occurs secondary to myocardial wasting, which leads to ventricular enlargement and fibrosis [68]. As previously mentioned, affected hearts do not always show signs of ventricular dilation. Another consequence of myocardial tearing and fragmentation is the dysregulation of ion influx/efflux in the heart [69, 70]. Notably, damages to the myocardial membrane disrupt Ca^{2+} homeostasis. Since Ca^{2+} acts as a second messenger in many cellular pathways, Ca^{2+} dysregulation results in multiple downstream abnormalities within myocardial tissue. One significant consequence of Ca^{2+} dysregulation includes increased reactive oxygen species (ROS) production and mitochondrial dysfunction. Elevated Ca^{2+} levels within cardiomyocytes activate the mitochondria to produce ROS which leads to apoptosis and further muscle wasting [69-79]. An important second messenger affected by Ca^{2+} dysregulation is nitric oxide (NO) which has roles in blood flow and vasoregulation [80, 81]. Increased intracellular Ca^{2+} concentrations also enhance the risk of Ca-dependent arrhythmias and cellular Ca^{2+} overload in DMD patients [69].

Cardiomyopathy in DMD patients is currently not curative. Therapies commonly used include: corticosteroids, angiotensin-converting enzyme inhibitors (ACEIs), angiotensin II receptor blockers (ARBs), beta-adrenergic receptor blockers, mineralocorticoid receptor antagonists and other interventions such as ventricular assist devices (VADs) or cardiac transplantation [9, 47, 82-92]. Treatment with corticosteroids has been shown to improve cardiac function. DMD patients who received steroids such as prednisolone/prednisone or deflazacort had significantly delayed onset of cardiomyopathy, less ventricular dilation and systolic dysfunction than those who did not. However, patients using corticosteroids can experience some long-term complications such as weight gain, bone fractures, cataracts, etc. [9, 10, 93-96], which limits their use for therapy. ACEIs and ARBs have proven effective in reducing left ventricular hypertrophy and fibrosis. Furthermore, initiation of beta-adrenergic receptor blocker treatment along with ACEIs has been demonstrated to improve left ventricular systolic function in DMD patients [88, 91, 97]. However, most of these studies have their inherent limitations, which makes it challenging for data interpretation as well as treatment application in clinical settings.

Antisense oligonucleotides for the treatment of DMD

At present, there is no cure for DMD. Current treatments are mostly palliative, aiming at alleviating the symptoms of the disease [11, 14]. Several promising approaches have been investigated such as utrophin up-regulation, viral gene therapy, cell-based therapy, antisense oligonucleotides (AOs) for exon skipping and,

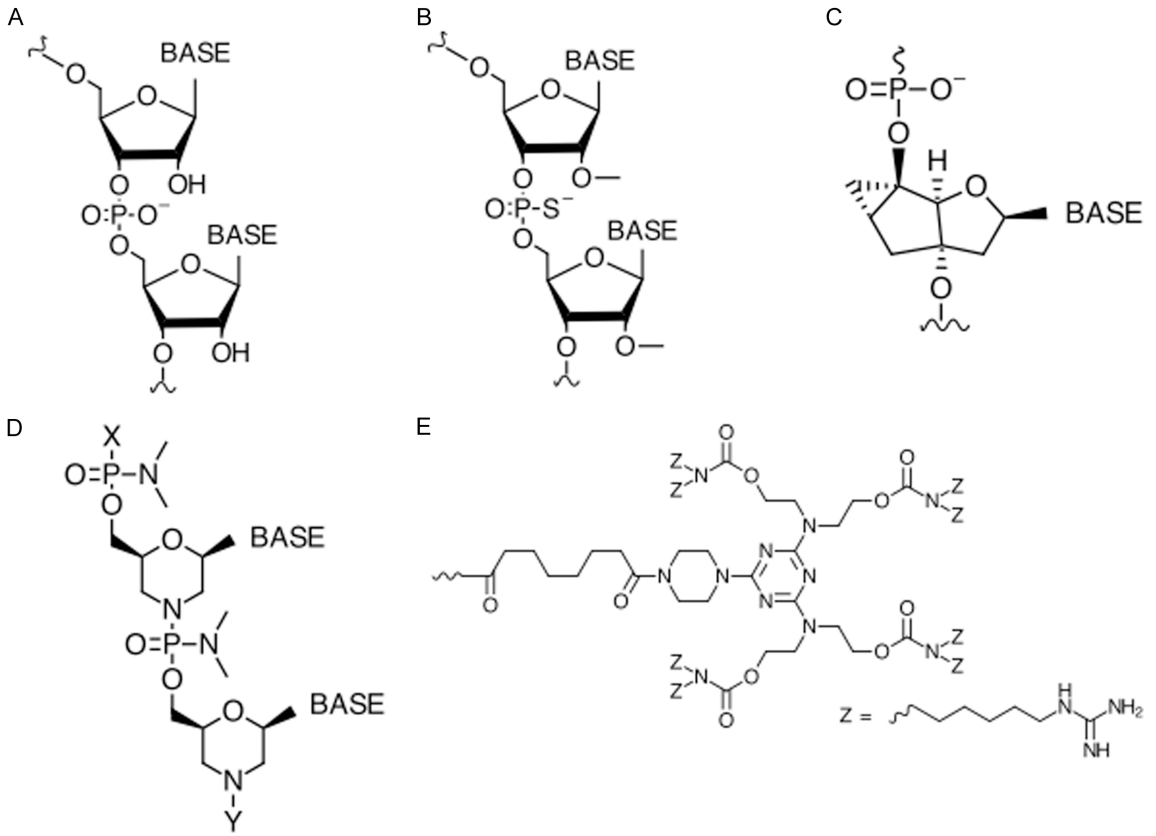

Figure 2. Chemical structures commonly used in antisense therapy. (A) RNA, (B) 2'-OMe-phosphorothioate, (C) Tricyclo-DNA, (D) Phosphorodiamidate morpholino oligomers. X or Y can be a peptide; Y can be an octaguanidine moiety (E) as seen in Vivo-morpholino.

most recently, CRISPR-Cas9-mediated gene editing [69]. In this review, we will discuss the use of AOs in the treatment of DMD with a specific focus on treating the heart.

AOs are short, synthetic nucleic acid sequences which bind to complementary target mRNA sequences and lead to either endonuclease-mediated transcript knockdown or splice modulation [98, 99]. AO-mediated exon skipping can correct the reading frame by removing an out-of-frame exon or exons from the *DMD* pre-mRNA, producing a truncated but partly functional dystrophin protein [100-102] (**Figure 1**). The first generation of AOs has the unmodified phosphoribose backbone, making them highly susceptible to degradation by nucleases [103, 104] (**Figure 2**). Due to their short half-life, first generation AOs rarely achieve sufficient intracellular concentrations to have a therapeutic effect [105, 106]. Second and third generation AOs contain chemically modified structures. These modifications not only increase AO resistance to nuclease degradation, but also enhance their pharmacological properties. Alterations at the 2' position of the ribose sugar have yielded a class of AOs with improved safety and efficacy profiles. 2'-*O*-methyl (2'-OMe) and 2'-*O*-methoxyethyl (2'-MOE) alkyl substituents are the most studied members of this group. In addition, the use of a phosphorothioate backbone which contains a sulfur in a non-bridging location on the phosphate backbone significantly improves AO nuclease resistance and binding affinity to serum proteins [98, 107-109]. Antisense chemistries that contain both a phosphorothioate backbone and a 2'-alkyl substituent, such as 2'-OMe-phosphorothioate (2'OMePS) AOs, have shown several favorable properties compared to their unmodified counterparts [105, 110-113]. The phosphodiester backbone can also be replaced with a polyamide structure made up of repeating N-(2-aminoethyl) glycine units, which has given rise to another class of AOs termed peptide nucleic acids (PNAs) [114]. This new class of AO can

sterically block splicing factors, inhibit ribosome recruitment and bind non-coding RNAs, thereby further expanding therapeutic potential [69].

Among these next-generation chemistries, phosphorodiamidate morpholino oligomers (PMOs) represent the most advanced use of antisense therapy for DMD. PMOs have the deoxyribose/ribose moiety replaced by a morpholine ring, and the charged phosphodiester intersubunit linkage replaced by an uncharged phosphorodiamidate linkage, making PMOs nuclease-resistant and charge-neutral [115, 116]. One of the challenges with using nucleic acid-based molecules as therapeutics is their stability and potential toxicity in cells. The advantage of being charge-neutral, as in the case of PMOs, is that this imparts an even greater resistance to nucleases, which typically target charged molecules. Additionally, due to the lack of charge, PMOs are safer since they are unlikely to activate Toll-like receptors, a class of receptors involved in producing innate immune responses against pathogenic material [117]. In fact, a PMO targeting *DMD* exon 51, called eteplirsen or Exondys 51 (Sarepta Therapeutics, Cambridge, MA, USA), was conditionally approved by the U.S. Food and Drug Administration (FDA) in 2016, and several PMOs targeting other *DMD* exons, including golodirsen or SRP-4053 (Sarepta Therapeutics) and NS-065/NCNP-01 (NS Pharma, Paramus, NJ, USA) are currently undergoing clinical trials [118-121].

Although AOs effectively rescue dystrophin expression in skeletal muscles, they are highly inefficient in cardiac muscles [122, 123]. Failure to restore dystrophin expression in the heart in the presence of rescued dystrophin in skeletal muscles can exacerbate cardiac issues due to increased patient voluntary activity [124, 125]. The major barrier to effective use of AOs in therapy is the delivery of antisense drugs to their intracellular targets. Upon reaching the cell surface, AOs are internalized by endocytosis. A variety of cell-surface receptors have been suggested to bind AOs and facilitate their entry into the cell including integrins, scavenger receptors and Toll-like receptors [126-128]. Regardless of the endocytosis route taken, AOs entering a cell will encounter an intricate network of membrane compartments that include early and recycled endosomes, late endosomes and multi-vesicular bodies (MVBs), and lysosomes. Most AOs are initially delivered to early endosomes. Subsequently, they can be trafficked to lysosomes for degradation or sequestered in late endosomes/MVBs. Antisense drugs trapped within endomembrane compartments are pharmacologically inactive as they remain separated from their target sites by membrane barriers [106, 129]. Nevertheless, a small portion of AOs escapes endosomes and only then can they reach their targets and become pharmacologically active. The concept of endosomal escape has been widely accepted as the most important barrier to the effective use of antisense drugs [127, 130]. Importantly for developing heart-effective AOs, the research found that AOs (particularly PMOs) tend to be trapped in endosomes in cardiomyocytes more than in skeletal muscle cells [131]. Since cardiomyopathy is the leading cause of death in DMD patients, researchers have looked into different approaches to enhance delivery of AOs to cardiac tissues. The next section will discuss some of the most promising approaches to improving AO efficacy for treating the heart.

Strategies to improve the efficacy of antisense drugs for the treatment of cardiomyopathy in DMD patients

Tricyclo-DNA

In the late nineties, a novel class of AOs known as tricyclo-DNAs (tcDNAs) was shown to display unique pharmacological properties. tcDNA is a conformationally-constrained DNA analog that deviates from the natural DNA structure by the presence of an ethylene bridge connecting C3' and C5' [132-134]. tcDNA shows enhanced RNA affinity and nuclease resistance, as well as the inability to elicit RNase H activity [135-138]. Furthermore, tcDNAs can spontaneously form defined nanoparticles which potentially improves delivery and cellular uptake compared to other antisense chemistries [139-141].

In a study by Goyenvalle and colleagues, a mouse model carrying a nonsense mutation in exon 23 of the *Dmd* gene (hereafter referred to as *mdx* mice) was injected intravenously with a 15 nucleotide long (15-mer) tcDNA-AO that targets the skipping of *Dmd* exon 23 [139]. Treatment with 200 mg/kg/week of the drug

for 12 weeks rescued up to 40% of wild type (WT) dystrophin expression in the heart. Additionally, tcDNA-treated mice showed significantly higher levels of dystrophin rescue, especially in the diaphragm and the heart, compared to those treated with PMO and 2'-OMe AOs at an equimolar dosage. Similar results were observed in a dystrophin/utrophin double knockout mouse model where treatment with weekly intravenous (IV) injections of 200 mg/kg of tcDNA over a period of 5-20 weeks restored 53% of WT dystrophin levels in the heart. Treatment with tcDNA also ameliorated cardiac systolic function as demonstrated by improved ventricular ejection fractions and shortening fractions in echocardiography.

One of the advantages of tcDNA is its increased RNA binding affinity. Due to this feature, shorter tcDNAs can be designed, reducing the mass of AOs administered and potential AO-induced toxicity while retaining their therapeutic effect. To investigate the efficacy of shorter tcDNAs, *mdx* mice were injected intravenously with a 13-mer tcDNA targeting *Dmd* exon 23 at 200 mg/kg/week for 12 weeks. The 13-mer tcDNA induced particularly high levels of exon skipping and restored dystrophin in the heart as shown by RT-PCR, Western blotting and immunostaining. The safety profile of the treatment also showed that tcDNAs were well-tolerated, with only minimal renal toxicity and liver inflammation observed [138]. Although tcDNAs have the potential to rescue dystrophin in cardiac tissue, treatment with these AOs requires high dosage and multiple dose administrations.

Peptides

Among the various delivery systems studied, cell-penetrating peptides (CPPs) have gained the most considerable interest. CPPs are composed of cationic and/or amphipathic amino acids that are capable of delivering a wide range of nucleic acid-based molecules both *in vitro* and *in vivo* [142-144]. These peptides either possess natural translocating properties or were engineered by combining different protein domains to have these properties [145]. Many studies have investigated peptide-conjugated AOs for the potential treatment of DMD. This review will discuss some of the most promising approaches, namely: arginine-rich peptides, B peptides, PNA/PMO internalization peptides (Pips), and phage peptides. Not all experiments are mentioned in this paper, but a selected few will be discussed for each approach.

The $(RXR)_4$ peptide (with R standing for arginine and X standing for aminohexanoic acid) was the first arginine-rich CPP tested in the *mdx* model of DMD [146]. In a study by Yin and colleagues, $(RXR)_4$ peptides were conjugated to PMOs targeting *Dmd* exon 23. A single IV injection at 25 mg/kg via tail vein resulted in ~50% exon 23 skipping in the heart of treated mice. A widespread, uniform distribution of dystrophin-positive fibers was also observed in cardiac tissue as shown by immunostaining. Western blot analysis demonstrated dystrophin restoration at levels between 10 and 20% of that found in the WT mouse heart. Even at lower doses administered (weekly injections at 6 mg/kg for 3 weeks), dystrophin restoration was still observed in cardiac tissue as shown by immunostaining and Western blotting.

Subsequently, another arginine-rich peptide, named the B-peptide, demonstrated high levels of exon skipping in various muscles including the heart. The B-peptide contains arginine, aminohexanoic acid and beta alanine (abbreviated B) in an $(RXRRBR)_2$ sequence [147-149]. A single IV injection at 30 mg/kg of B-peptide-conjugated PMO (PPMO) restored 94% of dystrophin expression in cardiac muscle fibers of treated *mdx* mice by immunohistochemistry, 50% of WT dystrophin levels by Western blot, and 63% exon 23 skipping by RT-PCR. Repeated treatment for 3 months at the same dose at biweekly intervals further improved dystrophin restoration in cardiac muscle. Skipped transcript levels increased to 72%. Immunohistochemistry and Western blot demonstrated dystrophin rescue at levels comparable to that in WT hearts. Dystrophin restoration was also observed in the muscles of the atria and large vessels such as the aorta or vena cava and the pulmonary arteries. PPMO treatment significantly improved cardiac function and prevented cardiac failure under dobutamine stress [150]. A PPMO cocktail was also shown to restore dystrophin expression in the myocardium and cardiac Purkinje fibers, and improve cardiac conduction abnormalities in a canine model of DMD [151].

Another promising class of CPPs is the Pips. Pips are a novel series of transduction peptides

developed from mutagenesis and functional studies of a derivative of Penetratin, with the six arginine residues added to the N terminus of the CPP (R6Pen) [152]. Penetratin is originally derived from the homeobox peptide of Drosophila Antennapedia. The CPPs in the Pip series are characterized by a central hydrophobic motif anchored on each side by arginine-rich sequences similar to that of the B-peptide which contains arginine, aminohexanoic acid, and beta-alanine residues. Among the highly efficient members of this series are Pip5 and Pip6, both of which have demonstrated high levels of dystrophin restoration in various muscles including the heart [153, 154]. A single IV injection of 25 mg/kg of Pip5e-PMO (a member of the Pip5 family) induced almost complete exon skipping in cardiac tissue where full length uncorrected transcript was barely detectable. Immunohistochemistry data revealed more than 90% of dystrophin-positive fibers in cardiac muscle. Dystrophin restoration was further confirmed by Western blot which revealed more than 50% of WT dystrophin levels in the heart [153]. Modifications to the central hydrophobic core of the Pip5 peptide gave rise to a novel derivative of the Pip series, consecutively named Pip6. When directly compared to Pip5e-PMO treatment, a member of the Pip6 family exhibited significantly higher dystrophin restoration in the heart of treated mice at very low doses (12.5 mg/kg). The Pip6 series have also been demonstrated to prevent exercise-induced cardiomyopathy following Pip-PMO treatment in dystrophic mice [154].

While the above-mentioned CPPs work well for neutral AOs like PMOs, it is challenging to conjugate positively charged peptides to negatively-charged AOs such as 2'OMePS due to the presence of strong electrostatic interactions. In an effort to find uncharged CPPs, Jirka and colleagues screened a phage display peptide library and identified a 7-mer peptide (P4) that significantly enhanced AO uptake in cardiac tissue [155]. Dystrophic *mdx* mice were injected subcutaneously with 200 mg/kg/week doses of 2'OMePS-P4 conjugate targeting mouse *Dmd* exon 23 for 6 weeks. Treatment with peptide-conjugated AOs resulted in enhanced exon skipping efficacy with a significant difference in cardiac tissue compared to naked AOs, possibly due to improved uptake in cardiac muscle. The safety profile of the peptide conjugate was also evaluated and no significant changes were detected for liver and kidney damage. This result is encouraging since the phage peptides could be used as an alternative to arginine-rich peptides, which have shown toxicity in higher animals [156-158].

Octaguanidine morpholino

In an effort to improve the efficacy of AO systemic delivery and to reduce the immunogenicity of cell penetrating peptides, dendrimeric octaguanidine moieties have been conjugated to PMOs to form a new class of AOs named Vivo-Morpholinos or vPMOs [150, 159].

The guanidine groups in vPMOs work similarly to the arginine-rich CPPs found conjugated to PMOs. As such, vPMOs have been shown to induce high levels of dystrophin expression especially in the heart [149]. Additionally, the combination of a synthetic scaffold and multiple unnatural side chains makes vPMOs unlikely to elicit an immune response, thus allowing for multiple administrations to maintain adequate levels of dystrophin in the body [160].

vPMOs have been shown to rescue cardiac dystrophin expression *in vivo* [161]. A single IV injection of a vPMO targeting mouse *Dmd* exon 23 (Vivo-ME23) at 6 mg/kg resulted in a strong dystrophin signal in a significant number of cardiomyocytes in *mdx* mice. Repeated treatment at the same dose for five times biweekly rescued dystrophin expression in more than 40% of cardiomyocytes as observed by immunostaining. Western blot showed dystrophin rescue to approximately 10% of normal levels in cardiac muscles. vPMOs have also been shown to restore dystrophin expression in a canine model of DMD, however, no systemic injection was performed so whether vPMOs can rescue dystrophin in canine cardiac tissues remains to be determined [162]. Regardless, these encouraging results represent an important step forward in our effort to overcome the challenges associated with the delivery of AOs to the heart.

Ultrasound and microbubbles

Another promising approach for improving AO delivery to cardiac tissue is the use of ultrasound in combination with contrast-enhancing microbubbles [163]. Microbubbles are used to carry the drugs to an area of interest, then

ultrasound is applied to burst the microbubbles, resulting in tissue-specific delivery of the therapeutic materials [164-167]. Ultrasound exposure improves the efficacy of intracellular delivery due to a phenomenon known as sonoporation or cellular sonification. This effect creates transient pores in the cellular plasma membrane by a process known as inertial cavitation, thus allowing bioactive materials to enter the cells. Inertial cavitation is enhanced by using microbubbles of contrast agents [164, 167-170]. AOs can be mixed with or incorporated into microbubbles in a number of ways such as binding to the microbubble shell or using site-specific ligands. The combination of ultrasound and microbubbles not only improves intracellular delivery but also reduces the amount of drugs used systemically thereby lessening the potential toxicity and side effects of treatment.

In order to investigate the microbubble properties that are important for influencing drug delivery efficacy *in vivo*, PMOs targeting *Dmd* exon 23 of the *mdx* mouse were injected systemically at 16 mg/kg via the tail vein [171]. PMOs were incorporated into three different commercially available microbubbles: Sonazoid, Optison, and SonoVue. Ultrasound was then applied to the heart for two minutes after injections. Treated hearts were collected 24 hours or one-week post injection. Of the three microbubbles tested, Sonazoid and Optison induced significant amounts of dystrophin-positive fibers in cardiac tissues. Treatments with PMOs only (no ultrasound or microbubbles) were ineffective in terms of restoring dystrophin in cardiomyocytes. Microbubbles were also found to increase AO delivery to the heart in a dose-dependent manner. Histological analysis showed no signs of tissue damage or observable morphological differences between samples treated with ultrasound and microbubbles and non-treated controls. Additionally, microbubble stability was determined to be the major factor affecting the efficiency of therapeutic drug delivery.

Nanoparticles

In order to improve AO stability and enable the use of lower AOs concentrations *in vivo*, AO delivery using nanoparticles was developed and demonstrated to be another promising approach. Two classes of nanoparticles, T1 and ZM2, have been shown to restore dystrophin expression in the heart of *mdx* mice [172, 173].

T1 nanoparticles are a type of cationic core-shell nanospheres, made up of a core of polymethylmethacrylate (PMMA) and surrounded by a shell of cationic groups, which facilitates the binding of charged AOs. In a study by Rimessi and colleagues, T1 nanoparticles were demonstrated to deliver 2'OMePS AOs and induce dystrophin rescue in body-wide striated muscles. AOs targeting the boundary sequences of exon and intron 23 of mouse *Dmd* (M23D) were conjugated with T1 nanoparticles, and delivered by weekly intraperitoneal (IP) injections at 2.7 mg/kg to *mdx* mice [172]. T1 nanoparticles showed a wide distribution in the body, including the heart. At one week post-injection, immunohistochemical analysis of treated mice showed the presence of dystrophin-positive cardiomyocytes in various areas of the heart. Dystrophin was however undetectable in cardiac tissues 6 weeks after the last injection. Total and skipped dystrophin transcript levels in the hearts of treated mice were also significantly increased with 80% and 16% more transcripts, respectively. Although dystrophin was undetected in the heart by Western blot, the results were positive considering a significantly reduced dose compared to previous studies. From a safety aspect, T1 nanoparticles may cause adverse effects due to their slow biodegradability, indicating a possibility of accumulative toxicity for long-term treatments [172]. Also, T1 nanoparticles can form small aggregates and are therefore not suitable for IV injections.

To improve the efficacy of T1 nanoparticles, a more efficient class of nanoparticles termed ZM2 was designed [173]. ZM2 nanoparticles are composed of a PMMA core and a random copolymer shell consisting of units derived from N-isopropylacrylamide+ (NIPAM) and reactive methacrylate-bearing cationic groups. *mdx* mice were injected IP weekly for 7 weeks at 7.5 mg/kg with M23D AOs loaded onto ZM2 nanoparticles. Immunohistochemical findings showed 3% by manual count and 6% by semi-automated count of dystrophin-positive signal in the heart. Dystrophin was also detectable, though faintly, in the heart of ZM2-M23D treated mice via Western blot. However, dystrophin was not detectable in the heart at 3 months post-injection as demonstrated in a follow-up study.

Polymers

Different series of polymers were investigated for their ability to improve the delivery of AOs *in vitro* and *in vivo*. The first class is a series of low molecular weight polyethylenimine (LPEI) conjugated pluronic copolymers (PCMs) [174]. Amphiphilic pluronic, poly (ethylene oxide)-b-poly (propylene oxide)-b-poly (ethylene oxide) (PEO-PPO-PEO triblock copolymer) has been used widely as a pharmaceutical adjuvant and a free adjuvant for gene delivery. Depending on the molecular weight, hydrophilic-lipophilic balance (HLB), and other characteristics of the pluronics, PCMs can have different AO delivery efficiencies and toxicities. In a study by Wang and colleagues, *mdx* mice were injected intravenously with 0.4 mg of PCMs and 2 mg of PMOs targeting the boundary sequences of exon and intron 23 of mouse *Dmd* gene (PMOE23), or 2 mg of PMOE23 only as the control. Two weeks after injection, dystrophin was not detectable in cardiac muscle of control mice, while immunohistochemistry showed membrane-localized dystrophin in more than 5% of cardiomyocytes in some areas of the heart from treated mice. Treatment also demonstrated no detectable toxicity in muscles, liver, and kidneys after systemic administration.

Another class of amphiphilic polymers, termed Z polymers, is constructed from Tween 85 and LPEI [175]. Based on preliminary results, two Z polymers Z7 and Z8 were selected for systemic injection. Dystrophic *mdx* mice were treated by IV injection with 1 mg Z7/1 mg PMO complex, or 0.5 mg Z8/1 mg PMO conjugate, or 1 mg PMO as a control. Dystrophin expression was not detected in the PMO-only group, however, Z8-PMO treated mice demonstrated membrane-localized dystrophin-positive fibers in some areas of the heart. No adverse effects were observed in systemic delivery under the experimental conditions. The mechanism/s by which amphiphilic polymers improve gene transfer is/are not fully understood. Their hydrophobic interaction with cell membranes could reduce membrane viscosity and facilitate the entry of AOs [173]. Amphiphilic polymers could also prevent nonspecific binding of AOs to charged extracellular components, thus increasing the effective concentrations of therapeutic drugs.

Conclusion

To summarize, with the exception of tricyclo-DNAs, naked AOs are generally ineffective in treating the heart. Conjugation to CPPs, especially arginine-rich peptides and the Pip series, appears to be a promising approach to improve AO uptake and dystrophin restoration in cardiac tissue. However, the practicality of CPPs in the clinical setting remains to be determined largely due to concerns over our complex immune system. Phage peptides and octaguanidine moieties were developed in an effort to reduce the immunogenicity of arginine-rich peptides, however, they have been less successful in rescuing dystrophin in systemic delivery of antisense drugs to the heart. Other non-covalent conjugation approaches such as the use of nanoparticles, polymers, or ultrasound and microbubbles are promising strategies to enhance the efficacy of AOs in cardiac tissue, especially for charged antisense chemistries. With a growing number of AO-based approaches being investigated, antisense therapy holds the potential to improve the treatments for DMD patients, with special focus on cardiac aspects of the disease.

Tricyclo-DNA clearly appears as a promising chemistry for the antisense therapy of DMD-associated cardiomyopathy due to its unique pharmacological properties and high therapeutic potential. Besides their ability to improve delivery and uptake into cardiac tissue, tcDNAs can also cross the blood-brain barrier at low levels after systemic administration [139]. Along with other distinctive properties, this outstanding feature opens up more therapeutic options for other neuromuscular and neurodegenerative disorders such as spinal muscular atrophy and Huntington's disease [135]. Although the safety profile of tcDNAs has been evaluated, observed toxicities were ascribed more to the phosphorothioate linkages rather than the tcDNA chemistry itself [138]. More extensive toxicological studies are recommended to assess the full safety profile of this promising chemistry. Given its positive performance in the preclinical phase, the evaluation of tcDNAs in human clinical trials seems to be the most logical next step. Similar to other chemistries, the outcome essentially depends on how well tcDNAs will be tolerated in patients.

An active body of research on DMD has significantly broadened our knowledge on the pathology as well as potential therapeutic treatments for the disease. To this day, DMD is still an invariably fatal disease that affects the whole body. It is, therefore, essential to focus on treatments for individual systems such as the heart. Advances in the understanding of antisense therapy have provided a strong foundation for the transition of AOs drugs from the lab bench to the clinic. However, delivery and uptake in cardiac tissue remain the key limiting factor in the bioavailability of AO-based therapies for the treatment of DMD. Perhaps more basic research into understanding the underlying mechanism of these processes is needed to overcome this major obstacle. Up to this point, clinical trials have focused mostly on the efficacy of AOs treatments on skeletal muscle, without much emphasis on the heart. Given how cardiomyopathy is currently the leading cause of death in patients with DMD, an increased focus on treatments for the heart is very much needed. In 2017, a clinical trial investigating the efficacy of a peptide-conjugated version of eteplirsen was initiated. The trial is expected to be completed in January 2019. With the promising results shown by peptide conjugated AOs in pre-clinical studies, it would be exciting to see the therapeutic outcome of the trial, particularly the cardiac aspects of it. Hopefully, these efforts will facilitate the progress of therapeutic development so that one day DMD will no longer be as devastating a disease as it is now.

Disclosure of conflict of interest

None.

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