Title
Therapeutic approaches for Duchenne muscular dystrophy

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Abstract

Duchenne muscular dystrophy (DMD) is a monogenic muscle-wasting disorder and a priority candidate for molecular and cellular therapeutics. While rare, it is the most common inherited myopathy affecting children, and so has been the focus of intense research activity. DMD can therefore be considered a pioneer disease for which a plethora of drug development programmes are currently underway, including exon skipping, stop codon read-through, gene replacement, cell therapy, and gene editing, among others. These efforts have led to marketing approval of four exon skipping antisense oligonucleotides and one stop codon read-through drug, with other approvals likely just over the horizon. Here we discuss the various therapeutic strategies that have been deployed to treat DMD, with a focus on therapies that aim to restore the function of the dystrophin protein. Lessons from these drug development programmes are likely to have a major impact on the DMD field, but also on molecular and cellular medicine more generally. Thus, DMD is at the forefront of future drug discovery efforts, with these experimental treatments paving the way for the development of therapies for other indications using similar mechanisms-of-action.

Keywords

DMD; Duchenne muscular dystrophy; dystrophin; exon skipping, micro-dystrophin

[H1] Introduction

DMD is a genetic muscle-wasting disease (X-linked recessive) and the most common inherited paediatric myopathy, affecting 1 in 3,500-5,000 live male births.[1] DMD is characterized by progressive muscle weakness, loss of ambulation around age ten, and is ultimately fatal due to cardiorespiratory failure around age thirty.[2–4] In addition to muscle wasting, commonly observed clinical features of the disease include scoliosis, joint contractures, and calf pseudohypertrophy.[5] DMD is caused by mutations that disrupt production of the dystrophin protein, the absence of which sensitizes muscle to contraction-induced damage.[6]

Standard of care for DMD patients is corticosteroid therapy (e.g. prednisone or deflazacort) which has demonstrated some limited efficacy in terms of prolonging ambulation and delaying disease progression,[7,8] through an anti-inflammatory mechanism. Importantly, long-term steroid use is associated with undesirable side-effects including Cushingoid symptoms, weight gain, growth delay, behavioral changes, and osteoporosis.[9] The latter is particularly concerning as accidental fractures in DMD patents often lead to a permanent loss in ambulation.[10] There are currently efforts to develop corticosteroid treatments for DMD with improved side effect profiles (e.g. vamorolone),[11] which have been discussed in detail elsewhere.[9]

Newer DMD therapeutic approaches have focused on efforts to restore dystrophin expression, of which there are multiple modalities. Drugs that can restore the dystrophin reading frame via antisense oligonucleotide-mediated exon skipping or stop codon readthrough have achieved regulatory approval. While these are major achievements for the DMD research and patient communities, the efficacy of these drugs is generally accepted to be very low. A variety of efforts are currently underway to enhance delivery of exon skipping drugs via novel chemical modification and conjugation to delivery-assisting moieties. Conversely, multiple gene replacement therapies, comprised of viral vector encoded truncated dystrophin variants, are in late stage clinical trials. Other

therapeutics strategies including cell therapy, gene editing, upregulation of compensatory genes such as utrophin, and combination therapies are also under investigation.

DMD is a key disease indication in the field of experimental therapeutics on account of constituting an unmet clinical need, devastating disease progression, relatively high incidence considering it is a rare disease, multiple recent regulatory approvals, and the plethora of molecular and cellular medicines that are being investigated for its treatment. Advances in DMD therapeutics will undoubtedly have an impact on therapeutics development in other areas of medicine, as similar modalities can be applied to other disease indications. Here we discuss the drug development landscape for DMD. This review is primarily focused on dystrophin restoration therapies, although other strategies will be considered briefly. Recent drug approvals, current clinical trial progress, improved delivery technologies, vector-associated safety issues, combination therapies, and more novel approaches will be discussed.

[H1] DMD Genetics and Pathophysiology

DMD is a dystrophinopathy, caused by genetic absence of the dystrophin protein, encoded by the *DMD* gene at Xp21. Dystrophin is located at the intracellular surface of the sarcolemma, where it acts as an organizing center for the dystrophin-associated protein complex (DAPC). Specifically, dystrophin binds to β-dystroglycan (DAG1) via an interaction towards its cysteine-rich domain at its C-terminus.[12] β-dystroglycan (DAG1) binds to α-dystroglycan (also encoded by the *DAG1* gene) that is exposed on the extracellular surface of the sarcolemma, where it interacts with a complex of laminins (LAMA2, LAMA5, LAMB1, and LAMC1). Loss of dystrophin results in disruption of the DAPC, and consequent mislocalization of many DAPC components from the sarcolemma,[12] and in many cases to reductions in their protein expression levels.[13,14] For example, the sarcoglycans (SGCA, SGCB, SGCG, and SGCD) are downregulated and mislocalized in dystrophin-deficient muscle.[13,14] Disruption of any one of these sarcoglycans is associated with various forms of limb-girdle muscular dystrophy (LGMD),[15] indicating that integrity of the DAPC is important for preventing muscle

pathology. Dystrophin also binds to filamentous γ-actin, intermediate filaments, and the microtubule network in the sarcoplasm via its N-terminus. As such, dystrophin forms a mechanical link between the extracellular matrix and the actin cytoskeleton.[16] The primary function of dystrophin is to serve as a 'shock absorber' that protects muscle from contractile damage.[6] Additionally, dystrophin is also involved in multiple signalling processes via DAPC interactions including nitric oxide (NO) signalling via the activity of nNOS, the MAPK pathway,[17] and MARK2 which has been reported to regulate muscle satellite (stem) cell polarity.[18]

Absence of dystrophin protein leads to DAPC disruption, sarcolemmal damage, increased Ca^{2+} influx, oxidative stress, and myonecrosis. Dystrophic muscle is characterized by foci of degeneration/regeneration, and persistent inflammation. During the early stages of disease, myofiber loss is balanced by compensatory regeneration driven primarily by satellite cells. In the advanced stages of disease, muscle quality declines as a consequence of extensive fibrosis and deposition of adipose tissue,[19] which both replace myofibers and generate a non-productive environment that is unable to support satellite cell-mediated regeneration (i.e. functional exhaustion). Importantly, evidence suggests that the number of satellite cells is not reduced in dystrophic muscle, and their regenerative potential is not diminished.[20,21]

The genomic locus encoding the *DMD* gene is the largest in the human genome (~2.2 Mb) and exhibits a high rate of *de novo* mutations. Common types of DMD-causing mutation include whole exon deletions (68%), exon duplications (11%), and nonsense mutations (10%).[22–25] While mutations can occur throughout the genomic region, they are concentrated at two hotspot regions located at exons 3-19 and exons 45-55.[26] The *DMD* gene consists of 79 exons (**Figure 1A**), many of which code for semi-redundant spectrin-like repeat domains (24 in total) in the central rod domain of dystrophin, many of which are redundant and dispensable for dystrophin function (**Figure 1B**). Importantly, whole exon deletions which do not disrupt the translation reading frame lead to an internally truncated dystrophin protein which retains partial functionality and is associated with Becker muscular dystrophy (BMD), a related dystrophinopathy.[27–30] BMD patients present with a wide-range of

severities, although the onset of symptoms is typically later and relatively mild compared to DMD. Life expectancy is longer than for DMD, with dilated cardiomyopathy appearing in the 4th decade of life.[31,32] BMD patients generate dystrophin protein at lower levels than in the case of DMD and/or produce a truncated, partially functional dystrophin on account of varying degrees of internal in-frame deletion.[29] In rare cases, some BMD patients with large internal dystrophin deletions are effectively asymptomatic.[27,28] These observations motivated the development of therapeutic dystrophin restoration strategies that aim to convert the severe DMD phenotype into the milder BMD situation.[33]

[H1] Dystrophin Restoration Strategies

Loss of dystrophin is the primary genetic cause of DMD, and so extensive research effort has been directed towards therapies that can restore dystrophin expression. A plethora of approaches have been tested, including splice correction (i.e. exon skipping) to restore the translation reading frame, stop codon read-through for the treatment of patients with nonsense mutations, gene replacement with truncated dystrophin transgenes, delivery of dystrophin-expressing myogenic cells (i.e. cell therapy), and gene editing to repair the *DMD* locus at the DNA level.

[H2] Exon Skipping

The leading dystrophin restoration strategy is currently exon skipping, whereby modulation of alternative splicing is utilized to restore the translation reading frame and promote the generation of a partially-functional, internally-deleted pseudo-dystrophin protein (**Figure 2**). Typically, this is achieved using steric block antisense oligonucleotide (ASO) drugs. These short (~20-30 nucleotide), single-stranded nucleic acid polymers interact with pre-messenger RNA transcripts via Watson-Crick base pairing and thereby influence splicing decisions by physically masking specific splicing signals. Exon skipping ASOs must each be designed to target a single specific exon, and so an individual drug can only ever have the capability of targeting a limited number of patients for which a given exon skip will restore the translation reading frame. Initial efforts focused on skipping of exon 51, which would be applicable to ~13% of all DMD patients[33] (e.g. those with whole exon deletions of *DMD* exons 50

or 52). The great promise of oligonucleotide therapeutics is that upon establishing platform chemistries and delivery strategies, novel drugs can be rapidly generated by the careful alteration of the constituent nucleotide sequence in order to target a different transcript. In the case of DMD, modification of ASO sequences can be deployed in order to target a wider range of DMD-causing mutations. So far, there are four FDA-approved ASO drugs designed to skip various DMD exons (**Table 1, Figure 3A**). These ASOs are all phosphorodiamidate morpholino oligonucleotides (PMO) (**Figure 3B**) developed by Sarepta Therapeutics (i.e. eteplirsen,[34] golodirsen,[35] and casimersen[36]) or NS-Pharma (i.e. viltolarsen[37–39]), which target the exons with the potential to treat the largest number of patients (collectively exons 45, 51, and 53). Both Sarepta and NS Pharma have pipelines with ASOs targeting the skipping of additional exons (collectively: exons 43, 44, 50, 52, and 55), although many of these programmes are still at the pre-clinical stage. Importantly, not all DMD-causing mutations are treatable with exon skipping approaches.[33] Since the FDA has not approved exon skipping as a class of drug but has instead required separate trials for each ASO, the targeting of rare mutations is unlikely to be commercially attractive enough for development by the pharmaceutical industry.

Notably, the European Medicines Agency (EMA) has declined to approve any of the exon skipping compounds described above,[40] based primarily on their low efficacy (and arguably marginal therapeutic benefit) and small trial sample sizes. Indeed, the approval of eteplirsen by the FDA was particularly controversial, leading to accusations of 'railroading at the FDA' and the resignations of several FDA review team members.[41–45] The mean dystrophin protein expression after 180 weeks of eteplirsen treatment was determined to be less than 1% of healthy dystrophin levels.[46] The efficacy of viltolarsen, golodirsen, and casimersen have been shown to be similarly modest.[36,37,47] Nevertheless, PMOs have been found to be remarkably safe in clinical trials[48,49], and doses of up to 3 g/kg are well-tolerated in mice.[50] Despite the low levels of dystrophin protein restored by these compounds, clinical trial participants treated with eteplirsen (the most studied exon skipping drug for DMD) have

maintained an attenuation in ambulatory decline over a treatment period of at least four years,[51] which is not consistent with the established progression of the disease.

Importantly, the four FDA approvals of PMO exon skipping drugs have not discouraged other companies from entering this space with enhanced chemistries and/or delivery technologies. The low efficacy of 'naked' PMO exon skipping drugs has motivated the development of improved delivery strategies, primarily based on bioconjugation.[52] The backbone linkages of PMOs are uncharged (unlike the majority of oligonucleotide therapeutics) which permits facile covalent conjugation to cell penetrating peptides (CPPs). CPPs are typically arginine-containing short peptides that facilitate interaction with the outer surface of the plasma membrane and glycocalyx, and which may to some extent promote endosomal escape. The resulting peptide-PMO (PPMO) conjugates have been shown to offer major increases in potency in pre-clinical DMD models compared to unconjugated, naked PMO.[53,54] PPMO conjugates are currently under investigation in two clinical programmes (**Table 1**). Firstly, Sarepta Therapeutics is conducting a phase II trial (NCT04004065, MOMENTUM) of vesleteplirsen (SRP-5051), which consists of the exon 51-targeting eteplirsen PMO sequence conjugated to the arginine-rich R_6Gly peptide (**Figure 3C**). Preliminary data from Sarepta suggests that vesleteplirsen exhibits greater drug exposure and exon skipping activity than eteplirsen at equivalent doses (>10% mean exon skipping, >6% dystrophin expression after treatment with 30 mg/kg/month for three months).[55] Secondly, PepGen Ltd is conducting a phase I clinical trial of PGN-EDO51 in healthy volunteers in Canada, based on novel PPMO technology developed by the Wood and Gait groups.[56] These conjugates were designed to balance exon skipping activity with renal toxicity, which has been reported to be a potential limitation of PPMO technologies.[57,58] PGN-EDO51 was found to be safe and well-tolerated in healthy volunteers, with dystrophin exon skipping observed at low levels, which was expected considering the relatively low dose (i.e. 2% mean exon skipping, after a single intravenous 15 mg/kg dose, assayed 28 days post injection).[59] Both Sarepta and Pepgen have reported cases of hypomagnesemia after PPMO treatment that require magnesium supplementation or which required no treatment, respectively.

Entrada Therapeutics are developing PPMO exon skipping conjugates based on cyclic peptides (i.e. enhanced endosomal escape vehicle, EEV) and are currently at the pre-clinical stage.[56]

Other ASO conjugation approaches are also under investigation. Avidity Biosciences is exploring an antibody-oligonucleotide conjugate approach (**Figure 3D**), with the leading compound AOC 1044, targeting *DMD* exon 44 skipping currently being investigated in the EXPLORE44 phase I/II clinical trial in healthy volouteers.[60] Similarly, Dyne Therapeutics is undertaking a first in human phase I/II clinical trial of DYNE-251, targeting skipping of *DMD* exon 51 in amenable DMD patients (NCT05524883). DYNE-251 consists of a PMO conjugated to a Fab fragment (**Figure 3E**) targeting the transferrin receptor (TFRC, TfR1) which is highly expressed in skeletal and cardiac muscle. Dyne recently reported pre-clinical exon skipping data in the *mdx* mouse using their FORCE platform.[61]

The prospect of multi-exon skipping using a cocktail of 10 octaguanidine dendrimer-conjugated PMO oligonucleotides (vivo-morpholinos) has been explored in a DMD mouse model lacking *Dmd* exon 52 (the *mdx52* mouse).[62] Skipping of 10 'hotspot' exons (*Dmd* exons 45-55) resulted in restoration of dystrophin expression and improvements in muscle function, although several skipped products were produced.[62] Importantly, skipping of these hotspot exons would be theoretically be applicable to ~63% of all DMD patients.[63] Whether such an approach can be translated for use in human patients remains to be demonstrated, as overall such multi-exon skipping strategies have thus far only proved minimally successful.

Wave Life Sciences recently initiated testing of an ASO designed to skip *DMD* exon 53 (WVE-N531) in a phase Ib/II clinical trial in 15 DMD boys (NCT04906460). Preliminary results from this trial after 6 weeks of treatment indicated substantial RNA level exon skipping, although dystrophin protein was below the lower limit of quantification.[64] WVE-N531 is chimeric stereopure steric block ASO that contains phosphorothioate (PS) and phosphoryl guanidine (PN) linkages, which reduce the overall charge of the oligonucleotide (**Figure 3F-J**).[65] This drug also includes stereospecific linkages at one

or more backbone linkages (both PS and PN linkages are chiral, unlike the analogous phosphodiester, PO, linkage) (**Figure 3I-J**). Control of backbone linkage stereochemistry has been shown to influence a multitude of oligonucleotide properties, including hydrophobicity, nuclease-resistance, target binding affinity, and splice-switching activity.[66] The notion that control of stereochemistry could be utilized to optimize ASO development is appealing, as ASOs with chiral centers in their backbone (which is the vast majority) in reality constitute racemic mixtures of hundreds of thousands of different molecules. Among this population there may be hyperfunctional molecules that could be synthesized in a stereopure manner, thereby offering a substantial increase in potency. However, the importance of stereopure ASO backbone linkages has also met with scepticism from some others in the oligonucleotide field.[67] Beneficial outcomes that are obtained in certain properties (e.g. target binding), may be counteracted by detrimental changes in other properties (e.g. uptake efficiency). Notably, Wave Life Sciences have also had a number of other high-profile failures of their stereopure ASO technologies for both their DMD and Huntington's disease programs.[68,69] Demonstration of efficacy for WVE-N531 will be important for supporting the continuation of stereopure ASO technology development.

Daiichi Sankyo is investigating renadirsen (DS-5141b) in a phase II trial in 8 participants (NCT04433234). Renadirsen is a 'mixmer' ASO consisting of 2′-*O*-Methyl and ENA (ethylene-bridged nucleic acid) residues with phosphorothioate linkages (**Figure 3G,K,L**) designed to skip *DMD* exon 45.[70] Tricyclo-DNA (**Figure 3M**) is an ASO chemistry that has been demonstrated to exhibit some limited activity for dystrophin restoration in the brain,[71,72] is being developed by SQY Therapeutics, and is expected to initiate a phase I/II clinical trial (Avance 1) in early 2023.

An alternative strategy is to use expressed exon skipping triggers (i.e. U1-snRNA and U7-snRNA) which can be delivered via adeno-associated viral (AAV) vectors (discussed in more detail below), which enable systemic delivery throughout the musculature.[73,74] Such an approach is currently under investigation in a phase I/II clinical trial (NCT04240314) sponsored by Nationwide Children's Hospital, USA, that aims to induce exon skipping of a duplicated *DMD* exon 2, the most commonly-

observed DMD-causing exon duplication.[23] The experimental therapeutic in this case (scAAV9.U7-ACCA) is a self-complementary AAV9 encoding four U7-snRNA exon skipping transgene cassettes (two each targeting the exon 2 splice acceptor and splice donor).[75] This approach is notable because exon skipping results in two possible beneficial splicing outcomes: (i) skipping of one copy of *DMD* exon 2 will result in the generation of full-length dystrophin (and therefore a significant advantage over other approaches that restore internally-truncated pseudo-dystrophins), or (ii) skipping of both copies of *DMD* exon 2. In the case of complete exon 2 exclusion, cap-independent translation occurs driven by an internal ribosome entry site (IRES) sequence located in exon 5, which generates a highly functional N-terminally-truncated dystrophin isoform.[76] Evidence from pre-clinical studies suggest that the second splicing outcome is dominant.[75] Preliminary findings from investigations in three DMD patients are promising.[77]

[H2] Stop Codon Read-through

Premature termination codons (PTCs) in the *DMD* gene result in the generation of truncated protein products and/or promote reductions in mRNA levels via the nonsense-mediated decay (NMD) pathway. Stop codon read-through therapies have therefore been developed which aim to promote ribosome miscoding of PTCs such that a random amino acid is incorporated and translation of the mRNA continues past the PTC, instead of translation termination occurring. Stop codon read-through approaches in the context of DMD are therefore expected to generate full-length dystrophin protein (albeit with a single internal amino acid change) (**Figure 4A,B**). Early work focused on the use of gentamicin (**Figure 4C**), an aminoglycoside antibiotic that binds in the aminoacyl-tRNA acceptor (A) site of the ribosome and interferes with codon-anticodon recognition. Gentamicin treatment showed some promise in pre-clinical studies,[78] although results in clinical trials were less impressive.[79–81] Notably, aminoglycosides are associated with renal and otic toxicities,[82] and clinical development of gentamicin was ultimately terminated.

PTC Therapeutics has developed an improved stop codon read-through compound, ataluren (PTC124, Translarna). Ataluren is an orally bioavailable small molecule (3,5-diaryl oxadiazole, **Figure 4D**) that

has no structural similarity with aminoglycosides antibiotics, exhibits no antibiotic activity, does not influence NMD target expression, and promotes read-through of PTCs but while not affecting normal termination codons.[83] In July 2014 Ataluren was granted conditional approval by the EMA[84] and is currently approved for use in nonsense-mutation DMD patients age 2 and older at a dose of 40 mg/kg/day. This approval was initially based on promising findings from a randomized, double-blind, placebo-controlled clinical trial (NCT00592553),[85] and conditional on the findings of a second phase III trial (ACT DMD, NCT01826487).[86] Neither of these trials met their primary endpoint of a statistically significant >30 m improvement in six minute walk distance (6MWD) at week 48 post treatment (relative to placebo-treated individuals). However, sub-setting of the data showed significant improvements in patients in the ambulation transition phase (i.e. those with baseline 6MWD of 300-400 m).[86] Importantly, the US FDA declined to approve ataluren based on the same data.[87] Subsequent studies have provided further evidence to support the efficacy of ataluren. A meta-analysis in which data from these similar trials was combined found that the improvement in 6MWD on ataluren did reach statistical significance (for both all intention-to-treat patients and the ambulation transition patient subset).[88] Nevertheless, controversy surrounding the approval of ataluren persists and a number of ataluren clinical trials are ongoing (phase II: NCT04336826, and phase III: NCT02369731, NCT01247207, NCT03179631). The mechanism-of-action for ataluren is currently unknown, and it was shown that this drug could bind and stabilize firefly luciferase, leading to an increase in its activity in reporter assays similar to those used to identify it as a stop codon read-through candidate.[89] Others have reported conflicting results using ataluren, such as a failure to show read-through activity using multiple reporter assays.[90] A convincing demonstration of dystrophin restoration in ataluren-treated patient muscle biopsies, and elucidation of the drug mechanism of stop codon read-through will help to assuage these concerns.

[H2] Gene Replacement Therapy

Classical gene therapy for DMD aims to introduce DNA that encodes a functional dystrophin protein into patient muscles. Transgene DNA is typically delivered using viral vectors, with AAV being the vector of choice. Treatment with viral gene therapy typically results in ‘immunization’ of the treated

individual against the vector, meaning that repeat administration of the therapy is precluded.[91] AAV is a single-stranded DNA parvovirus, that is generally considered to be non-pathogenic in humans and has been widely used for gene therapy applications, with two AAV-based gene therapy products reaching marketing approval for non-DMD indications (e.g. intrathecal injection of zolgensam for the treatment of spinal muscular atrophy (SMA) and subretinal injection of luxturna for the treatment of Leber congenital amaurosis). Importantly multiple AAV serotypes exhibit tropism for skeletal and cardiac muscle.[92–94] However, the AAV genome has a maximum packaging capacity of ~4.8 kb, and so is insufficient to deliver the full-length *DMD* cDNA in a single vector. (The mature mRNA for the major *DMD* muscle transcript isoform is ~14 kb, with a ~11 kb reading frame). Efforts have therefore focused on the generation of dystrophin minigenes, in which non-essential internal domains are deleted, inspired by the example of a very mildly-affected patient who expressed a internally-truncated but functional pseudo-dystrophin protein (**Figure 5**).[27] This minigene (with 46% of the normal dystrophin protein coding region) was shown to prevent pathology in the *mdx* mouse and the deletion was further extended to generate micro-dystrophin genes.[95] As such, gene replacement therapies for DMD have focused on the delivery of various micro-dystrophin transgenes (sometimes also called 'mini-dystrophin'). Micro-dystrophins typically lack large portions of the central rod domain (and therefore the majority of multiple spectrin-like repeat domains are also missing), but interactions between the DAPC and the cytoskeleton are maintained, thereby preserving the primary function of dystrophin. The majority of micro-dystrophin constructs lack the C-terminal (CT) domain, the inclusion of which has been shown to confer little additional therapeutic benefit.[96] There are currently five micro-dystrophin drugs in clinical trials, sponsored by Sarepta, Pfizer, Solid Biosciences, Genethon (in partnership with Sarepta), and REGENXBIO. These constructs differ in terms of (i) the micro-dystrophin structure, (ii) choice of promoter, and (iii) the AAV serotype used (**Table 2**). AAV-mediated delivery of micro-dystrophin has been shown to improve dystrophic pathology in various mouse and canine DMD models,[97–100] suggesting that these micro-dystrophin genes might be sufficient to convert the DMD phenotype to a milder Becker clinical course.

SRP-9001 (developed by Sarepta) is the AAV-micro-dystrophin gene therapy that is at the most advanced stage, and for which the most information is publicly available. SRP-9001 (also called rAAVrh74.MHCK7.micro-dystrophin) utilizes a codon-optimized human micro-dystrophin minigene driven by a synthetic MHCK7 promoter, consisting of the MCK promoter fused with the MCK and α-MHC enhancers to promote high expression levels specifically in skeletal and cardiac muscle.[101] This transgene cassette is delivered using an AAV variant derived from rhesus macaques (AAVrh74) which exhibits strong skeletal and cardiac muscle tropism,[102] and for which seroprevalence of neutralizing antibodies is low in DMD patients.[103] A phase I/2a clinical trial (NCT03375164) in four DMD patients treated with 2×10^{14} vg/kg of SRP-9001 reported expression of dystrophin 12 weeks post injection, and improved NSAA (North Star Ambulatory Assessment) scores and serum CK levels up to 1 year post treatment.[104] Three of these treated patients underwent further analysis by quantitative magnetic resonance imaging and spectroscopy which illustrated an improvement in muscle fat fraction and transverse relaxation time (qT_2, which is affected by inflammation and fat infiltration) values for patients treated with SRP-9001 compared with a natural history cohort.[105] Multiple further clinical trials of SRP-9001 are ongoing (phase I open label extension: NCT03375164, phase II randomized placebo controlled: NCT03769116, phase I: NCT04626674, and phase III double-blind, randomized, placebo-controlled: NCT05096221), with some preliminary data available through press releases and conference presentations.[106–108] Based on the promising findings from across its clinical programs, Sarepta submitted a Biologic Licence Application for SRP-9001 to the FDA, which was granted Priority Review status in November 2022.[109] If the FDA opinion is positive, SRP-9001 will be the first approved gene therapy product for DMD.

Unfortunately, other micro-dystrophin gene therapy development programs have not run so smoothly. In December 2021, Pfizer announced the death of a 16 year old non-ambulatory trial participant with advanced disease treated with a high dose (2×10^{14} vg/kg) of PF-06939926 in an open-label phase Ib trial (NCT03362502), leading to a temporary FDA hold on the drug.[110,111] A randomized, double-blind, placebo-controlled phase III trial of PF-06939926 (NCT04281485, CIFFREO) is ongoing. No peer reviewed findings from these studies are currently available, but claims of relatively high levels

of micro-dystrophin expression (24-50%, by anti-peptide antibody enriched, immunoaffinity liquid chromatography tandem mass spectrometry assay) and significant functional improvement in the phase 1b trial have been reported in a Pfizer press release.[112] However, a number of treatment-related serious adverse events have been reported related to muscle weakness and myocarditis, leading to a protocol amendment to exclude patients with any mutations affecting *DMD* exons 9 to 13, or deletions affecting both exons 29 and 30, and a 7 day hospitalization period after treatment adminstration.[113,114] A collaborative working group that combined the data and experience from Pfizer, Sarepta Therapeutics, Solid Bioscience, and Genethon (together with experts from academia) was established to address the potential safety issues with micro-dystrophin gene therapy.[115] The observation that the worst serious adverse events occurred only in patients who carried deletions of dystrophin encoding regions that are present in the micro-dystrophin transgene protein, suggested that a T cell-mediated immune response is responsible,[115] and provides a scientific rationale for excluding such patients from clinical trials.

Similarly, a phase I/II trial (NCT03368742, IGNITE) of SGT-101 (Solid Biosciences) has been placed on hold by the FDA twice, as a consequence of serious adverse events in a patient in the high dose (2×10^{14} vg/kg) cohort, including thrombocytopenia, complement activation, reduced red blood cell count, acute kidney injury, and cardio-pulmonary insufficiency.[116] Clearance to continue was given following several protocol amendments, including improvements to the AAV manufacturing process to remove the majority of empty viral capsids. Prophylactic measures to minimize immune reactions were also implemented (i.e. treatment with eculizumab and a C1 esterase inhibitor), together with an increase in corticosteroid dose in the first month after SGT-001 injection.[117] No peer reviewed data on the safety and efficacy of SGT-001 are currently available, but dystrophin levels of up to 17.5% (by western blot) of healthy levels (but which was below the limit of quantification in one patient) and improvements in 6MWD, NSAA score, and pulmonary function tests have been reported in a Solid Bioscience press release.[118] An improved DMD micro-dystrophin gene therapy product (SGT-003) with improved tropism for muscle and heart, and reduced liver delivery is being developed by Solid Bioscience and is currently at the IND (investigational new drug) stage.

Notably, there have been several other deaths following treatment with high dose AAV in a clinical trial for X-linked myotubular myopathy (NCT03199469),[119] a trial for Sanfilippo syndrome (NCT03612869),[120] and in two patients treated with the zolgensma (an FDA-approved AAV gene therapy for spinal muscular atrophy).[121] High dose AAV9 therapies (i.e. 2×10^{14} vg/kg) have also been reported to induce severe hepatic and neurological toxicities in nonhuman primates and piglets.[122] However, even higher doses of AAV micro-dystrophin gene therapies have been administered to DMD patients (SGT-001 at 2×10^{14} vg/kg, and PF-06939926 at 3×10^{14} vg/kg)[112,123], suggesting that some patients may be more susceptible to severe toxic effects than others. It is clear that a re-evaluation of the safety of high dose AAV therapies is warranted.

Aside from safety issues associated with high dose AAV, gene replacement therapy for DMD faces a number of additional challenges. Indeed, as many as ~40% of humans are already positive for anti-AAV antibodies as a consequence of natural exposure,[124] which creates a key challenge for the application of AAV-derived vectors in DMD patients. The presence of these antibodies is typically an exclusion criterion in gene therapy trials. The expression of micro-dystrophin in DMD patient muscle has the potential to the generate non-self antigens, leading to an anti-transgene immune response. The relative failure of the first clinical trial of AAV-micro-dystrophin was attributed to such an anti-dystrophin T cell response.[125] Evidence from pre-clinical studies suggests that the anti-dystrophin antibody response can be avoided through the co-treatment with immunomodulatory drugs such as rituximab and VBP6.[126] The success of AAV-micro-dystrophin therapy is predicated on long-term expression of the therapeutic transgene. AAV vector genomes do not integrate into the host DNA, but are instead maintained as episomal chromatin with the potential for long-term persistent transgene expression.[127,128] However in practice, AAV viral genomes are progressively lost from treated dystrophic muscle meaning that micro-dystrophin expression is expected to progressively diminish, leading to a recurrence of dystrophic pathology.[129] Epigenetic silencing of the micro-dystrophin transgene cassette may also contribute to a loss of expression over time.[130] Intriguingly, Mollard *et al*.

recently reported a reduction in AAV transgene expression in post-regeneration mouse muscle, suggesting that dystrophic pathology itself may constitute a limitation to effective therapy.[131]

[H2] Cell Therapy

Cell therapies for DMD aim to treat the disease via the transplantation of dystrophin-expressing pro-myogenic cells into patient muscle. Such therapies may involve cells derived from healthy, histocompatible donors (i.e. allogenic), or via patient-derived cells that are genetically-corrected in order to express dystrophin *ex vivo* (i.e. autologous). Healthy cells are injected via intramuscular injection (which can involve multiple injections per muscle) or systemic administration, whereby the transplanted cell population expands, undergoes myogenic differentiation, and fuses to generate new myotubes and/or integrate with existing or regenerating myofibers. Initial results in the *mdx* mouse demonstrated that implanted healthy neonatal muscle progenitor cells (i.e. myoblasts) can fuse with pre-existing *mdx* myofibers and render them dystrophin positive.[132,133] Subsequently, multiple other cell sources have been explored for cell transplantation, including satellite cells,[134] bone-marrow-derived myogenic cells,[135] side population cells,[136] mesoangioblasts,[137] pericytes,[138] CD133+ cells,[139] and induced pluripotent stem cells.[140] Despite promising results in pre-clinical studies, results in human patients using these approaches have been relatively disappointing. For example, DMD patients treated with a series of high density injections of normal myoblast allotransplants (under tacrolimus immunosuppression) exhibited dystrophin expression (ranging from the presence of a single dystrophin-positive myotube to positivity in 26% of myofibers) that was mostly restricted to the area surrounding the injection sites.[141] In contrast, CD133+ cells introduced via intramuscular injection failed to fused with the myofibers in DMD patient muscle.[139] Similarly, intra-arterial injection of mesoangioblasts derived from HLA-matched healthy donors resulted in the detection of dystrophin expression, but no functional improvement.[137]

Cell therapies for DMD face a number of important challenges.[142] In many cases, obtaining sufficient numbers of cells to treat all muscles will be difficult (especially for cell types like satellite cells). Transplanted cells may face immune rejection, and typically large numbers of the donor cells die

shortly after injection.[143] Delivery also constitutes a major challenge, as cells may exhibit reduced potential for migration, or aggregate inside blood vessels following a failure to extravasate with the potential for pulmonary embolism or accumulation in filter organs.[138] Delivery to disease-critical muscles such as the diaphragm is also particularly challenging.

A novel alternative cell therapy approach using cardiosphere-derived cells (CDCs) has been pioneered by Capricor Therapeutics. Allogenic CDCs derived from healthy donors have been administered to DMD patients via intracoronary[144] and intravenous[145] routes of administration. While these have the potential to express wild-type dystrophin protein, this is not the goal of the therapy *per se*. Instead, CDCs are hypothesized to release extracellular vesicles containing cargo molecules that exert anti-inflammatory and anti-fibrotic effects. In a recent double-blind, placebo-controlled, phase II clinical trial (NCT03406780, HOPE-2) late ambulatory DMD patients were intravenously injected every 3 months with 1.5×10^8 CDCs (i.e. CAP-1002) for a total of four administrations.[145] CAP-1002-treated patients exhibited a slowing of the loss of upper limb function, together with improvements in cardiac structure and function.[145] An open-label extension study is ongoing (NCT04428476), and a phase III trial (NCT05126758, HOPE-3) is currently recruiting.

ENCell is currently conducting a phase I clinical trial (NCT05338099) in DMD patients for its stem cell therapy EN001 based on the transfer of Wharton's jelly (umbilical cord)-derived mesenchymal stem cells.

[H2] Gene Editing

With the re-purposing of the CRISPR/Cas9 system for gene editing in mammalian cells, there has been intense interest in deploying this technology for the treatment of DMD.[146] In its simplest configuration, the CRISPR/Cas9 system consists of the Cas9 endonuclease, which is capable of inducing double-strand DNA breaks, and a single guide RNA (sgRNA) that acts to program the Cas9 such that it cuts at a specific DNA sequence. Notably, the use of multiple guides enables the possibility for multiplex gene editing.[147] The host cell DNA damage repair machinery is of key

importance for the success of CRISPR/Cas9 therapies, with the non-homologous end joining (NHEJ) pathway being the most relevant for the purposes of this review.

Multiple CRISPR/Cas9-based strategies have been proposed for the treatment of DMD. The first demonstrations of CRISPR/Cas9-mediated correction in the context of DMD utilized an exon excision approach.[148–150] This strategy is conceptually similar to exon skipping, whereby two double-strand breaks are induced in intronic sequences flanking a target exon (initially demonstrated for *Dmd* exon 23 in the *mdx* mouse) using a pair of sgRNAs. The resulting lesions are joined via the NHEJ pathway and the intervening DNA removed. Such a corrected locus would now be 'permanently exon skipped' as the preceding exon (i.e. *Dmd* exon 22) will be spliced onto the following exon (i.e. *Dmd* exon 24).

Myofibers that re-express dystrophin are believed to experience a selection advantage, meaning that they are progressively enriched over time.[151] Furthermore, early treatment in dystrophic mice is more effective than treatment at the adult stage, possibly as the former approach leads to tolerization towards dystrophin-associated antigens. The editing of satellite cells is desirable, as the resulting corrected stem cells will continue to add myonuclei with the potential to express dystrophin during growth and regeneration throughout the life of the treated individual. However, there have been conflicting reports regarding the potential of AAV vectors to transduce satellite cells.[148,152–154]

Excitingly, multiplex gene editing enables the simultaneous deletion of multiple exons (analogous to multi-exon skipping described above), with the potential for a single therapy that could be applied to a large proportion of patents.[140,155] An alternative approach is the single cut strategy, in which the targeted introduction of an indel is used to either disrupt a splicing motif (e.g. exon splicing enhancer or splice site) or to reframe a transcript.[151] Multiple other CRISPR-based technologies are under investigation for the treatment of DMD including base editing,[156] prime editing,[157] CRISPR activation (CRISPRa),[158] and *ex vivo* gene correction using homology dependent repair (HDR).[159]

Vertex Pharmaceuticals and Sarepta Therapeutics are currently developing CRISPR/Cas9-based therapies for DMD, although both are at the discovery/pre-clinical phase. However, the most clinically advanced DMD CRISPR therapy is CRD-TMH-001 developed by a non-profit organization, Cure Rare Disease, in collaboration with the University of Massachusetts. Few details are publicly available about CRD-TMH-001 other than it is designed to activate dystrophin expression using a CRISPRa approach, and that Cure Rare Disease has multiple other personalized CRISPR-based therapies in pre-clinical development.[160] An *n*=1 clinical trial (NCT05514249) with CRD-TMH-001 has been initiated, but Cure Rare Disease recently announced in October 2022 that the single 27-year old trial participant had died.[161] The cause of death, and whether the treatment had been administered, have not been publicly disclosed.

The majority of CRISPR/Cas9 therapeutics for DMD rely on the use of AAV vectors for delivery of the gene editing apparatus, and as such, are subject to all of the limitations of these delivery vectors described above (especially considering the high doses of vectors that will be required). In addition, there are multiple additional CRISPR/Cas9-specific limitations that must be addressed before such strategies can be translated into new therapies. Firstly, deleterious off-target editing events must be carefully considered. Non-productive on-target editing also has the potential to corrupt myonuclei, leading to a patchy pattern of dystrophin in treated myofibers.[162] Pre-existing anti-Cas9 antibodies and T cells are relatively common in the general population,[163–165] which may further limit the applicability of these therapies.

[H1] Utrophin Upregulation

Utrophin (*UTRN*) is an autosomal (6q24) paralogue of dystrophin which is expressed during fetal development, at the neuromuscular and myotendinous junctions, and during muscle regeneration.[166–168] The 395 kDa utrophin isoform is upregulated in the muscles of dystrophin-deficient mouse models (e.g. *mdx*, *mdx52*)[13,14] and DMD patients,[169] where it relocates to the sarcolemma and can bind to DAPC components.[170] These observations suggest that utrophin can substitute for dystrophin to some

extent, and that its upregulation in dystrophic muscle may serve as a compensatory and protective mechanism. Accordingly, dystrophic pathology is severe in the dystrophin/utrophin double knock-out (dKO) mouse,[171,172] and genetic overexpression of full-length utrophin (by 3-4 fold) on an *mdx* background was sufficient to prevent the development of dystrophic pathological features.[173,174] Upregulation of utrophin is a particularly attractive therapeutic strategy as a single approach could be used to treat all patients regardless of mutation type. Furthermore, the *UTRN* gene is invariably unaffected in DMD patients, and its expression is ubiquitous,[175] meaning that patients are already tolerized to UTRN-associated antigens.[176] Ubiquitous overexpression of a utrophin transgene in mice was also found to be non-toxic.[175]

The leading strategy for utrophin upregulation is the use of small molecule modulators. The drug ezutromid (SMT C1100, developed by Summit Therapeutics), a 2-arylbenzoxazole, was identified in a screen of small molecules that promoted transcriptional activation of the Utrophin-A promoter.[177] Daily oral administration with this compound improved muscle pathology in the *mdx* mouse.[178] Investigation of ezutromid in an open-label phase II clinical trial (NCT02858362) showed evidence of utrophin upregulation, and reduced muscle turnover after 24 weeks of treatment,[179] but this effect was not present at 48 weeks.[180] Development of ezutromid was discontinued based on these disappointing findings. Subsequent investigation revealed that ezutromid binds to, and acts as an antagonist of the aryl hydrocarbon receptor (AHR), and that other AHR antagonists similarly promoted utrophin expression,[180] suggesting that this could be utilized for future drug development. It has been proposed that the lack of sustained efficacy of ezutromid is a consequence of its cellular metabolism leading to reduced drug exposure,[180] and that such metabolism of future utrophin modulator drugs could be avoided through medicinal chemistry optimization (Angela Russell, personal communication). Similarly, second generation utrophin up-regulator compounds that are derivatives of ezutromid have been reported with improved activity (such as SMT022357).[181]

The sequence of utrophin is highly similar to that of dystrophin.[166,167] As such, internally-truncated utrophin minigene variants have been generated (analogous to the micro-dystrophin approach

described above).[182,183] To this end, transgenic micro-utrophin expression resulted in improvements in histopathology and reduced serum CK levels in *mdx* mice[182] and various canine models.[176] Similarly, AAV-delivered micro-utrophin constructs improved muscle function and increased lifespan in the severely-affected dKO mouse.[184]

Several other approaches have been explored for utrophin upregulation in pre-clinical models including CRISPRa,[158] deletion of miRNA target sites in the *UTRN* 5′ UTR,[185] and artificial transcription factors.[186]

Notably, there are important differences in the functionality of utrophin and dystrophin, which suggest that the former may be insufficient to fully compensate for the absence of the latter. Specifically, utrophin is not capable of anchoring nNOS at the sarcolemma,[187] or rescue the disordered pattern of the microtubule network that is observed in dystrophic myofibers.[188] Furthermore, there is also evidence that the functional benefit of utrophin minigenes is substantially less than that of full-length dystrophin.[173]

[H1] Other Therapeutic Approaches

A multitude of other disease-modifying approaches for DMD have been investigated which target the various pathological features of DMD. These include anti-inflammatory, vasodilating NO-donor, modulation of Ca^{2+} handling, anti-fibrotic, antioxidant, and myostatin pathway blockade strategies. A detailed discussion of these strategies is beyond the scope of this review, and they have been reviewed elsewhere.[189,190] However, two promising strategies are of note, given their interesting and distinct mechanisms of action. Givinostat is a small molecule histone deacetylase inhibitor (HDACi) developed by Italfarmaco as an epigenetic therapy for DMD. A recently completed randomized, double-blind, placebo-controlled phase III trial of givinostat (NCT02851797, EPIDYS) reported slowed disease progression in ambulant DMD boys in the treatment arm. Eighteen months of givinostat treatment was reported to result in improved performance (i.e. reduced decline) in timed

function tests, muscle strength analysis, and fat infiltration in the vastus lateralis muscle as measured by magnetic resonance spectroscopy, according to a press release from Italfarmaco.[191] HDAC activity is elevated in dystrophic muscle as a consequence of impaired NO signalling,[192] resulting in widespread chromatin level alterations in gene expression that contribute to DMD pathology. Previously, givinostat-mediated HDAC inhibition was shown to improve muscle histopathology in the *mdx* mouse,[19] and in a phase I/II clinical trial (NCT01761292) in DMD boys.[193]

Edgewise Therapeutics is developing EDG-5506, an orally bioavailable, small molecule inhibitor of myosin that is specific to type II (fast twitch) fibers while not active against type I (slow twitch) fibers. A phase II clinical trial of EDG-5506 in DMD patients (NCT05540860, LYNX) is currently recruiting. Fast twitch myofibers are more susceptible to contraction-induced damage in DMD,[194,195] and individuals with inactivating variants in fast myosin (i.e. *MYH2*) exhibit mild proximal muscle weakness and typically do lose ambulation.[196] As such, EDG-5506 is designed to protect dystrophic muscle by inhibiting the contraction of fast twitch myofibers, and thereby paradoxically reducing muscle strength. Edgewise is pursuing clinical trials in both DMD and BMD patients.[197]

[H1] Combination Therapy

It is increasingly apparent that there will likely be no ‘one size fits all’ therapy for DMD. The diversity of DMD-causing genetic insults means that a degree of personalization will be required to address specific mutation types. However, dystrophin restoration alone may also be insufficient to correct the disease in patients with established pathology. For example, the progressive decline in muscle quality that is the result of chronic inflammation and fibro/fatty degeneration means that there may be relatively few fibers present in which to restore dystrophin at the time that treatment is administered. As such, combination therapies capable of simultaneously restoring dystrophin expression and addressing the downstream molecular and cellular pathologies occurring in dystrophic muscle are desirable.[198] Notably, the majority of novel DMD treatments are technically combination therapies as most patients are subject to chronic steroid regimens. However, there is a paucity of pre-

clinical studies on the combination of experimental therapies with clinically-relevant glucocorticoid co-treatment.[199]

While restoration of dystrophin protein expression by exon skipping has now been reported in numerous pre-clinical studies, achieving the kind of protein levels observed in dystrophic animals in human patients constitutes a significant challenge. The combination of various dystrophin restoration strategies may lead to a synergistic benefit. For example, the combination of ASO-mediated, and AAV-U7-snRNA-mediated exon skipping strategies was shown to lead to reduced AAV vector loss and prolonged dystrophin expression.[200] AAV is a single-stranded DNA virus, and so second strand synthesis is required before a therapeutic transgene can be expressed. This results in a time delay between injection of the virus and therapeutic dystrophin rescue, during which time the muscle turnover associated with dystrophic pathology results in the loss of AAV vector genomes from treated muscle, and therefore a reduction in therapeutic efficacy.[129] Pre-treatment of *mdx* mice with antisense oligonucleotides (PPMO conjugates) resulted in a transient restoration of high levels of dystrophin and concomitant stabilization of muscle turnover, after which the therapeutic AAV vectors were injected 2 weeks later.[200] This pre-treatment strategy resulted in a ten-fold increase in dystrophin protein expression when compared to AAV-treated mice alone (measured after 6 months).[200] Importantly, PPMO pre-treatment also enhanced AAV-mediated micro-dystrophin therapy,[200] meaning that this combination strategy is likely to be beneficial for enhancing any therapy which relies on AAV transduction. It will be interesting to determine if such an approach can be used to improve the efficacy of CRISPR-Cas9-mediated dystrophin recovery.

Conventional pharmacological means have also been used to enhance the efficacy of exon skipping, including co-treatment with so-called skipping enhancer drugs like dantrolene,[201] and with various HDAC inhibitors.[202]

Similarly, the possibility of combining dystrophin re-expression and utrophin up-regulation has also been investigated in the *mdx* mouse.[203] Genetic overexpression of utrophin combined with PPMO

treatment resulted in a complete restoration of muscle function to wild-type levels that was not observed for either approach in isolation (as measured by force drop measurements in isolated extensor digitorum longus muscles).[203] Both dystrophin and utrophin can co-exist at the sarcolemma suggesting that this approach could be used in DMD patients. However, very high levels of utrophin transgene expression in a wild-type mouse resulted in a decrease in dystrophin expression, suggesting that these two proteins compete for a finite number of occupancy sites at the sarcolemma.[203] However, this is not likely to be an issue for therapy as (i) such high levels of utrophin are very unlikely to be achieved, and (ii) the two proteins co-localize at the sarcolemma when expressed at levels which prevent pathology in the mouse.

Cell therapy has also been combined with AAV-micro-dystrophin therapy in the dystrophic CXMD$_J$ dog model.[204] Co-treatment of AAV with bone marrow-derived mesenchymal stromal cells (MSCs) resulted in an improvement in the dystrophic phenotype in the single dog tested, which was attributed to the immunomodulatory properties of the MSCs.[204]

Dystrophin restoration therapies might be augmented via combination with a microRNA (miRNA) inhibition strategy. miRNAs are small RNA molecules that typically regulate gene expression by binding to partially complementary mRNAs and repressing translation and/or inducing target transcript degradation.[205] For example, miR-31 is highly upregulated in dystrophic muscle[13,206,207] and has a target site in the 3′ untranslated region (UTR) of the dystrophin mRNA.[208] This miRNA:target interaction is not expected to have any effect in the dystrophic condition, as dystrophin protein is not expressed. However, when dystrophin expression is restored via exon skipping, the high levels of miR-31 limit the degree of protein recovery.[208] Inhibition of miR-31 using expressed miRNA sponges or anti-miRNA oligonucleotides resulted in enhanced dystrophin rescue after exon skipping using the U1 snRNA system in the *mdx* mouse.[208] Similarly, miRNA regulation of dystrophin expression was shown to account for differences in dystrophin protein levels between BMD patients with varying levels of disease severity,[209] suggesting that miRNA inhibition might be further exploited to maximize dystrophin rescue in a therapeutic context. Notably, miRNAs which regulate utrophin expression have

also been identified,[210] and masking of a let-7c site on the utrophin 3′ UTR has been shown to induce functional improvement in the *mdx* mouse by inducing utrophin protein up-regulation.[211] Another miRNA, miR-29, has been shown to supress the expression of pro-fibrotic factors in dystrophic muscle, such that synthetic mimics of this miRNA could be utilized for therapeutic purposes. However, the combination of such an approach with a dystrophin restoration therapy has not been tested to date.

Many other approaches have combined dystrophin restoration with strategies to improve muscle quality. For example, co-delivery of two AAV vectors, one encoding micro-dystrophin and the other encoding the muscle isoform of insulin like growth factor 1 (*Igf1*), resulted in synergistic benefits that were not observed for either vector in isolation.[212] Specifically, stabilization of myofiber turnover and protection against contraction induced injury (attributed to expression of micro-dystrophin) was accompanied by an increase in muscle mass and strength (attributed to the *Igf1* transgene).[212] The combination of exon skipping and myostatin blockade (to increase muscle mass) has also been explored in several studies.[213–215] However, myostatin blockade strategies have so far proven ineffective in DMD clinical trials, which is likely due to the already low circulating levels of myostatin in DMD patients.[216]

[H1] Conclusions and Perspectives

The genetic insult underlying DMD is relatively simple, and yet the goal of restoring gene expression has proven to be a substantial challenge. While there are multiple drugs that have achieved marketing authorization in various jurisdictions, these are applicable to only small subsets of patients, and expert opinion on whether these offer therapeutic benefit is mixed. It is clear that better therapies are still needed. Improved ASO delivery technologies based on peptide, antibody, and Fab fragment ASO-bioconjugation strategies, together with novel nucleic acid chemistries, have the potential to overcome the low efficacy of naked PMO-based ASOs, although renal toxicity must be considered carefully. By contrast, therapies based on gene replacement, utrophin upregulation, and disease modifying

approaches are in theory ‘mutation agnostic’ with widespread applicability. However, this notion has been challenged recently, as patients with certain mutation types may be susceptible to anti-transgene T cell immune toxicity. Furthermore, the high diversity of DMD causing mutations means that personalized medicine approaches will likely be needed to treat all patients. In many cases, there may be insufficient patients to undertake conventional clinical trials. Alternative approaches include the use of Bayesian statistics to predict the pathological trajectory within a single patient, or *n*=1 clinical trials.

The success of dystrophin restoration therapies is likely to be dependent on three key factors: (i) That the total amount of dystrophin restored. Evidence from pre-clinical models suggests that >15% of wild-type levels are required for functional correction,[217] and levels greater than 10-20% were associated with less severe pathology in BMD patients.[218,219] (ii) The quality of dystrophin produced. Therapies such as exon skipping, micro-dystrophin gene therapy, and CRISPR exon deletion/skipping result in generation of internally-truncated pseudo-dystrophins that are expected to exhibit reduced functionality relative to full-length wild-type dystrophin. The degree of internal truncation differs between therapeutic strategies, and so should be considered carefully. (iii) The correct localization of dystrophin at the sarcolemma. We have recently demonstrated the importance of uniform sarcolemmal dystrophin for stabilizing turnover in dystrophic muscle.[220] This is important because the various dystrophin restoration strategies can lead to distinct patterns of sarcolemmal coverage.[162,221]

While it is often assumed that re-expression of functional dystrophin protein in the muscles of DMD patients is presumed to correct the disease, combination therapies may be required that are able to both correct the initial genetic insult and to address the myriad molecular pathologies occurring in dystrophic muscle. This is especially important in the case of patients with low overall muscle quality and as a consequence of established pathologies/chronic disease. However, performing clinical trials for combination therapies is likely to be highly complex, especially if the individual therapies to be combined are only minimally efficacious in isolation. It is therefore likely that such approaches

therapies will necessarily consist of already-approved single therapies. Facilitating such a therapy may require cooperation between pharmaceutical companies with distinct intellectual portfolios.

It is likely that the micro-dystrophin gene therapy SRP-9001 developed by Sarepta will be the next treatment to achieve marketing approval for DMD. The approval of a gene therapy product for DMD would constitute an enormous step forward for the DMD field, and would follow-on from the highly successful drug zolgensma for the treatment of SMA. While zolgensma is a truly life-changing treatment, there has so far be no equivalent breakthrough for DMD. Importantly, early therapeutic intervention in SMA has been shown to be much more effective than treatment later in life.[222] Whether such early intervention, before the onset of pathology, in the case of DMD is beneficial is currently unknown. Identifying applicable DMD patients for early treatment/trial participation would necessitate the implementation of newborn screening programmes. Notably, there is delay in diagnosis of 2.2 years for DMD patients, which has largely remained unchanged over the last 3 decades.[223] Implementation of newborn screening has the potential to identify patients for early treatment, and decrease this diagnostic delay.

In conclusion, there are a plethora of molecular medicine approaches that are under investigation, or approved for use, in DMD patients. However, there is still a need for improved therapies with higher efficacy and which are applicable to a wider group of patients. In particular, treatments that can correct cardiac pathology are needed to maximally benefit patients. Additionally, treatments that are effective for the whole lifetime of a patient, or which can at least be repeat administered are also desirable. Knowledge gained from current drug development programmes, and especially from clinical data, will be crucial for the ongoing development of therapies for DMD, but will also be highly useful in the development of treatments for other disease indications.

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Author contributions

The manuscript was conceived by KED and TCR. The first draft was written by TCR. All authors researched data for the article. All authors contributed substantially to discussion of the content and edited the manuscript before submission.

Competing interests

KED is a member of the scientific advisory board of Sarepta Therapeutics. MJAW is an advisor and shareholder in PepGen Ltd and Evox Therapeutics. TCR declares no financial competing interests.

Display Items

Name	Company	Chemistry	Target Exon	Approval/ stage
Eteplirsen	Sarepta Tx	PMO	51	**FDA**
Viltolarsen	NS Pharma	PMO	53	**FDA/Japan**
Golodirsen	Sarepta Tx	PMO	53	**FDA**
Casmiersen	Sarepta TX	PMO	45	**FDA**
Vesleteplirsen	Sarepta TX	PPMO (R_6Gly)	51	Phase II
WVE-N531	Wave Life Sciences	PS/PN stereoselective	53	Phase Ib/II
Renadirsen	Daiichi Sankyo	2'OMe/ENA mixmer	45	Phase II
AOC 1044	Avidity Bioscience	PMO-Antibody conjugate	44	Phase I/II
DYNE-251	Dyne Tx	PMO-FAb fragment conjugate	51	Phase I/II
ENTR-601-44	Entrada Tx	PPMO (EEV)	44	Pre-clinical
PGN-EDO51	PepGen Ltd	PPMO (EDO)	51	Phase I
SQY51	SQY Tx	Tricylco-DNA	51	Phase I/II (in 2023)

Table 1

Exon skipping drugs: approved and in clinical development.

Name	Company	Micro-dystrophin	AAV Serotype	Promoter	Approval/ stage
SRP-9001	Sarepta Tx	ΔR4-23/ΔCT	AAVrh74	MHCK7	Phase III/BLA
PF-06939926	Pfizer	ΔR3-19/20-21/ΔCT	AAV9	hMSP	Phase III
SGT-101	Solid Bio	ΔR2-15/R18-22/ΔCT	AAV9	CK8	Phase I/II
GNT 0004	Sarepta Tx/Genethon	Undisclosed	AAV8	Undisclosed	Phase I/II/III
RGX-202	REGENXBIO	Undisclosed (Includes CT)	AAV8	Spc5-12	Phase I/II

Table 2

Micro-dystrophin gene replacement therapies in clinical development.

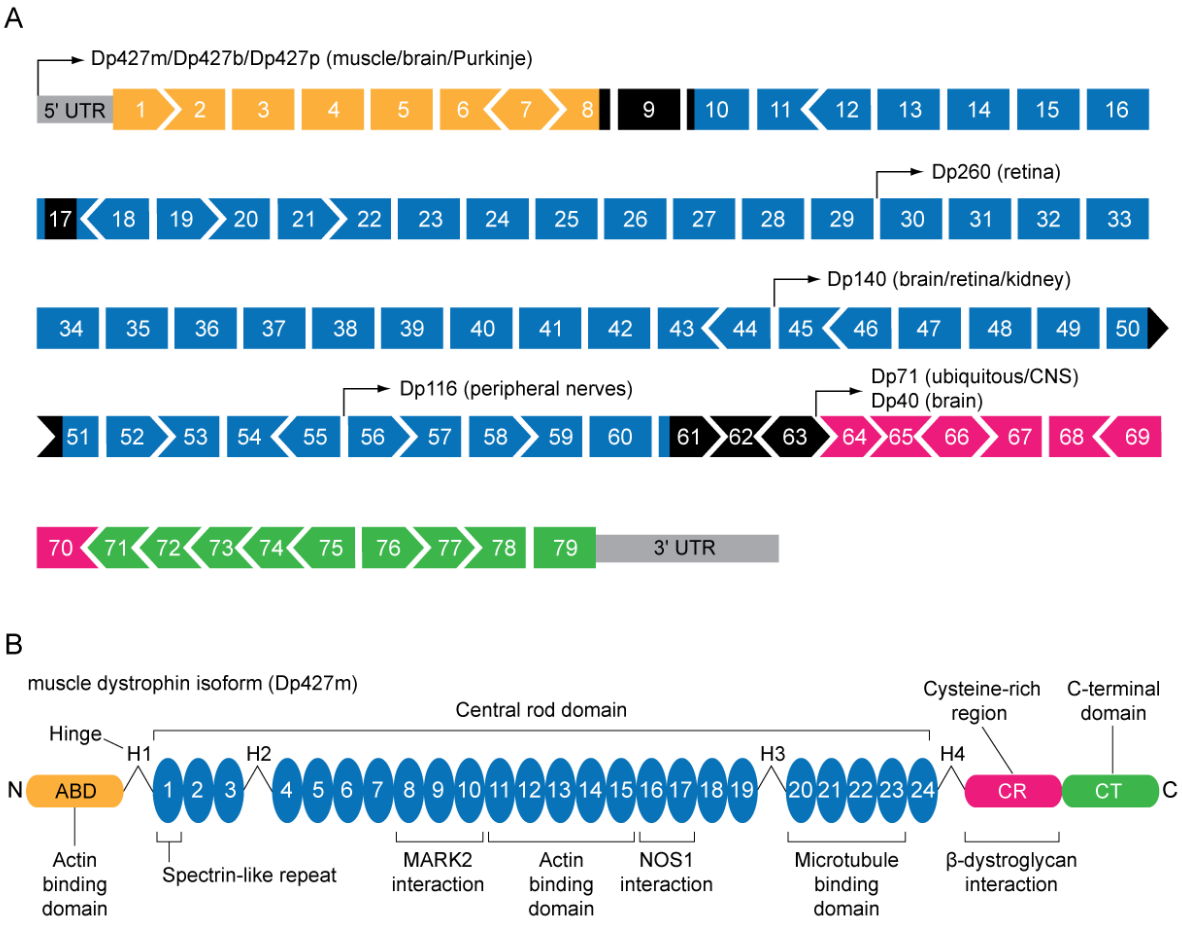
A
Dp427m/Dp427b/Dp427p (muscle/brain/Purkinje)
5' UTR
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
Dp260 (retina)
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
Dp140 (brain/retina/kidney)
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
Dp116 (peripheral nerves)
Dp71 (ubiquitous/CNS)
Dp40 (brain)
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
66
67
68
69
70
71
72
73
74
75
76
77
78
79
3' UTR
B
muscle dystrophin isoform (Dp427m)
Central rod domain
Hinge
H1
H2
H3
H4
Cysteine-rich region
C-terminal domain
N
ABD
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
CR
CT
C
Actin binding domain
Spectrin-like repeat
MARK2 interaction
Actin binding domain
NOS1 interaction
Microtubule binding domain
β-dystroglycan interaction

Figure 1

The dystrophin gene and protein.

(**A**) Schematic of *DMD* gene. Each exon is color-coded according to the protein domain that they encode: orange for the actin binding domain (ABD), blue for the central rod domain, black for hinge regions, magenta for the Cysteine-rich region (CR), and green for the C-terminal domain (CT). Exon shapes indicate how the triplet base code is distributed across the exons such that an in-frame mature DMD transcript is produced. The locations of the transcription start sites for the various dystrophin protein isoforms are indicated. (**B**) Structure of the full-length muscle isoform of dystrophin (Dp427m). Key interactions between dystrophin and other binding partners are indicated.

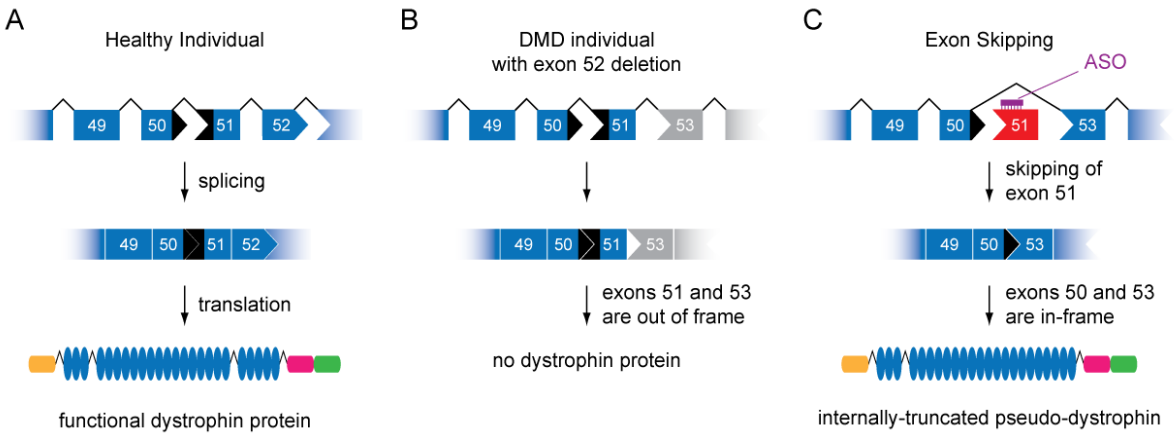
A
Healthy Individual
49
50
51
52
splicing
49
50
51
52
translation
functional dystrophin protein
B
DMD individual
with exon 52 deletion
49
50
51
53
49
50
51
53
exons 51 and 53
are out of frame
no dystrophin protein
C
Exon Skipping
ASO
49
50
51
53
skipping of
exon 51
49
50
53
exons 50 and 53
are in-frame
internally-truncated pseudo-dystrophin

Figure 2

Restoration of dystrophin expression by exon skipping.

(**A**) In a healthy individual, the *DMD* gene undergoes splicing to excise intronic regions from the mature *DMD* mRNA transcript. A functional dystrophin protein is generated via the translation of this transcript. The schematic shows a zoomed-in region of the DMD gene/transcript covering exons 48 to 53, which encodes spectrin-like repeat domains (shown in blue) and a hinge region (H3, shown in black). (**B**) In DMD individuals, mutations (often whole exon deletions) disrupt the translation reading frame of the *DMD* transcript. Here a relatively common DMD-causing mutation is shown, in which exon 52 is deleted. As a result, exons 51 and 53 are out-of-frame, leading to failure to generate dystrophin protein. Out-of-frame exons are shown in grey. (**C**) Treatment with an antisense oligonucleotide (ASO) targeting an exon splicing enhancer motif in DMD exon 51 induces skipping of this exon by effectively hiding it from the spliceosome. As a result exon 50 and exon 53 are spliced together, resulting in restoration of the dystrophin translation reading frame. Following translation, an internally-truncated (i.e. lacking the H3 domain) pseudo-dystrophin is generate that retains a degree of functionality.

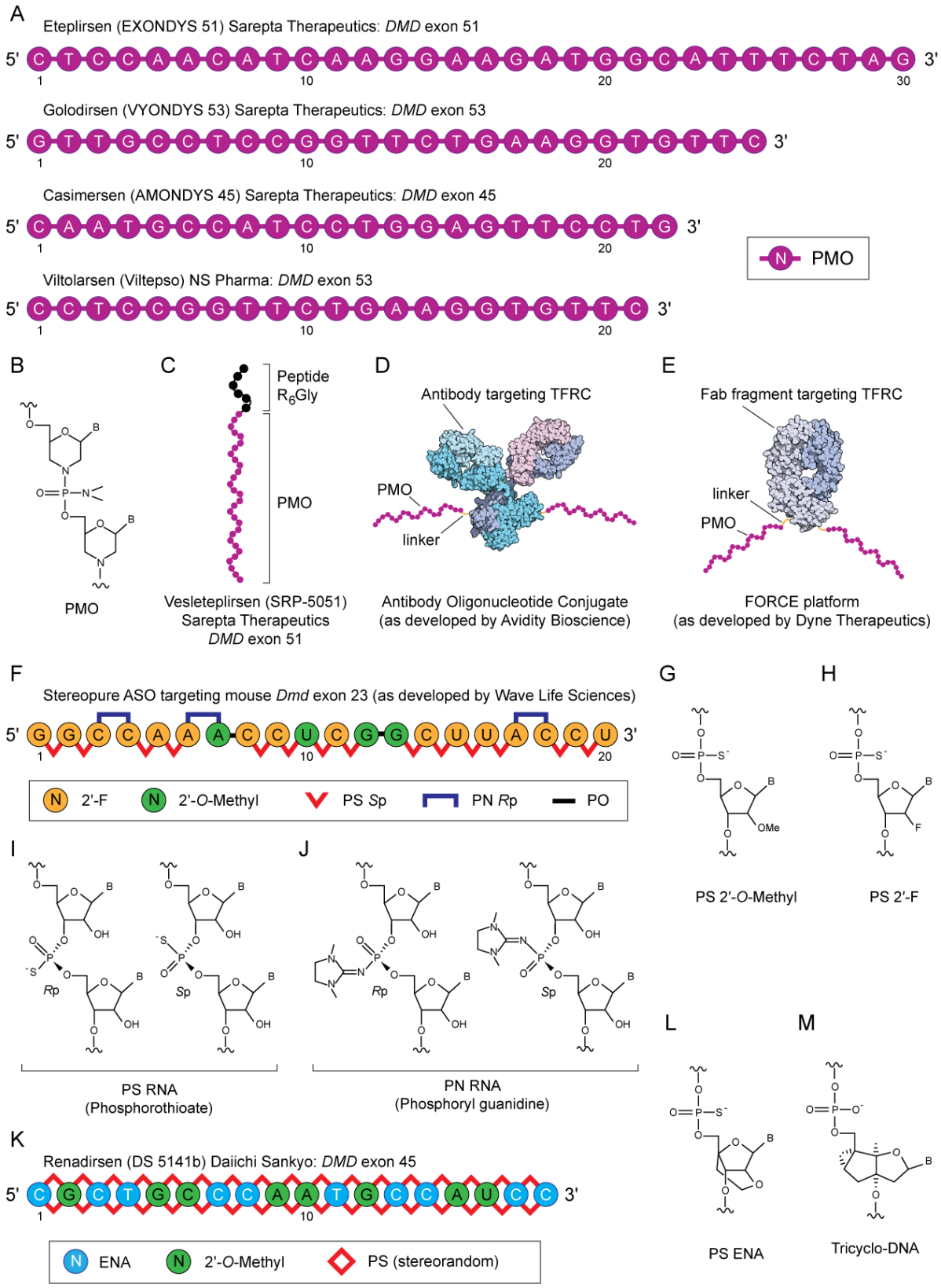
A
Eteplirsen (EXONDYS 51) Sarepta Therapeutics: DMD exon 51
5' C T C C A A C A T C A A G G A A G A T G G C A T T T C T A G 3'
1 10 20 30
Golodirsen (VYONDYS 53) Sarepta Therapeutics: DMD exon 53
5' G T T G C C T C C G G T T C T G A A G G T G T T C 3'
1 10 20
Casimersen (AMONDYS 45) Sarepta Therapeutics: DMD exon 45
5' C A A T G C C A T C C T G G A G T T C C T G 3'
1 10 20
Viltolarsen (Viltepso) NS Pharma: DMD exon 53
5' C C T C C G G T T C T G A A G G T G T T C 3'
1 10 20
N PMO
B
PMO
C
Peptide
R6Gly
PMO
Vesleteplirsen (SRP-5051)
Sarepta Therapeutics
DMD exon 51
D
Antibody targeting TFRC
PMO
linker
Antibody Oligonucleotide Conjugate
(as developed by Avidity Bioscience)
E
Fab fragment targeting TFRC
linker
PMO
FORCE platform
(as developed by Dyne Therapeutics)
F
Stereopure ASO targeting mouse Dmd exon 23 (as developed by Wave Life Sciences)
5' G G C C A A A C C U C G G C U U A C C U 3'
1 10 20
N 2'-F
N 2'-O-Methyl
PS Sp
PN Rp
PO
G
OMe
PS 2'-O-Methyl
H
F
PS 2'-F
I
Rp
Sp
PS RNA
(Phosphorothioate)
J
Rp
Sp
PN RNA
(Phosphoryl guanidine)
K
Renadirsen (DS 5141b) Daiichi Sankyo: DMD exon 45
5' C G C T G C C C A A T G C C A U C C 3'
1 10
N ENA
N 2'-O-Methyl
PS (stereorandom)
L
PS ENA
M
Tricyclo-DNA

Figure 3

Antisense oligonucleotide therapies for Duchenne muscular dystrophy.

(**A**) Chemical composition of FDA-approved exon skipping phosphorodiamidate morpholino oligonucleotide (PMO) compounds (eteplirsen, golodirsen, casimersen, and viltolarsen, developed by Sarepta Therapeutics and NS Pharma). (**B**) Chemical structure of PMO chemistry. Schematics of; (**C**) a peptide-PMO (PPMO) conjugate (vesleteplirsen, developed by Sarepta), (**D**) an antibody-PMO conjugate (developed by Avidity Bioscience), (**E**) a Fab fragment-PMO conjugate (developed by Dyne Therapeutics). (**F**) Chemical composition of a stereopure ASO targeting mouse *Dmd* exon23 developed by Wave Life Sciences. This compounds also includes phosphodiester (PO) linkages, and is chemically similar to WVE-N531 (exact composition not publicly disclosed). Chemical structure of; (**G**) phosphorothioate 2′-*O*-Methyl RNA (PS 2′-*O*-Methyl), and (**H**) phosphorothioate 2′-fluoro RNA (PS 2′-F), (**I**) *R*p and *S*p stereoisomers of phosphorothioate (PS) linkages, and (**J**) *R*p and *S*p stereoisomers of phosphoryl guanidine (PN) linkages. (**K**) Chemical composition of renadirsen (developed by Daiichi Sankyo). **(**I) Chemical structure of; (**L**) phosphorothioate 2'-O,4'-C-ethylene-bridged nucleic acid (PS ENA) and (**M**) tricyclo-DNA. The IgG (1IGY) and Fab fragment (5FUZ) (Fab fragment) structures were downloaded from the Protein Data Bank.

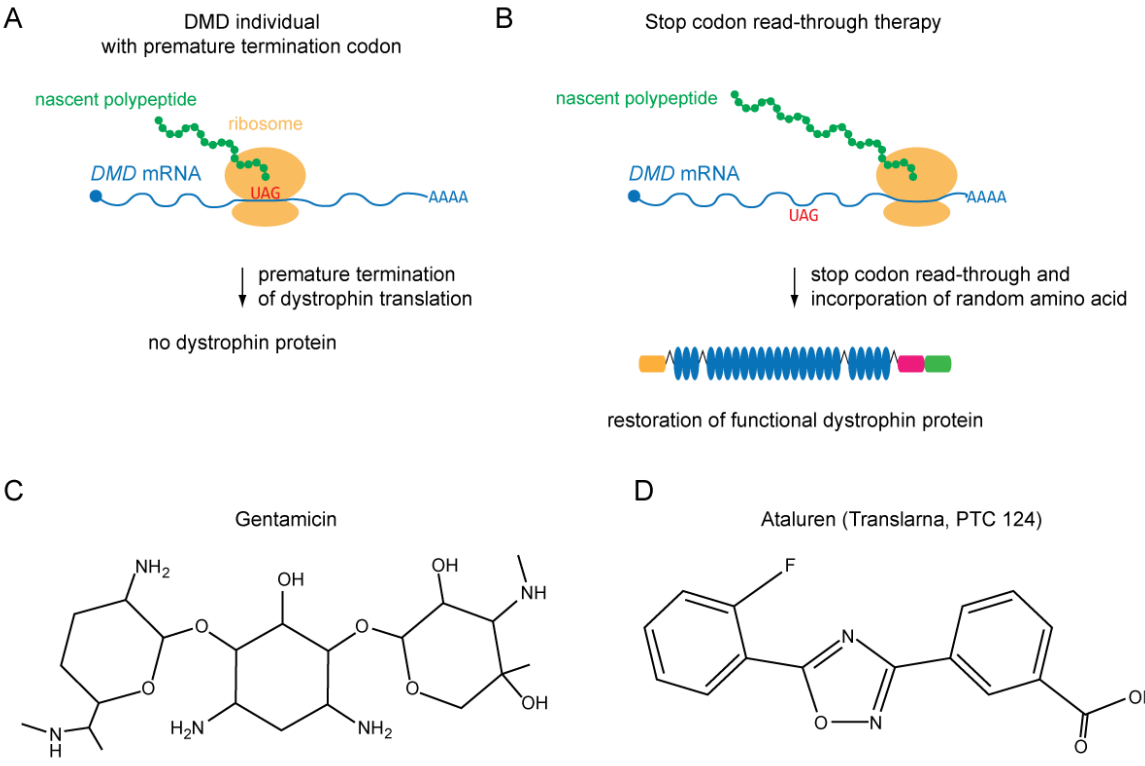
A
DMD individual
with premature termination codon
nascent polypeptide
ribosome
DMD mRNA
UAG
AAAA
premature termination
of dystrophin translation
no dystrophin protein
B
Stop codon read-through therapy
nascent polypeptide
DMD mRNA
UAG
AAAA
stop codon read-through and
incorporation of random amino acid
restoration of functional dystrophin protein
C
Gentamicin
D
Ataluren (Translarna, PTC 124)

Figure 4

Restoration of dystrophin by stop codon read-though therapy.

(**A**) Nonsense mutations that introduce premature stop codons in the DMD mRNA result in premature termination of translation by the ribosome and a failure to generate full-length, functional dystrophin protein. (**B**) Treatment with a stop codon read-through drug (e.g. ataluren) results in the incorporation of a random amino acid at the premature termination codon site. As such, the ribosome can process through the premature stop codon and expression of full-length dystrophin protein is restored. Chemical structures of (**C**) gentamcin, and (**D**) ataluren (developed by PTC Therapeutics).

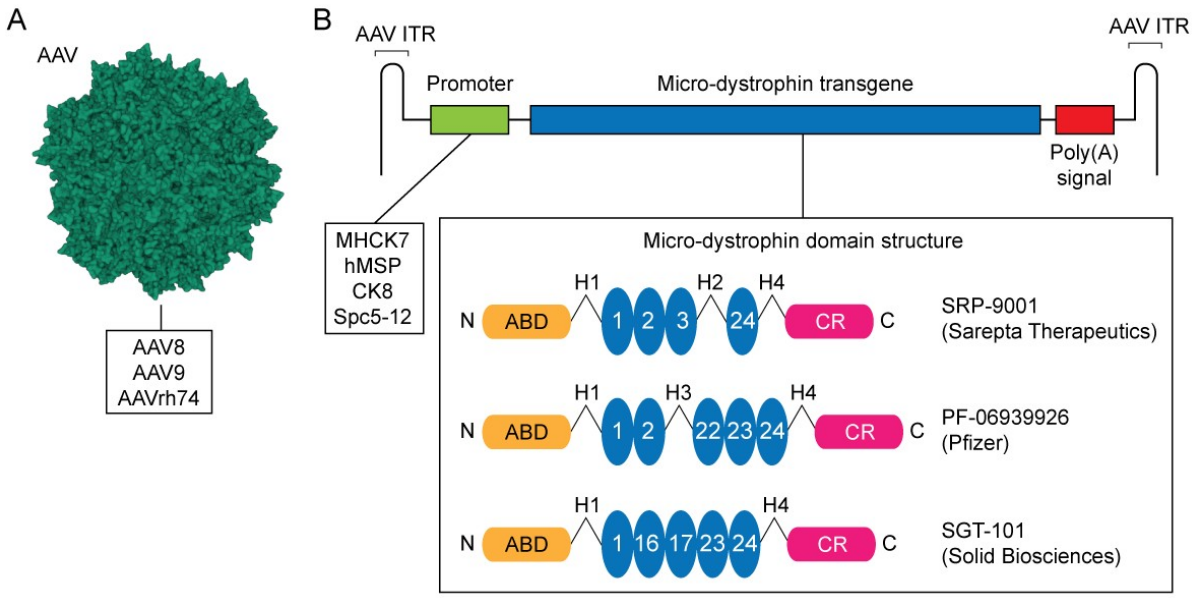
A
AAV
AAV8
AAV9
AAVrh74
B
AAV ITR
Promoter
Micro-dystrophin transgene
AAV ITR
Poly(A)
signal
MHCK7
hMSP
CK8
Spc5-12
Micro-dystrophin domain structure
N
ABD
H1
1
2
3
H2
24
H4
CR
C
SRP-9001
(Sarepta Therapeutics)
H3
22
23
PF-06939926
(Pfizer)
16
17
SGT-101
(Solid Biosciences)

Figure 5

Micro-dystrophin gene replacement therapy.

(**A**) Micro-dystrophin gene therapy vectors consist of adeno-associated viral (AAV) particles of which there are a number of muscle-tropic serotypes. (**B**) Schematic of micro-dystrophin AAV genomes showing the flanking inverted terminal repeat (ITR) regions, a muscle-specific promoter, the micro-dystrophin transgene, and a Poly(A) transcription termination signal. Promoter variants and micro-dystrophin domain structures are indicated for three of the gene therapy products currently in clinical development. The AAV particle structure (4RSO) was downloaded from the Protein Data Bank.