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2. 実用新案登録
なし

3. その他、特記事項
なし

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The American Journal of Pathology, Vol. 179, No. 1, July 2011

DOI: 10.1016/j.ajpath.2011.03.050

Review

Restoring Dystrophin Expression in Duchenne Muscular Dystrophy Muscle

Progress in Exon Skipping and Stop Codon Read Through

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The identification of the Duchenne muscular dystrophy gene and protein in the late 1980s led to high hopes of rapid translation to molecular therapeutics. These hopes were fueled by early reports of delivering new functional genes to dystrophic muscle in mouse models using gene therapy and stem cell transplantation. However, significant barriers have thwarted translation of these approaches to true therapies, including insufficient therapeutic material (eg, cells and viral vectors), challenges in systemic delivery, and immunological hurdles. An alternative approach is to repair the patient's own gene. Two innovative small-molecule approaches have emerged as front-line molecular therapeutics: exon skipping and stop codon read through. Both approaches are in human clinical trials and aim to coax dystrophin protein production from otherwise inactive mutant genes. In the clinically severe dog model of Duchenne muscular dystrophy, the exon-skipping approach recently improved multiple functional outcomes. We discuss the status of these two methods aimed at inducing *de novo* dystrophin production from mutant genes and review implications for other disorders. ***(Am J Pathol 2011, 179:12–22; DOI: 10.1016/j.ajpath.2011.03.050)***

Dystrophin Replacement: From the Outside, or Inside?

Duchenne muscular dystrophy (DMD) is the most common neuromuscular disease and affects all world populations equally. The cause of this genetic disease is loss of a single protein, dystrophin, in all types of muscle (ie, skeletal, cardiac, and smooth) and in neurons.[1,2] The loss of protein function is the consequence of mutations in the large *DMD* gene. The gene contains 79 exons distributed over 2.3 million bp of genetic real estate on the *X* chromosome; however, only approximately 14,000 bp (<1%) is used for translation into protein (coding sequence).[3] The 99.5% of intronic junk must be spliced out of the 2.3 million bp initial heteronuclear RNA transcript to lead to the mature 14,000 bp mRNA that includes all key information for dystrophin protein production. Patients with DMD have mutations in the gene that prevent the appropriate construction of the mRNA and/or the production of the dystrophin protein, and all patients with DMD show marked dystrophin deficiency in their muscle.[4]

During the past 25 years since gene and protein identification, dozens of innovative experimental therapeutic approaches for DMD have emerged; many are transitioning to clinical trials. These include slowing the progression of the disease by immune modulators (eg, steroids

Supported by The Foundation to Eradicate Duchenne, CureDuchenne, the Department of Defense (Congressionally Directed Medical Research Program; grant W81XWH-09-1-0599), and grants from the National Institutes of Health (R24HD050846; P50AR060836).

Accepted for publication March 21, 2011.

Disclosures: A.A.L. is employed by Santaris Pharma A/S, which develops oligonucleotide therapeutics for diseases other than DMD; is a consultant on a fee-for-services basis for Prosensa; was formerly in a consulting relationship with AVI BioPharma; and is shareholder in Isis Pharmaceuticals, which is an oligonucleotide therapeutics company not presently working on DMD.

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and transforming growth factor-β inhibitors), inducing or introducing proteins that may compensate for dystrophin deficiency in the myofiber (eg, utrophin, biglycan, and laminin), or bolstering the muscle's regenerative response (eg, myostatin and activin 2B). A parallel approach places dystrophin back into patient muscle.

There are two general tactics to introducing dystrophin back into dystrophin-deficient muscle: introducing a new more functional gene into the patient or repairing the patient's own gene in some manner. Gene therapy using viral vectors[5,6] and stem cell transplants[7] has been used for exogenous gene delivery. Despite extensive research, including limited clinical trials,[8,9] these approaches have failed to produce clinically significant levels of dystrophin in the muscle of patients with DMD. Key obstacles include delivery problems [ie, getting the stem cell or viral vector to the right place in the large target organ (muscle)], immunological barriers, and production issues (obtaining adequate amounts of cells or viruses to treat a patient). Therefore, clinical progress in gene therapy and cell transplantation has been slow.

On the other hand, approaches to coax dystrophin production out of the patient's own disabled gene have been more impressive. A key to the more rapid advance is the development of small-molecule drugs for gene repair that overcome problems with target organ delivery, production, and immune response.

In this review, we discuss progress and the remaining hurdles in small-molecule drug approaches for gene repair in DMD.

Turning Duchenne into Becker: Exon Skipping

With the characterization of the dystrophin gene, it was quickly recognized that patients with a clinically milder dystrophy, Becker muscular dystrophy, showed mutations of the same dystrophin gene as boys with Duchenne dystrophy.[10,11] The molecular explanation for the often dramatic clinical differences was framedness. Although the muscle of patients with DMD could not put together what was left of the dystrophin gene into a serviceable (translatable) mRNA (it was out of frame), patients with Becker dystrophy had mutations in which the rest of the gene could still be used effectively and produce translatable mRNA (in frame).

A model for therapeutics emerged in which a patient diagnosed as having clinically severe DMD might be converted to having the milder Becker dystrophy at the molecular level, by restoring the framedness [eg, turning an out-of-frame mutation into an in-frame (multiple of three) mutation]. This occurred spontaneously in some patients with DMD who appeared to have a frameshift nonsense mutation on genomic DNA but were able to rescue some dystrophin production by skipping an additional exon, bringing the resulting mRNA back into frame.[12–14] The same spontaneous exon-skipping process is observed in many muscle biopsy specimens from patients with DMD and in *mdx* mouse muscle in the form of revertant fibers [ie, a small proportion (<1%) of strikingly positive myofibers in a background of complete dystrophin deficiency].[15–17]

The therapeutic strategy using this concept was dubbed exon skipping, in which antisense oligonucleotides (AOs) were designed to modulate the splicing of the dystrophin gene of a patient with DMD, resulting in mRNA transcripts that are Becker-like (ie, able to make some level of functional dystrophin) (Figure 1). AOs are short nucleic acid sequences designed to selectively bind to specific mRNA or pre-mRNA sequences to generate small double-stranded regions of the target mRNA. By binding to these critical regions and forming double strands at key sites where the spliceosome or proteins of the spliceosome would normally bind, the mutated (frameshifting) exons are skipped and the remainder of the pre-mRNA is edited correctly in frame, albeit shorter. AOs were designed to target specific exons (eg, exon 51 drug PRO051 in Figure 1) and tested in the *mdx* mouse model[19,20] and then in cultures of muscle from patients with DMD.[21] In these systems, they diffused into the dystrophic myofibers and then into the nucleus, where they bound the unspliced pre-mRNA, modulated splicing, and restored dystrophin expression.

Why Do AOs Work Better in DMD Compared with Other Previous Clinical Applications?

Antisense drug development for human disease has been pursued for approximately 20 years, and AOs have been tested clinically in >90 clinical trials (*http://www.clinicaltrials.gov/ct2/results?term=antisense,* last accessed March 1, 2011). Of these trials, 40 have been completed, involving >2000 patients, targeting cancer, inflammatory disease, and other indications.[22,23] Despite this impressive effort, only one AO has been approved by the Food and Drug Administration (Vitravene, an intraocular injection to inhibit cytomegalovirus retinitis in immunocompromised patients; Isis Pharmaceuticals, Carlsbad, CA), and this drug is no longer marketed.

Why have so many of the AO drug programs failed, and why might AO treatment in DMD work better? Excellent literature reviews have indicated the significant biological barriers to antisense efficacy, including uptake and sequestration in the reticuloendothelial system, significant barriers to achieving sufficiently high intracellular concentrations in target cells because of endothelial, basement membrane, and cell membrane barriers, and intracellular sequestration in phagolysomes or in oligoprotein complexes. In addition, there is the challenging requirement that to produce pharmacological activity, a large fraction of many RNA targets needs knocking down (>90%) before biochemical efficacy is realized.[24] For DMD, the disease itself seems to have navigated some of these major hurdles, with a dramatic improvement in biochemical efficacy relative to other indications. There are two key differences with AO applications to DMD, and these result in an approximate 100-fold improvement in efficacy compared with previous AO applications. First, AO drugs in all other indications are designed to knock down (inhibit) the target, whereas the goal in DMD is to

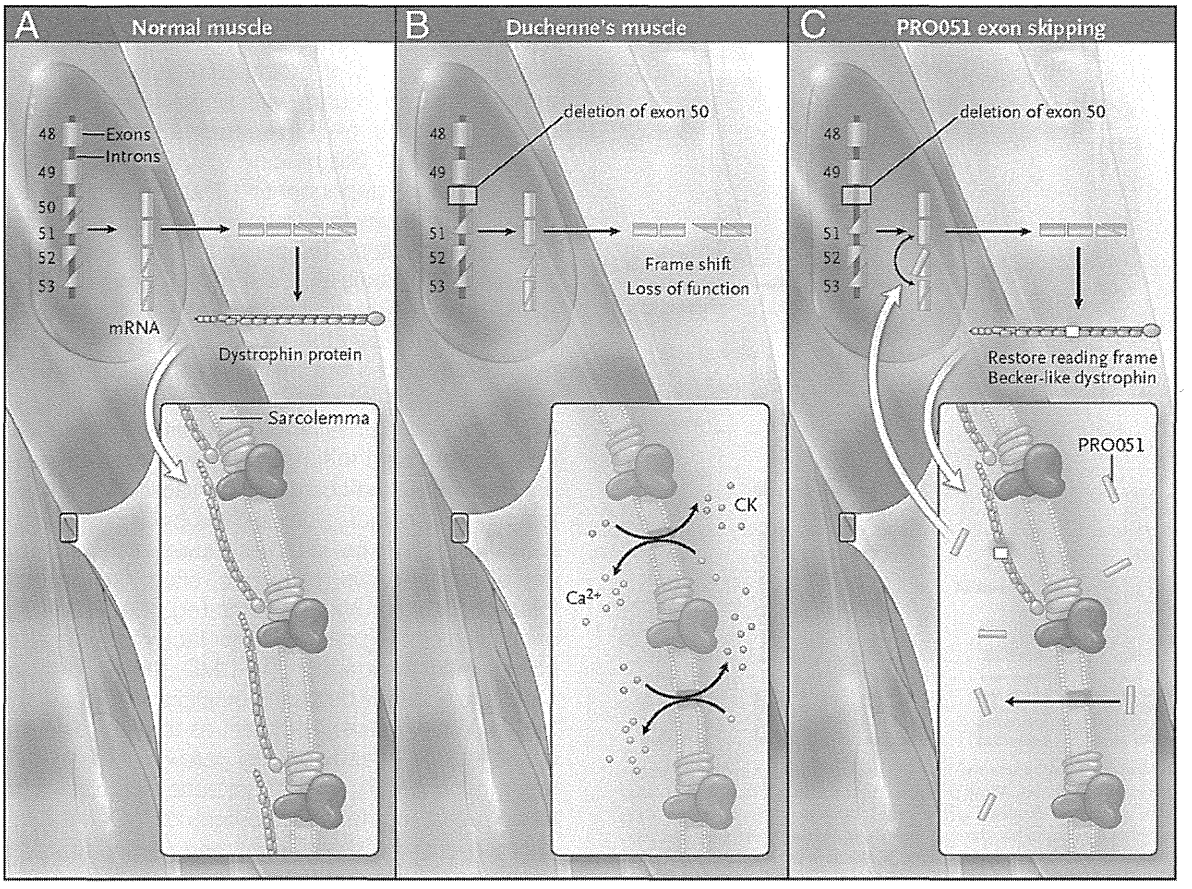

Figure 1. Mechanism of action of AO exon-skipping drugs. **A:** Dystrophin gene splicing in healthy muscle, in which all 79 exons are precisely spliced together to maintain the protein translational reading frame (only exons 48 to 53 are shown). **B:** A patient with DMD with a deletion of exon 50. The remaining exons are spliced together, but there is a disruption of the reading frame, disabling the ability of the mRNA to produce any dystrophin. Consequently, there is a dystrophin deficiency in muscle and unstable plasma membranes. CK indicates creatine kinase. **C:** The mechanism of action of PRO051, an AO drug targeting exon 51. The exon 51 sequence (adjacent to the missing exon 50 sequence) is skipped, so that the mRNA splices exon 49 to 52. The new deletion is able to be translated into semifunctional Becker-like dystrophin, resulting in partial repair of the myofiber plasma membrane. Reproduced with permission from Hoffman (copyright 2007, Massachusetts Medical Society).[18]

rescue (knock up or increase) the target. Second, the membranes of DMD muscle are leaky as a result of the underlying pathophysiological features, facilitating a route of entry for AOs into myofibers.[25] Indeed, i.v.delivered AO's show very poor delivery to normal muscle, while dystrophic muscle or i.m. injection in normal muscle shows excellent delivery (Figure 2).[26]

Regarding previous knockdown AO approaches, it is approximately 10 times harder to effectively knock down a target than it is to knock up a target (as in DMD). In a knockdown model, the goal is to take 100% of the protein down to approximately ≤10% to achieve the desired biochemical loss of function. For example, in cancer, where an oncogene is targeted by an AO, the AO would need to bind approximately 90% of the mRNA target to bring protein expression down to 10% and oncogenic transformations are generally not a single-gene disorder. For DMD, the goal is to restore expression of the target gene to >10%, but this translates into needing to hit only approximately 10% of mRNA targets with the drug (bringing protein expression from 0% to 10%). Thus, knockdowns need to hit 90% of targets, but DMD knock ups need to hit only 10% of targets (a 10-fold difference).

Then, there is an additional advantage of dystrophic muscle providing easier access for the AO into myofibers. All previous AO applications have had trouble achieving adequate concentrations of drug within the cell. The major barrier to AO drugs is the cell plasma membrane. AOs typically do not traverse membranes well, and efforts to make the drugs more cell permeable tend to increase toxicity. Patients with DMD have unstable plasma membranes in their muscle fibers, which effectively provide a leaky entry for drug delivery (Figure 1). Although it is challenging to quantify this delivery advantage in DMD muscle, the cell permeability defect may increase drug delivery by a factor of ≥10. Consistent with this model of unstable membrane delivery, systemic AOs delivered to healthy muscle do not show effective delivery, indicating that the dystrophic process is a requirement for sufficient drug delivery (Figure 2).

Taken together, the 10-fold increase in cell delivery because of unstable membranes and the 10-fold relaxed requirements for hitting mRNA targets cumulatively give AO used in DMD a 100-fold advantage compared with other clinical applications of antisense.

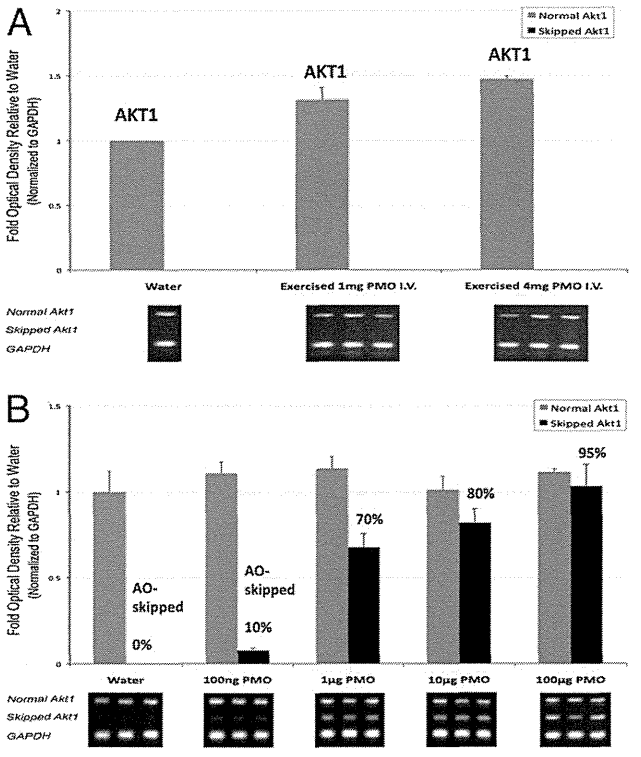

Figure 2. Morpholino AOs achieve myofiber delivery through bulk flow across unstable plasma membranes. Many publications have shown that morpholinos delivered i.v. achieve unexpected efficacy for modulating splicing within dystrophic myofibers, presumably through bulk flow across unstable dystrophic plasma membranes. Herein, we test this model using i.v. versus i.m. delivery of a morpholino in healthy murine muscle. **A:** 0 mg (water) or 1 or 4 mg morpholino was given in an i.v. bolus in healthy mice, and drug delivery to myofibers was assayed by exon skipping in the Akt1 mRNA (skipped Akt1). No detectable exon skipping was observed in healthy skeletal muscle (0%). **B:** As a positive control, the same morpholino was delivered by i.m. injection in saline (0, 0.1, 1, 10, and 100 μg). The saline destabilizes the myofiber membranes, and efficient dose-related exon skipping is observed (skipped Akt1). GAPDH indicates glyceraldehyde-3-phosphate dehydrogenase.

AO Medicinal Chemistry and Preclinical Safety

Organisms have fairly sophisticated inflammatory responses directed against exogenous DNA or RNA. Genetic material coming into the body from the outside is assumed to be infectious; as a result, DNA or RNA is immunostimulatory or proinflammatory. Oligonucleotides activate innate immunity, with single-stranded oligonucleotides binding to toll-like receptor 9 or other receptors of innate immunity. This binding tends to be both sequence and chemistry dependent.[27] Thus, AO drugs must be disguised in a way to circumvent surveillance and inflammatory responses. Typically, this is accomplished by avoiding CG motifs that are more common in bacterial DNA and by using medicinal chemistry that keeps the G, A, T, and C bases the same (so they can bind to the target sequence) but replacing the ribose-phosphodiester backbone with different chemistries (Figure 3) that evade immune surveillance. In addition, medicinal chemistry can be used to further enhance cell uptake. In general, increasing the charge of the backbone increases protein binding, including cell surface binding, making it more likely that the AOs get into cells. However, increased charge can also make AOs more toxic, often through facilitating interactions with other proteins (eg, the tenase complex of intrinsic clotting cascade[28] or factor H in the alternative complement cascade).[29] In DMD, the need to increase charge to enhance delivery is ameliorated (AOs do not have the same cell delivery problem as in other disorders) because there is already a leaky gateway into the cell. There are two commonly used backbone chemistries that are being used in the development of AO for DMD, one charged and the other uncharged (described later), and each has its pros and cons.

2′-O-Methyl Phosphorothioate

Candidate drugs using this chemistry keep the ribose ring intact but add moieties to both the ribose ring and the phosphodiester linkage between riboses in the AO chain. This is the chemistry of choice in the Prosensa/GlaxoSmithKline DMD drug development program (Prosensa Therapeutics, Leiden, the Netherlands). The toxicity and clinical safety of phosphorothioate oligonucleotides as a class have been well characterized in preclinical studies and human clinical trials of candi-

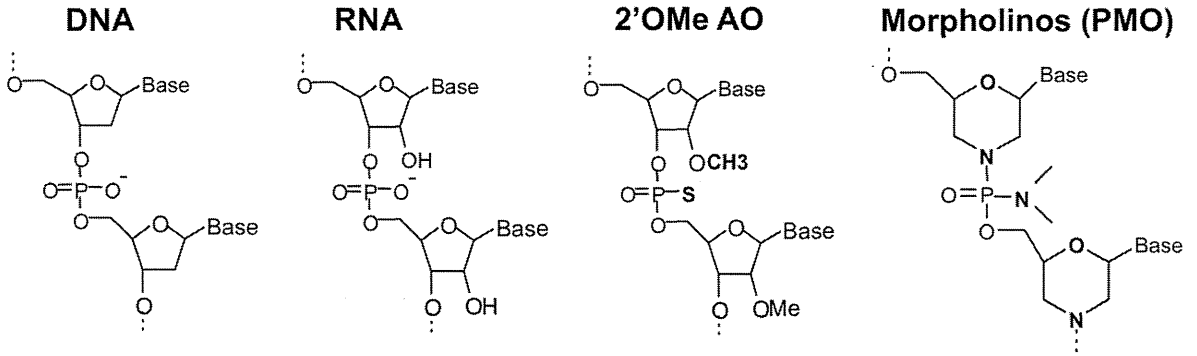

Figure 3. Backbone chemistries of nucleic acids and antisense drugs. Normal DNA and RNA has ribose rings (sugar moieties) attached by phosphodiester linkages, and one of four bases (G, A, T, C for DNA and G, A, U, C for RNA) is attached to the ribose and participates in sequence-specific base pairing with other nucleic acid strands. The AO drug chemistries modify the backbone to make the drugs more stable and less toxic. The 2′OMe AO adds a methyl group to the ribose ring and a sulfur residue to the phosphodiester linkage. The morpholino (PMO) chemistry makes many more changes, replacing the ribose with a nitrogenous morpholine ring; amine groups replace the phosphodiester linkage. Despite the relatively dramatic chemical changes to the PMO backbone, the spacing between the bases is similar to DNA and RNA and does not disrupt base pairing with other nucleic acid strands.

date AO drugs developed for several conditions.[30–34] Some of these studies have used modification of the 5′ and 3′ ends, with 2′-O-methoxy ethyl–modified ribose to make the drugs more resistant to degradation by nucleases. AOs can prolong the intrinsic clotting pathway (activated partial thromboplastin time) and increase complement split products in the monkey, but this appears to be dose dependent, with clinically significant levels occurring at relative high plasma peak concentrations (>50 μg/mL).[35] Human phase 1 safety studies have shown concentration-dependent effects on coagulation and complement, with the maximum tolerated dose by 24-hour infusion being approximately 20 mg/kg.[36] The observed adverse effects appear to be transient. Similar to other 2′-substituted AOs, the most prominent end-organ finding for phosphorothioate AOs in the monkey has been the presence of granules in the proximal tubular epithelial cells of the kidney, most likely from the uptake by phagocytosis of filtered oligonucleotide.[37] Regarding applications to DMD, phosphorothioate chemistries (2′OMe) have the great advantage of extensive preclinical and clinical experience.

Morpholino

This is the chemistry of choice in the AVI BioPharma DMD program (AVI BioPharma, Bothell, WA). The key advantage of the morpholino chemistry compared with phosphorothioate is the superior therapeutic window. Morpholino AOs have been dosed i.v. in monkeys to 320 mg/kg per week and in rodents to 960 mg/kg per week, with no evidence of dose-limiting toxicities.[38] As noted later, the 2′OMe drug PRO051 showed proteinuria at 6 mg/kg per week using s.c. doses in patients with DMD, whereas a similar morpholino drug showed no proteinuria at doses to 320 mg/kg per week using i.v. delivery in monkeys.

The major disadvantages are the much lower clinical experience with morpholino chemistry. There have been three clinical trials completed involving 39 patients with morpholino antisense, compared with 40 trials and 2000 patients in completed trials with other antisense AO chemistries (*http://www.clinicaltrials.gov*).

Phosphorodiamidate morpholino oligomers (PMOs) are a class of backbone modification that has a morpholino ring as a replacement for the furanose, with phosphorodiamidate linkage connecting the morpholino nitrogen atom with the hydroxyl group of the 3′ side residue (Figure 3). This backbone modification sets this class of AOs apart from most other modifications, and the synthesis of these AOs is unique. Until recently, this chemistry was not in the public domain for therapeutic applications. As a result, only modest progress has been made in improving the purity, capacity, and cost of goods for these AOs.

AOs synthesized from morpholinos retain high sequence specificity and strong binding to the target RNA. They are sufficiently dissimilar from native RNA and DNA in that they are not recognized by host RNA or DNA or degrading enzymes, thus making them more stable. In animal models, AOs synthesized from morpholinos (PMOs) do not cause complement activation at high serum concentrations after repeated (weekly) i.v. administration (approximately 1 g/kg per week i.v.; AVI BioPharma poster, *http://www.avibio.com/wp-content/uploads/2010/10/AVI-4658-WMS-Preclin-Poster-101510.pdf*, last accessed March 1, 2011). PMOs are highly water soluble, are not subject to metabolic degradation, and do not activate the toll-like receptors, the interferon system, or the NF-κB–mediated inflammation response.[39]

Toxicity studies have been performed in both mouse (12 weekly i.v. or s.c. injections to 960 mg/kg per dose) and monkey (12 weekly i.v. or s.c. injections to 320 mg/kg per dose). No evidence of liver or kidney dysfunction was seen, although there was histological evidence of accumulation in the proximal renal tubules, a finding seen with most AOs. Clinical trials of PMOs are under way in the UK and are about to begin in the US; thus, clinical safety data for DMD are limited.

Additional Chemistries and Technologies for Exon Skipping

Although the approaches previously described are promising, alternative strategies are being developed to address some potential limitations. Alternatives include the development of methods and chemistries to i) increase potency to reduce the amount of drug that will need to be manufactured and delivered to patients; ii) permit delivery to nonskeletal muscle target tissues, such as the heart; and iii) mitigate the need for repeated parenteral administration (eg, weekly or monthly i.v.).

One approach is to increase the charge of the AO through addition of residues along the backbone or at either end. Examples of modifications of the end of the AO include the peptide-modified PMO or morpholino[40] and guanidium dendrimer (vivo morpholino).[41] Another approach is to add targeting peptides (ie, small amino acid sequences that can interact with the muscle fiber membrane).[42,43]

Although each of these modifications to the backbone increases potency, the modifications also tend to bypass the holes in membrane delivery that unstable DMD membranes afford and, thus, lose this disease-specific advantage in DMD. They also tend to increase toxicity because they may bind to plasma proteins or cell surface proteins on nonmuscle cells (or vasculature or blood cells) and generate undesired off-target effects. Although alternative chemistries will be a continued focus for research, it is likely that efficacy in DMD will first be proved using the existing PMO and 2′OMe chemistries.

Another alternative approach is to perform exon skipping using gene therapy instead of AOs.[44–46] Herein, specific mRNA splicing molecules (ie, U7 or U1 RNA) are designed to splice out extra exons; these customized U7 drugs are coded within gene therapy viral vectors. The muscle is infected with the virus, the U7 drugs are expressed, and the drugs work efficiently at driving the desired in-frame spliced products. A critical advantage of the U7 approach is that one treatment may last a lifetime because the gene therapy vectors seem stable in muscle and continue to express the U7 RNA. A disadvantage of this approach is that it requires

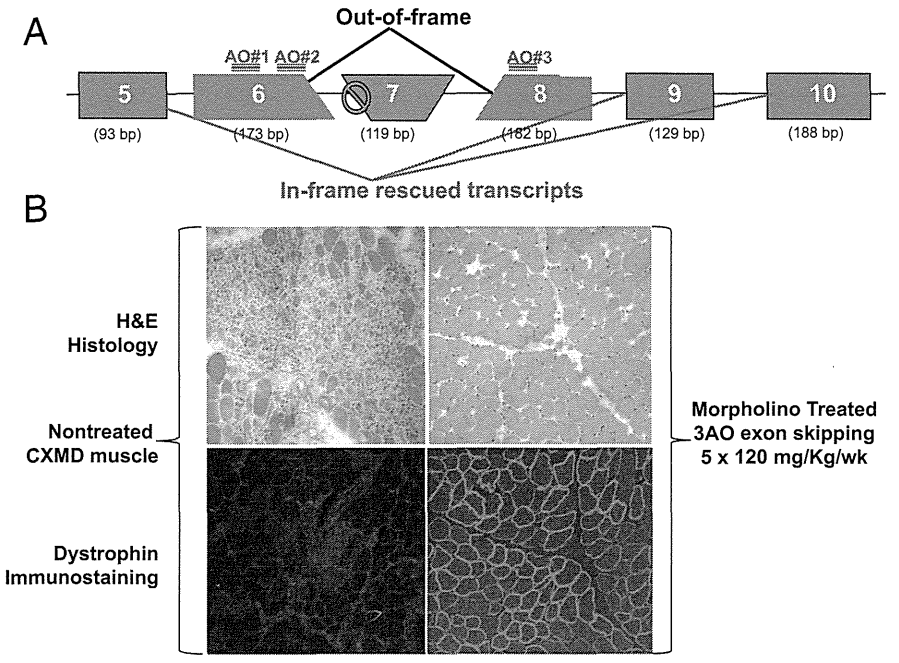

Figure 4. Delivery of multiple PMO drugs to a dog model of DMD skips multiple exons and results in *de novo* dystrophin production. **A:** Schematic of dog gene structure. The sporadic golden retriever dystrophin gene mutation is a splice-site mutation of exon 7 (red symbol). This forces the exclusion of exon 7, whereby the dystrophic dog muscle splices exon 6 to 8, but these exons do not share the same reading frame (out of frame). AOs covering exons 6 and 8 were designed (AOs 1, 2, and 3) to block inclusion of exons 6 and 8, leading to in-frame rescued transcripts (exons 5 to 9 or 5 to 10). **B:** Histological features and matched dystrophin immunostaining of AO-treated dystrophic dogs (**right**) and controls [nontreated canine X linked muscular dystrophy (CXMD) muscle; **left**]. Nontreated muscle shows necrosis of myofibers and inflammatory cell infiltration, whereas AO-treated muscle shows no inflammation or necrosis. Dystrophin protein is absent in the nontreated muscle, whereas the AO-treated muscle shows high amounts of membrane dystrophin, comparable to healthy muscle. Adapted with permission from Yokota et al (copyright 2009, John Wiley & Sons).[56]

viral gene therapy; as previously noted, gene therapy of DMD has faced persistent hurdles of immune response, viral production, and systemic delivery.[47]

Evidence for Efficacy of AO Exon Skipping: Preclinical and Clinical Studies

Animal Studies

The premise for exon skipping in DMD has been well studied in the *mdx* mouse model. In the early part of this decade, several laboratories established the fact that delivering sequence-specific AOs can induce exon skipping, which reestablishes the reading frame of dystrophin mRNA in myogenic cell cultures.[19,48–50] After these early findings, the AOs could be delivered via i.m. injection and could induce dystrophin expression to near–normal levels in most muscle fibers; this was accompanied by functional improvement.[51] Most recently, systemic delivery of AOs by i.v. injections can induce exon skipping and dystrophin expression up to levels found in healthy muscle. In addition, after three i.v. injections at weekly intervals, enhanced dystrophin expression was detected in every skeletal muscle examined.[52] Regarding dose-response and dosing schedules, single injections at a high dose (3 g/kg) show robust dystrophin expression and relatively long persistence of protein rescue.[53] These preclinical data suggest that i.v. delivery might show good efficacy at a frequency of three to four doses per year, rather than the weekly doses used in most current preclinical and clinical studies.

An oft-quoted adage is that academic medicine has generated thousands of highly efficacious mouse drugs and far fewer effective human drugs. Demonstration of efficacy in a large animal model typically engenders more confidence in human applications. Therefore, work[54,55] has been performed in the dog model of DMD that has a mutation in exon 7 of the dog dystrophin gene. Dogs with DMD represent a particularly stringent test of exon skipping, in that: i) they typically show rapidly progressive disease, often leading to death by 6 months; ii) the nature of the dog mutation requires skipping of two exons to bring the transcript back into frame; and iii) because the dog deletion is near the beginning of the dystrophin protein (actin binding site), this may be more biochemically disabling to the protein than more central deleted regions (Figure 4). In these studies, three morpholino AO drugs were codelivered to dogs with DMD to achieve exon skipping, using high doses of up to 200 mg/kg i.v. per week.[56] Given the size of the dogs, these studies required production of a large amount of AO drug.

Despite the stringency of the model, all of the three dogs tested showed stabilization or improvement of multiple functional, imaging, and histological parameters (Figure 4). Dystrophin production was increased to an average of approximately 20% in all skeletal muscles, and no toxicities were observed despite the high cumulative exposure. The dystrophin amounts varied considerably from muscle to muscle, and, consistent with murine studies, systemic delivery to the heart was poor.

Clinical Studies

The first human studies were published from a private/public partnership in Leiden, the Netherlands, between the university and Prosensa Therapeutics.[57] The AO drug, PRO051, was against exon 51 of the human dystrophin *(DMD)* gene and used phosphorothioate (2′OMe) chemistry (Figure 1). In a phase 1 safety study completed in 2007, single i.m. doses of PRO051 were safe and well tolerated in four patients with DMD who were aged 10 to 13 years and were selected on the basis of mutational

status, muscle condition, and positive response to exon skipping 51 in their cultured cells *in vitro*. A biopsy specimen of the injection site that was obtained 4 weeks later showed evidence of *de novo* dystrophin expression.

Data from an investigator-initiated clinical trial in London, UK, using a single i.m. injection of morpholino AO (AVI BioPharma) were published in 2009.[58] The investigators used an AO sequence that was similar, but not identical, to that used in the previous Dutch trial but switched to the newer morpholino chemistry. In this phase 1 study, AVI-4658 was given to seven patients with DMD (aged 12 to 18 years) as an i.m. injection in the extensor digitorum brevis. Two boys received a low dose of 0.09 mg in 900 μL, and five boys received 0.9 mg in 900 μL. Each boy received a saline injection in the contralateral extensor digitorum brevis. Muscle biopsy specimens were obtained before treatment and at 3 or 4 weeks and examined for dystrophin production. AVI-4658 was well tolerated, and no dose-limiting toxicities were observed. Treated patients had evidence of induced dystrophin production in a dose-responsive manner.

In both i.m. studies, the amount of dystrophin in treated muscle, measured by immunoblot, was low (approximately 1% to 5%) versus levels in healthy muscle. Although immunoblotting is a good method for determining the average levels of dystrophin in the tissue, it has less sensitivity compared with dystrophin immunostaining, which is able to identify individual fibers expressing relatively low levels of dystrophin. Work is ongoing to evaluate and standardize the optimal methods for use in clinical trials. In addition, the amount of dystrophin expression that correlates with clinical response is not established. From early genotype-phenotype studies[59,60] of dystrophinopathies, dystrophin immunoblot levels >10% of normal may be necessary for clinical activity; neither i.m. study consistently reached this level.

An open-label dose-ranging study[61] of the PRO051 2′OMe drug in 12 patients was recently reported. Patients with DMD were given five weekly s.c. doses, ranging from 0.5 to 6 mg/kg, with muscle biopsy specimens obtained at both 2 and 7 weeks after the initiation of treatment. Both the 2- and 7-week biopsy specimens showed drug-induced dystrophin mRNA splicing and protein production, although the levels of dystrophin by immunoblot appeared lower than might be needed for altering clinical symptoms. There was no clear dose-response relationship between dystrophin immunostaining and drug doses. All patients were then enrolled into a 12-week extension study using the peak dose (6 mg/kg per week). At the conclusion of the extension study, patients seemed to perform better on a 6-minute walk test, suggesting clinical efficacy. Because biopsy specimens were not obtained after the 12-week extension study, it was not possible to correlate molecular efficacy with apparent clinical efficacy; and because the study was open label and not placebo controlled, the improvement in functional outcomes needs to be interpreted cautiously. Nevertheless, this study provided sufficient evidence for GlaxoSmithKline to initiate a 1-year, phase 3, blinded placebo-controlled study of 6 mg/kg per week s.c. dosing in 180 patients; the study enrolled patients at 14 sites in seven countries as this article was being written (*http://clinicaltrials.gov/ct2/show/NCT01254019?term=duchenne&rank=4*, last accessed March 1, 2011).

A key issue for success of high-dose antisense drug delivery is the achievement of a balance of toxicity and efficacy (therapeutic window). As previously described, there are well-documented toxicities that limit human dosing to approximately 20 mg/kg, yet both mouse and dog studies suggest that ≥40 mg/kg may be required for sufficient dystrophin production. In the GlaxoSmithKline/Prosensa dose-ranging study, all 12 patients enrolled experienced proteinuria and an elevated urinary α1-microglobulin level at week 12 of the extension period, suggestive of kidney toxicity. Renal proximal tubuli accumulate oligonucleotides through drug reabsorption, and it will be important to monitor kidney toxicity in the ongoing 12-month phase 3 study.

AVI BioPharma has performed a dose-escalation study in the UK with systemically administered AVI-4658. Although not yet published, data have been presented in press releases and at meetings. The study included six cohorts given 12 weekly i.v. doses, ranging from 0.5 to 20.0 mg/kg per dose. At the highest dose, one patient is reported to have *de novo* dystrophin production, with approximately 50% of fibers testing positive for dystrophin by immunostaining (AVI BioPharma news release, *http://investorrelations.avibio.com/phoenix.zhtml?c=64231&p-irol-newsArticle&ID=1433350&highlight=*, last accessed March 1, 2011); however, this likely translates to approximately 20% of total dystrophin muscle content by immunoblotting. The response of patients to a similar dose has been variable, and large interpatient variability may become a theme in exon skipping. There are at least two likely reasons for differences in interpatient response to a similar dose. First, i.v. doses are typically calculated based on weight of the patient (mg/kg); the peak serum dose, at which the drug can permeate through the leaky DMD myofiber membranes, may be more important. Thus, drug doses may need to be calculated more by body mass index or some other means of approximating blood volume, rather than simply by patient weight. Second, the *de novo* dystrophin produced by exon skipping is Beckerlike (not normal); researchers have observed that there can be remarkable interpatient variability in muscle dystrophin content, despite patients having the same in-frame deletion. For example, patients with Becker dystrophy who share a common exon 45 to 47 deletion can vary widely in the amount of dystrophin in their muscle by immunoblot and the severity of the histopathological features (Table 1).[62]

The preclinical and clinical data available thus far suggest that exon skipping may hold significant promise as a candidate treatment for DMD (although the response may be variable). However, these studies are early and clinical development is ongoing. Prosensa, in partnership with GlaxoSmithKline, has announced work on AO, targeting additional exons. AVI has an investigational new drug with the Food and Drug Administration and is expected to begin enrolling patients in trials in the US in 2011.

Table 1. Variability in Dystrophin Amount and Severity of Histopathological Features in Patients with Becker Muscular Dystrophy Who Share the Same In-Frame Deletion

Patient no.	Age at biopsy (years)	CPK level (U/L)	Histopathological features (severity of dystrophy)	Immunoblot (%)	Immunostaining
31	9	9760	Very mild	80	+++
32	7	NA	Moderately severe	5	++
33	1	3000	NA	50	++++
34	37	2844	Mild	20	+++
35	29	692	Mild	50	+++
36	38	NA	Severe	5	++
37	43	NA	Moderate	5	++
38	20	9543	Very mild	30	+++
39	13	NA	Moderately severe	80	++
40	59	NA	Moderate	30	++

Data are adapted from Kesari et al.[62] The gene mutation was an exon 45 to 47 deletion for all patients.
CPK, serum creatine phosphokinase; NA, not available; ++, moderate intensity; +++, moderately high intensity; ++++, high intensity (similar to normal controls).

Regulatory Pathway for AO Drugs

Exon skipping in DMD presents some unique challenges and may serve as a test case for personalized medicine, in which drugs are customized to a patient's genetic fingerprint. The exon 51 drug would only be applicable to relatively few patients with DMD. Indeed, drugs against five exons would be needed before even half of the patients with DMD could be treated with exon skipping. As each drug is developed, the number of patients available for that drug becomes smaller, for an already rare disorder. If each exon is considered a new drug requiring the full battery of toxicology and preclinical and clinical studies, then the time for development and costs represent a significant challenge. Some of the populations are so small that achieving statistical significance in a clinical trial will not be possible. Because some mutations will require simultaneous delivery of multiple drugs, as was the case with the dog model (Figure 4), the problem is compounded.

AO drugs in development for DMD have been granted Orphan Drug Designation by the Food and Drug Administration, which is designed to facilitate the development of these (and other) drug candidates.[63] This designation provides certain tax credit and marketing incentives to sponsors. Although Orphan Drug Designation does not change the requirements for drug approval, these drugs may also qualify for a 6-month priority review.[64] Although the challenges are significant (as previously described), at least two companies have launched clinical trials of AO products; these products will begin to define the regulatory path forward. Also, regulatory and scientific agencies, parent advocates, and academic researchers in the US and Europe are working to define the key issues and potential solutions in AO drug development for DMD.

One concept that has received some attention is based on an assumption that AOs of a given chemistry will have a common safety profile (preclinical and clinical) and that they will have a common pharmacokinetic profile. If this turns out to be the case, then cumulative data on the initial exon-specific drugs may allow a more streamline preclinical toxicology package. Also, if biomarkers, such as qualitative dystrophin expression, can be validated and correlated with clinical outcomes in initial trials, they could hypothetically be used in studies of later exon-specific drugs (particularly when a given mutation occurs in a few boys). After the first exon-specific drugs (eg, two drugs) are subjected to the standard battery of preclinical and clinical tests, using existing paradigms for drug approvals in rare life-threatening orphan diseases, subsequent exon-specific drugs (and perhaps multidrug combinations) would be approved, with a reduced battery of testing. This process reduces the cost and time to bring all exonic drugs to all patients with DMD. This concept is similar to the concept used in the annual release of the influenza vaccine. After approval of a given manufacturer's vaccine, in subsequent years, the seasonal vaccine (often with a composition that is different from that studied for initial approval) is released (approved) based on a smaller, but well-defined, set of parameters. Regardless of the pathway to approval, given that the number of boys with DMD available for study prelicensure will be limited, it is likely that postapproval studies and long-term follow-up of treated patients will be required.

Another issue in AO drug development for DMD is the selection of clinical trial end points based on an understanding of the natural history of DMD and (as previously discussed) standardized consensus methods for dystrophin protein measurement (biochemical outcome measures). The outcome measure that has previously been used for drug approval in other areas has been a 6-minute walk test. The TREAT-NMD European network has formed an international effort with the US Wellstone Center network to address clinical outcome measures in clinical trials, and publications are expected within the next year. One of the issues with the existing test is that it limits registration trials to ambulatory boys. Additional end points for boys in most need of treatment (nonambulatory) are needed, such that this group of patients can benefit from participation in clinical trials and so that nonambulatory boys will be included in the drug approval process.

Finally, approval of AO drugs for DMD will require refinements in production and potency. As previously mentioned, current estimations of the dose and regimen needed for treatment of a boy with DMD suggest that it

may involve ≥10 i.v. injections per year, with a cumulative annual dose of >10 g of AO drug. If we assume that these doses will be tolerated, the current production costs of morpholino drugs are high and the GMP production capacity is limited. 2′O-methyl chemistries are more widely available and less expensive. For morpholinos, one approach to decrease the high cost of production of large amounts of drug is to increase potency so that less drug is needed per patient. Some promising approaches to increase potency have been reported in mouse models, in which the AOs are modified to more efficiently enter cells or by codelivery of small molecules or nanoparticles that enhance AO uptake or splicing efficiency.[65–69] However, these drugs show new toxicities relative to the naked unmodified morpholino backbone; and it may be challenging to achieve an appropriate therapeutic window, despite the higher potency.

Premature Stop Codon Read Through: Gentamicin and Ataluren (PTC124)

In approximately 10% to 15% of boys with DMD, the disease is caused by a point mutation that causes a change in a triplet codon, so that it no longer codes for an amino acid but instead codes for a stop signal (nonsense codons UAA, UAG, or UGA). Translation of the dystrophin protein is prematurely stopped, and the short fragment is nonfunctional and/or degraded. A promising therapy for nonsense mutation DMD is ataluren (PTC Therapeutics, South Plainfield, NJ), an orally delivered small molecule designed to selectively induce ribosomal read through of premature stop codons but not normal termination codons. Ataluren was developed after gentamicin, an aminoglycoside, promoted read through in mammalian models and in the *mdx* mouse model but presented lack of potency and potential toxicity and administration issues.[70] These proof-of-concept experiments led researchers to use high-throughput screening methods to identify compounds that suppressed the early, but not normal, termination codons; and did not present the potency, toxicity, and administration issues associated with gentamicin. In *mdx* mice and muscle cell cultures from patients, ataluren, a nonaminoglycoside, promoted dystrophin production in primary muscle cells in humans and in *mdx* mice expressing dystrophin nonsense alleles. In addition, ataluren restored striated muscle function in *mdx* mice within 2 to 8 weeks of drug exposure.[71]

PTC Therapeutics has completed phase 1 clinical trials with ataluren and is finishing data analysis of its phase 2 studies. In phase 1, ataluren, delivered as a single or multiple doses, was safe and well tolerated and supported the initiation of phase 2 trials. A total of 62 healthy adult male and female volunteers were treated in phase 1.[72] In phase 2, 38 patients with DMD were given ataluren at one of three dose levels for 28 days. The drug was safe and well tolerated, with infrequent adverse events. Plasma concentrations correlating to activity in preclinical models were found at the middle and high doses. In addition, patients receiving ataluren showed qualitative increases in muscle dystrophin expression and reductions in serum creatinine kinase levels. These patients are being followed up in an open-label long-term safety study. In April 2008, a phase 2b study was initiated; by February 2009, the study had full enrollment by 173 patients with nonsense mutation DMD at 37 sites in 11 countries. This randomized, double-blind, placebo-controlled study had three arms, with approximately 55 patients per arm: placebo, low dose (10 mg/kg), and high dose (20 mg/kg) (PTC Therapeutics, *http://www.parentprojectmd.org/site/DocServer/2010-04-16_Final_Summary_of_Ataluren_Data_at_AAN.pdf?docID=9461*, last accessed March 1, 2011). Inclusion criteria permitted both steroid- and non–steroid-treated patients, a broad age range, and patients showing both Duchenne and Becker phenotypes. As a result, there was considerable range in disease progression. Neither drug-treated arm reached significance for the primary clinical outcome measure (a 30-m increase in the 6-minute walk test), although the low-dose cohort showed a promising trend toward clinical improvement. Dystrophin data have not been reported, and there have been no formal announcements of if or how clinical testing will continue.

Ataluren is in clinical trials for three other genetic disorders: cystic fibrosis (phase 3), hemophilia A and B (phase 2), and methylmalonic acidemia (phase 2). However, no new trials are listed for DMD; and the future of the drug in patients with muscular dystrophy is uncertain.

Summary

Small-molecule drugs to coax dystrophin production from mutated genes in DMD have emerged as the most promising molecular therapeutics. Both exon skipping using AOs and stop-codon read through (PTC124) have entered clinical trials, and preliminary results are encouraging. Both approaches are mutation specific and can be thought of as personalized medicine. Should clinical efficacy be demonstrated for exon skipping, then it will be important to have an efficient path for approval of other exon-specific drugs in the same class (chemistry) to bring this to most patients with DMD.

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Current Status of Pharmaceutical and Genetic Therapeutic Approaches to Treat DMD

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Duchenne muscular dystrophy (DMD) is a genetic disease affecting about one in every 3,500 boys. This X-linked pathology is due to the absence of dystrophin in muscle fibers. This lack of dystrophin leads to the progressive muscle degeneration that is often responsible for the death of the DMD patients during the third decade of their life. There are currently no curative treatments for this disease but different therapeutic approaches are being studied. Gene therapy consists of introducing a transgene coding for full-length or a truncated version of dystrophin complementary DNA (cDNA) in muscles, whereas pharmaceutical therapy includes the use of chemical/biochemical substances to restore dystrophin expression or alleviate the DMD phenotype. Over the past years, many potential drugs were explored. This led to several clinical trials for gentamicin and ataluren (PTC124) allowing stop codon read-through. An alternative approach is to induce the expression of an internally deleted, partially functional dystrophin protein through exon skipping. The vectors and the methods used in gene therapy have been continually improving in order to obtain greater encapsidation capacity and better transduction efficiency. The most promising experimental approaches using pharmaceutical and gene therapies are reviewed in this article.

Received 22 December 2010; accepted 3 March 2011; published online 5 April 2011. doi:10.1038/mt.2011.59

INTRODUCTION

Muscular dystrophies are characterized by progressive degeneration and weakness of multiple muscle groups depending on the specific dystrophy. Duchenne muscular dystrophy (DMD) is an X-linked pathology due to the absence of dystrophin in muscle fibers.[1,2] The first symptoms of the disease appear during early childhood, usually before 3 years of age, and death occurs in the mid to late twenties.

The dystrophin gene, called *DMD* gene, extends over 2.4 megabases of the X chromosome, thus ~90 times the size of most genes. It contains 79 exons that code for a 14kb mRNA.[3,4] Its translation generates a large protein of 3,685 amino acids with a molecular size of 427 kDa[5] called dystrophin. This protein is localized beneath the sarcolemma of the muscle fibers.[6]

Dystrophin can be divided into four main regions (**Figure 1a**). The N-terminal domain interacts with actin filaments.[7] The central rod domain also links to actin filaments[8] and, in addition, to neuronal nitric oxide synthase (nNOS).[9] This enzyme is implicated in several physiological functions of the muscle such as its regeneration and its contraction.[10] The central domain also contains four hinge regions that provide flexibility.[11] The third region is the cystein-rich domain that interacts with the sarcolemmal β-dystroglycan, which in turn interacts with the transmembrane α-dystroglycan.[12] The dystrophin C-terminal region is associated with α-, β-, and γ-syntrophins.[13–15] Since dystroglycans and syntrophins are also linked to other proteins, dystrophin thus interacts with many proteins in a complex called dystrophin-associated glycoprotein complex (DGC) (**Figure 1b**).[16–19] The main function of dystrophin is to stabilize and link the muscle fiber cytoskeleton to the membrane. The lack of functional dystrophin results in the loss of the DGC, thereby rendering the muscle fibers less resistant to mechanical stress.[16,20]

In DMD, the *DMD* gene mutations almost always result in a premature stop codon due to frameshift mutations or nonsense mutations. There are >4,700 different mutations divided into three main categories: deletion of one or more exons, duplication of one or more exons and small mutations. Depending on the cohorts studied, the proportion of these categories varies from 60 to 80% for deletions, from 7 to 11% for duplications and from 10 to 30% for more subtle DNA changes including nonsense mutations, splice-site mutations, and small insertions/deletions that disrupt the reading frame.[21–26] As mentioned, most of the

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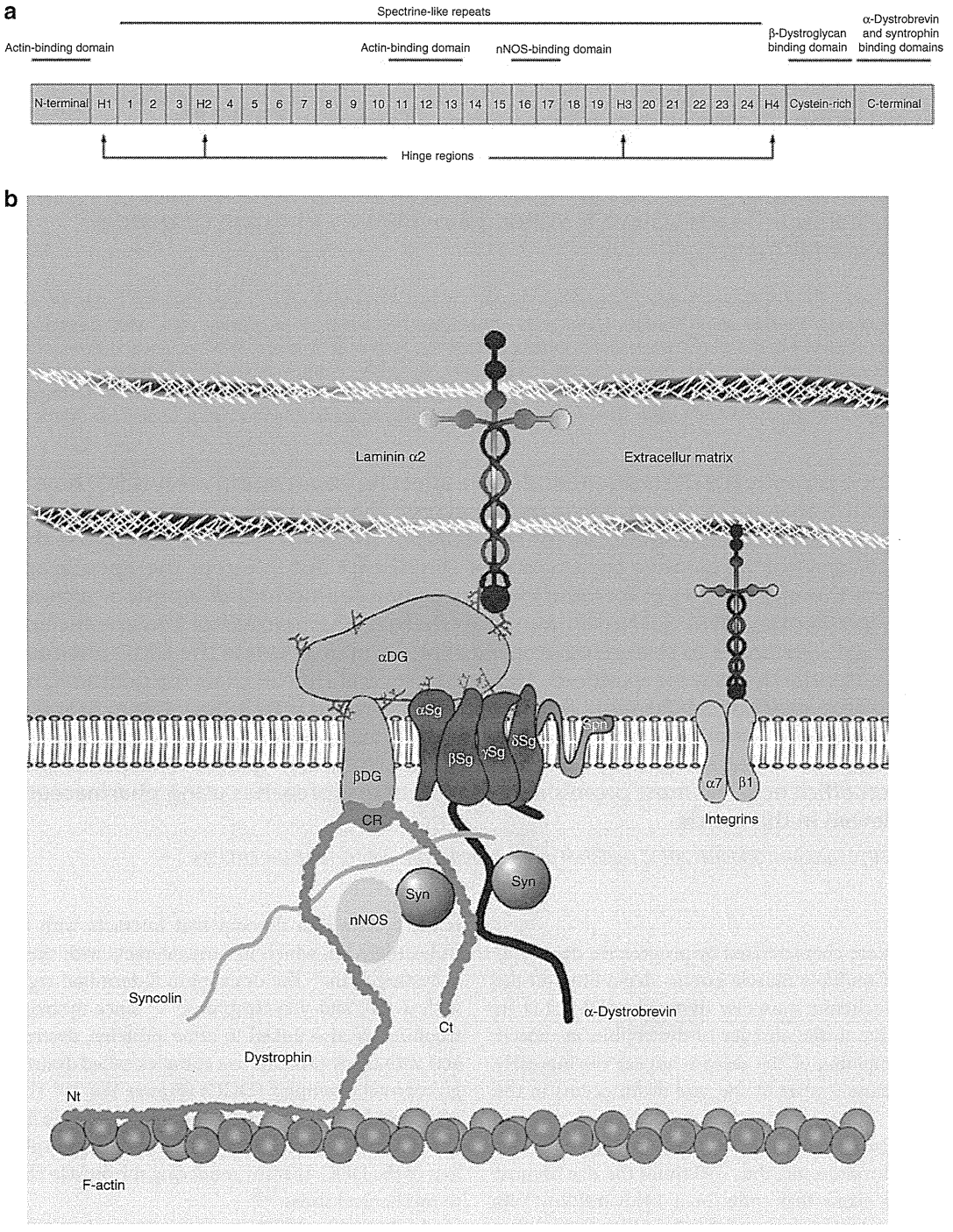

Figure 1 The dystrophin protein. (**a**) Schema representing the four main domains of dystrophin: the N-terminal part, central rod domain (containing 24 spectrin-like repeats and four hinge domains), cystein-rich region and the C-terminal part. The protein binding domains are also indicated. (**b**) Diagram of the dystrophin-associated glycoprotein complex (DGC). This complex includes dystrophin with its C-terminal (Ct), cysteine-rich (CR), and N-terminal (Nt) regions as well as proteins associated in this complex. DG, dystroglycan; nNOS, neuronal nitric oxide synthase; Sg, sarcoglycan; Syn, syntrophin. Modified from Odom *et al.*[19]

deletions in the *DMD* gene result in a frameshift.[27] Those that do not produce a frameshift result in the production of an internally deleted dystrophin and give rise to a dystrophy called Becker muscular dystrophy (BMD).[28] The BMD phenotype varies according to the functional loss of the missing exons but is generally less severe than DMD.[29–31] For example, a deletion in the rod domain will often be less severe than a deletion in N-terminal. The life expectancy of BMD patients is also variable: some may suffer life threatening complications in their late twenties and have a similar life expectancy as DMD patients whereas many live a normal lifespan beyond 50 years of age.

DMD symptoms are very severe. Thus, even if there are currently no curative treatments for this disease, the medical monitoring and the care coverage of these patients contribute to

prevention of some complications and to improvement in their quality of life. For that purpose, the follow-up of patients must be considered at various levels: rehabilitation, cardiac, pulmonary, orthopedic, psychosocial, and nutrition.[32,33]

Following the initial open-label trials of corticosteroids, the potential benefit of prednisone was clearly demonstrated >20 years ago in a double-blind randomized controlled trial for 6 months in a study of >100 boys.[34] Subsequent reports showed equal benefit using deflazacort, a sodium-sparing steroid.[35] These results were confirmed by other studies (see refs. 32,36,37 for an exhaustive list of these studies). Long-term follow-up of open-label administration of corticosteroids reveals prolonged ambulation for about 2 years. In addition, the lower prevalence of scoliosis through the use of long-term corticosteroid treatment represents a significant change in the natural progression of DMD.[38] Prednisone prescription to DMD patients is now openly authorized in many countries but many patients are forced to stop taking the drug because of unwanted side effects that include weight gain, bone demineralization, vertebral compression fractures, hypertension, and/or behavior disorders.

Besides the DMD patient's follow-up, different therapeutic approaches are currently in development to improve the DMD phenotype. This review focuses more specially on the current status of pharmaceutical and of gene therapy approaches in DMD. We have not reviewed the different potential cell therapies for DMD; however, some *ex vivo* gene therapies have been included.

PHARMACEUTICAL APPROACH

The great advantage of a pharmacological approach is that nearly all drugs can be delivered systemically (orally, intravenously, subcutaneously) and thus will reach and potentially treat all muscles which is critical for clinical success in DMD. However, the development and testing of new drugs for the DMD population is far from being a simple task.

Dystrophin restoration approaches

Stop codon read-through. About 10–15% of DMD patients have a mutation that converts an amino acid into a premature nonsense codon, while the rest of the mRNA is unaffected.[21–26] Some drugs have been shown to enable stop codon read-through by introducing an amino acid at the premature stop codon to continue the mRNA translation. This phenomenon called "stop codon read-through" has been intensively investigated.

Gentamicin: Gentamicin is an aminoglycoside antibiotic interacting with the translational machinery (40S ribosomal subunit) when it recognizes a stop codon.[39–41] This interaction induces the introduction of an amino acid at stop codons in the mRNA and thus allows the translational machinery to continue the mRNA translation.[42,43] It specially occurs in premature stop codons since the context of nucleotide sequences surrounding nonsense mutations and regular stop codons are different.[44] Gentamicin was tested as a therapeutic approach for DMD. When used in dystrophic (*mdx*) mice, this drug induced up to 20% dystrophin-positive fibers.[45] After this positive result, two clinical trials on DMD and BMD patients were undertaken. However, the results were moderate[46,47] as was also the case for some further studies in animals.[48,49] Recently, a clinical trial showed that a 6 months gentamicin administration resulted in up to 15% dystrophin expression in three DMD patients, lower percentages in three other patients, and no expression in the remaining patients.[50] The different results obtained in mouse and in human are probably due to the presence of different gentamicin isomers, which are not all equally potent in inducing read-through[41] and since each gentamicin batch consists of a mix of different isomers, some batches may be more effective than others.

Given that gentamicin has variable effects and exhibits some toxicity, less toxic effective derivatives of this drug need to be developed for an effective DMD treatment.

Ataluren: Ataluren (PTC124) is a new molecule recently identified by PTC Therapeutics (South Plain Field, NJ). It is presumed to work similarly to gentamicin except that PTC124 binds to the 60S ribosomal subunit.[51] Its efficiency is comparable to gentamicin in mouse: between 20 and 25% dystrophin-positive fibers were observed in treated *mdx* mice.[52] Three phase II clinical studies began on DMD and BMD patients but these studies were halted prematurely on March 2010 since the predetermined primary outcome (30 m improvement compared to placebo in the 6-minute walk test) was not reached[53] while ataluren was generally well tolerated in DMD patients.[54] No information is available concerning the dystrophin expression in treated muscles.

Even though gentamicin and ataluren have shown good efficiency in the *mdx* mouse model, the clinical studies that have been done up to date showed that these drugs still need further improvements before they can be used clinically in DMD patients.

Exon skipping. In BMD patients, dystrophin is internally deleted, but still partially functional due to the presence of the essential N- and C-terminal domains. Using antisense molecules which were able to interfere with splicing signals, the skipping of the targeted specific exons in the dystrophin pre-mRNA can restore the open reading frame and allow the expression of an internally deleted but functional dystrophin in DMD patients (Figure 2). These molecules are small synthetic modified RNAs or DNAs called antisense oligonucleotides (AOs) able to bind specific intronic or exonic sites of pre-mRNA. Annealing to selected splice motifs, the AO essentially masks the targeted exon from the splicing machinery, thereby promoting specific exon exclusion from the mature mRNA. Two types of AO are mainly used: 2′-O-methyl-phosphorothioate (2OMP) and phosphorodiamidate morpholino oligomer (PMO) (**Supplementary Figure S1**).

2′-O-methyl-phosphorothioates: 2OMPs contain around 20 nucleotides and are obtained by modifying the classic synthesis of oligonucleotides.[55] The first modification is the replacement of the negatively charged oxygen by sulfur. The second one is the methylation of the hydroxyl group at the 2nd position of ribose. These modifications make the AOs more resistant to nucleases, improve their affinity for RNA, provide favorable pharmacokinetic properties and prevent RNase H to induce cleavage of RNA:RNA hybrids.[56–58]

Several 2OMPs designed to target several human *DMD* exons were tested with success in DMD patient-derived myotubes.[59,60] In