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Readthrough Strategies for Suppression of Nonsense Mutations in Duchenne/Becker Muscular Dystrophy: Aminoglycosides and Ataluren (PTC124)

Richard S. Finkel, MD

Abstract

Nucleotide changes within an exon may alter the trinucleotide normally encoding a particular amino acid, such that a new “stop” signal is transcribed into the mRNA open reading frame. This causes the ribosome to prematurely terminate its reading of the mRNA, leading to nonsense-mediated decay of the transcript and lack of production of a normal full-length protein. Such premature termination codon mutations occur in an estimated 10% to 15% of many genetically based disorders, including Duchenne/Becker muscular dystrophy. Therapeutic strategies have been developed to induce ribosomal readthrough of nonsense mutations in mRNA and allow production of a full-length functional protein. Small molecule drugs (aminoglycosides and ataluren [PTC124]) have been developed and are in clinical testing in patients with nonsense mutations within the dystrophin gene. Use of nonsense mutation suppression in Duchenne/Becker muscular dystrophy may offer the prospect of targeting the specific mutation causing the disease and correcting the fundamental pathophysiology.

Keywords

Codon; nonsense; dystrophin; drugs; investigational; pediatric

Introduction

Duchenne muscular dystrophy is the most common neuromuscular disorder of childhood. An X-linked disorder, the disease occurs predominately in young boys, and has an incidence of approximately 1 in 3,500 live born males.¹ A small subset of patients is classified as having Becker muscular dystrophy, a phenotypically milder form in the continuum of the disease that is usually associated with a later manifestation of symptoms and a slower rate of decline in motor, respiratory and cardiac function.² Patients with Duchenne muscular dystrophy develop progressive muscle weakness that typically leads to deterioration of ambulation in the first decade, wheelchair dependency in the early second decade, followed by the development of scoliosis, further loss of limb function and respiratory and cardiac failure in the late second decade. Corticosteroids (prednisone and deflazacort) are the only medications shown in randomized clinical trials to have benefit in Duchenne muscular dystrophy.³ They prolong ambulation on average by 2 to 3 years, reduce the incidence of severe scoliosis, and temper pulmonary and cardiac decline in the second decade.

Address correspondence to : Dr Richard S. Finkel, Division of Neurology, The Children’s Hospital of Philadelphia, 34th St. and Civic Center Blvd., Philadelphia, PA 19104; phone: (215) 590-2763; fax: (215) 590-2223; Finkel@email.chop.edu.

Conflict of interest statement: This manuscript describes off-label use of gentamicin and also discusses ataluren (PTC124), an investigational drug currently in clinical development. The Children’s Hospital of Philadelphia has received funding from PTC Therapeutics to support the time and effort of Dr Richard Finkel to participate in clinical trials of ataluren and to serve on the company’s medical advisory committee. Dr Finkel also serves as an advisor for DuchenneConnect, without compensation.

Significant side effects, however, often occur with use of chronic corticosteroids and limit their utility.^{4,5} With improvements in clinical management of Duchenne muscular dystrophy and its complications, most of these patients now live into young adulthood.⁶ Still, most patients succumb to cardiopulmonary complications in the third decade.⁷ There remains a need for medications that target the fundamental pathophysiology of Duchenne muscular dystrophy, reverse or prevent the decline in muscle function, and avoid the burden of chronic corticosteroid therapy.⁸

The Duchenne muscular dystrophy gene and the encoded gene product, dystrophin, were identified in 1987 by Kunkel and co-workers.^{9,10} Dystrophin is a cytoskeletal protein that links actin, a component of the contractile apparatus of the muscle cell, to a complex of proteins in the sarcolemmal plasma membrane, and is important for muscle cell stability.¹¹ It is hypothesized that dystrophin is required to absorb the force generated when the muscle fiber contracts, and thereby limits damage to the cell membrane.¹²

As with many genetically based diseases, Duchenne/Becker muscular dystrophy is caused by a number of different types of mutations. The approximate distribution of mutations in patients with Duchenne muscular dystrophy includes deletions (~65%) or duplications (~7%) of one or more exon, small insertions or deletions within an exon (~7%), single nucleotide point mutations (~20%), and splice site or intronic mutations (<1%).¹³ Those mutations that inappropriately result in generation of a termination codon are termed nonsense mutations, also termed premature stop or premature termination mutations. There are 3 types of nonsense mutations in mRNA: UAG (“amber”), UGA (“opal”), and UAA (“ochre”). Messenger RNA containing a nonsense mutation is often degraded rapidly through the process of nonsense-mutation-mediated decay.¹⁴ In addition, the presence of the nonsense mutations hinders protein production. During translation of the mRNA, nonsense mutations cause the ribosome to release the nascent peptide, which is usually non-functional and degraded.

The “reading frame rule” predicts that mutations that disrupt the ribosome from reading the proper sequence of trinucleotides encoding an amino acid (“out-of-frame”) result in no functional dystrophin being produced and a Duchenne muscular dystrophy phenotype. Those who retain the reading sequence (“in-frame”) generate a shortened but partly functional protein and a milder Becker muscular dystrophy phenotype.¹⁵ Exceptions to this reading frame rule are observed for up to 9% of dystrophin mutations.¹³ Interestingly, nonsense mutations also may also result in a Becker muscular dystrophy phenotype.¹⁶ This may be due to the fact that in nature these premature stop mutations may be “leaky,” allowing a very low level of full-length protein to be produced.¹⁷ The neighboring nucleotide appears to be important to the context of how the nonsense mutation is interpreted by the ribosome.¹⁸ A UGA trinucleotide nonsense mutation appears to be the most permissive, in an in vitro setting, while a UAA is the most stringent. These observations have generated the hypothesis that drugs that suppress nonsense mutation can promote a higher level of readthrough by exploiting this natural tendency, and generate a full-length functional protein.

The concept of personalized medicine has emerged from the increasing understanding that genetic variations can influence both toxic and beneficial responses to drug therapy. A patient’s genotype can be predictive of risk to medication, eg, patients with a particular HLA allele, HLA-B*1502, are at a higher risk for Stevens-Johnson syndrome when exposed to carbamazepine.¹⁹ Alternatively, knowledge of a patient’s genotype may allow selection of patients for application of mutation-specific therapies. Such genetic modulation approaches are distinct from gene correction strategies such as gene replacement therapy, and may target the mutation directly or modify the amount of full-length transcript produced by

altering a regulatory suppressor or enhancer factor. These techniques, in the aggregate, may be applicable in over 90% of all Duchenne mutation dystrophy mutations.²⁰

Two main strategies are now in clinical testing. As discussed elsewhere in this issue, exon skipping strategies for Duchenne/Becker muscular dystrophy provide an attractive means of targeted therapy. With this approach, a short, specific oligonucleotide segment is administered. The oligonucleotide binds to a homologous target region of mRNA in an exon that neighbors the mutation, alters splicing, and causes the faulty exon to be excluded during translation. This “antisense oligonucleotide” treatment increases the size of the original mutation, but can transform a mutation from out-of-frame to in-frame, such that the ribosome can then read through the defective region to the 3' end of the message and generate a shortened but stable protein with some biological function. In the case of Duchenne muscular dystrophy this would result theoretically in a Becker muscular dystrophy phenotype. Single exon skipping is predicted to benefit approximately 50% of Duchenne muscular dystrophy mutations, and multi-exon skipping, over 90%.²⁰ A library of multiple oligonucleotides will be needed to cover the full assortment of different dystrophin mutations among the 79 exons. This strategy holds great promise and is being explored currently in human clinical trials.^{21,22}

Readthrough strategies for nonsense mutations take a different approach and may be applicable in ~13% of patients with Duchenne/Becker muscular dystrophy.²³ With this strategy, small molecule drugs are administered that introduce a conformational change in the mRNA and allow the ribosome to insert an amino acid at a UGA, UAG, or UAA premature stop codon site during translation.^{24, 25} Such nonsense mutation suppression therapy is selective for premature stop codons relative to normal termination codons at the 3' end of the gene because the geometry of the mRNA at these 2 sites is different.^{14, 26} Drugs that induce suppression of these nonsense mutations results in an increase in the readthrough of the premature stop signal and production of full-length protein. The lowest quantity of full-length dystrophin that is required to achieve normal functional muscle stability is not known, but a reduction to 30% of normal has been seen without apparent skeletal muscle weakness.²⁷ Induction of lesser amounts of dystrophin may allow amelioration of symptoms or may temper disease progression. Unlike exon skipping, where a specific oligonucleotide needs to be constructed for each exon that is to be skipped, a single small molecule drug that reads through nonsense mutations can theoretically address all such mutations within the entire coding region of the gene.

History of Readthrough of Premature Stop Mutations

The history of readthrough of premature stop mutations in eukaryotes begins in 1979, with 2 papers describing suppression of these mutations by aminoglycosides.^{28,29} Several of these antibiotics were tested for relative capacity to read through premature stop mutations.³⁰ These observations led to testing of gentamicin, initially in a cystic fibrosis cell line,²⁴ then in the *mdx* mouse, an animal model for Duchenne muscular dystrophy that fortuitously harbors a UAA premature stop mutation.³¹ This *mdx* proof-of-concept study demonstrated expression of muscle fiber dystrophin in vitro and in vivo that was ~20% of normal, and showed that dystrophin was properly localized to the sarcolemma. These muscle fibers showed increased resistance to eccentric contraction injury. A decrease in the leakage of creatine kinase from muscle into blood was also observed, suggesting reduced muscle cell fragility.

Human Studies Using Gentamicin

On the basis of these findings, human trials of intravenous gentamicin were undertaken. The initial study was a small pilot effort performed by Wagner and colleagues³² In this trial, 4

patients (2 described as having Duchenne muscular dystrophy and 2 as having Becker muscular dystrophy) were administered daily gentamicin 7.5 mg/kg intravenously for 2 weeks. Over this short period of drug exposure, drug activity, as assessed by muscle dystrophin expression and muscle strength, was not detected. No renal or ototoxicity was observed. Politano and colleagues then administered IV gentamicin to 4 subjects with Duchenne/Becker muscular dystrophy. These investigators used a treatment regimen comprising 2 six-day courses of therapy separated by an intervening period of 7 weeks. They demonstrated an increase in dystrophin expression in 3 of 4 subjects in end-of-treatment biopsies and identified no toxicity.³³

Malik and colleagues have recently completed a more extensive study in Duchenne muscular dystrophy.³⁴ The subjects were divided into 4 cohorts in 2 groups and compared: (1) 14-days of daily IV gentamicin (7.5 mg/kg/day) in boys with nonsense mutations to a matched control group of Duchenne muscular dystrophy boys with a deletion mutation, and (2) 6 months of intravenous gentamicin (7.5 mg/kg) given once/week versus twice/week in Duchenne muscular dystrophy boys with nonsense mutations. Pre- and posttreatment muscle biopsies were performed for dystrophin expression analysis by immunostain and Western blot. In this safety study, all subjects were carefully monitored for adverse effects and in all 4 cohorts there were no persistent findings of nephrotoxicity or ototoxicity. All subjects were screened for risk of gentamicin-induced ototoxicity by testing for the A1555G mutation in 12S rRNA gene of mtDNA and excluded if this was identified.

The initial 14-day portion of the study demonstrated a reduction in serum creatine kinase levels to approximately 50% of baseline, whereas the controls had no significant change, supporting the specificity of gentamicin action to those with nonsense mutations. Activity levels during this inpatient study were similar to those in the home setting. The creatine kinase levels in the nonsense cohort returned to near baseline levels within 1 month of stopping the drug. In the 6-month treatment group, comparing once- with twice-weekly gentamicin infusions, creatine kinase levels similarly declined. Dystrophin expression in muscle was increased from baseline in those subjects who had some level of baseline dystrophin production but not in those with a complete absence of protein. This suggests gentamicin suppression of the nonsense mutation is more effective when the mutation is “leaky.”

In 2 subjects, dystrophin levels increased to 13% to 15% of normal levels and in one of these subjects there was a stabilization of strength and forced vital capacity during the 6 months of treatment, hinting at some clinically meaningful response. Response to gentamicin in this study was not correlated with the type of nonsense mutation or by the adjacent fourth nucleotide. Interestingly, and of possible clinical importance, was the finding of immunogenic dystrophin epitopes in the posttreatment biopsies. This occurred in subjects who had no measurable dystrophin in the pre-treatment biopsy but not in those with some baseline production. This finding suggests that in patients with a full null mutation, the newly produced full-length dystrophin protein is recognized as foreign and generates an immune response. T-cell activation targeted the region of the protein generated distal to the nonsense mutation site, ie, the novel portion of the gene not previously translated. This has broad implications for any strategy that generates a dystrophin transcript with novel nucleotides, including ataluren and exon skipping.

Several issues make the use of gentamicin problematic. First, there is a narrow therapeutic window between the dose sufficient to generate optimal dystrophin expression and that which can cause renal and ototoxicity. Second, the need for regular intravenous administration and monitoring of drug levels and safety laboratory parameters adds to the burden of the treatment. Third, there are multiple forms of gentamicin, with significant

variation in their potential to promote dystrophin expression.³⁵ To address these concerns, novel aminoglycosides³⁶ and non-aminoglycosides³⁷ are being explored as safer alternatives.

Ataluren (PTC124)

Ataluren (formerly known as PTC124) was discovered by PTC Therapeutics in a high-throughput drug screening program designed to identify compounds that specifically induce ribosomal readthrough of NM in mRNA. The goal was to find small molecules that could be given orally, possessed favorable pharmacokinetic properties, and had a favorable safety profile. More than 500,000 compounds from a chemical library were screened in both cell-based and cell-free systems. Several chemical scaffolds were identified that induced nonsense mutation suppression. From these lead scaffolds, a medicinal chemistry effort was undertaken to synthesize molecules that had the best combination of efficacy and pharmaceutical characteristics. Ataluren was identified as an orally bioavailable compound with potent nonsense suppression activity. This was demonstrated by increased protein expression and function in 2 nonsense mutation-mediated animal models. Ataluren was shown to induce full-length functional dystrophin in the *mdx* mouse¹⁹ and full-length functional cystic fibrosis transmembrane conductance regulator in a mouse harboring a human nonsense-mutation-containing transgene.²⁰

Like gentamicin, ataluren works at the level of the ribosome to induce readthrough of premature stop codons in mRNA. However, chemical footprinting studies indicate that the 2 molecules bind at different ribosomal locations on different ribosomal subunits.³⁸ Table 1 summarizes the comparison of these 2 drugs. Ataluren was tested in the *mdx* mouse in much the same way as gentamicin had been previously evaluated.²⁵ In vitro dose-response studies in *mdx* myotubes demonstrated a dose response, with maximal expression at ataluren drug levels of 10 ug/mL. In vivo administration of the drug orally and intraperitoneally to *mdx* mice over periods ranging from 2 to 8 weeks generated dystrophin expression in skeletal, cardiac, and diaphragmatic muscle, although there was some variation among muscles sampled. The eccentric contraction test of isolated muscle fibers from ataluren-treated animals showed protection from muscle damage. Serum creatine kinase levels in the ataluren-treated animals declined during drug treatment. These results were encouraging and led to initiation of studies in humans.

Human Experience with Ataluren (PTC124)

Human exposure to ataluren was first evaluated in a phase 1 study in healthy adult human volunteers.³⁹ Data from this study established that orally administered ataluren was palatable, rapidly absorbed, achieved desired blood levels when given with or without food, and was generally well-tolerated at doses exceeding those required for in vitro and in vivo nonsense mutation suppression. To address drug selectivity, peripheral blood mononuclear cells were evaluated in study subjects receiving high doses of ataluren; at drug levels that induced premature stop codon readthrough in an in vitro assay, no evidence of protein elongation that would suggest normal termination codon readthrough was observed.²⁵

A phase 2 study in 44 adult cystic fibrosis patients treated with PTC124 has been published.⁴⁰ Here, a reduction of the transepithelial nasal potential difference of the chloride channel was used as a pharmacodynamic response to the drug and indicated suppression of the nonsense mutation in the cystic fibrosis transmembrane conductance regulator gene. In the first group, subjects were treated with ataluren 16 mg/kg/day, in 3 oral doses, for 14 days. Sixteen of 23 subjects demonstrated a reduction in the transepithelial nasal potential difference. In the second group, treated at 40 mg/kg/day, 8 of 21 subjects demonstrated a response. These findings, along with a favorable safety profile, have led to a phase 3

efficacy study that has recently started recruitment (clinicaltrials.gov identifier NCT00803205). Preliminary data are also available in abstract form from a phase 2a proof-of-concept study performed in 38 boys with Duchenne/Becker muscular dystrophy.⁴¹ Participants in this study underwent dystrophin gene sequencing to ensure that Duchenne/Becker muscular dystrophy resulted from a nonsense mutation. Ataluren was administered for 28 days in 3 cohorts of mainly ambulatory patients: 16, 40, and 80 mg/kg/day in 3 daily doses. The primary objective of this study was to see whether an increase in full-length dystrophin expression in muscle could be identified as a pharmacodynamic response to drug. Primary muscle cells, obtained from pre-treatment muscle biopsies, showed dose-dependent increases in dystrophin expression in response to in vitro ataluren treatment for 12 days, suggesting the potential for nonsense mutation suppression if sufficient tissue concentrations are achieved. This dystrophin expression was seen at concentrations that paralleled the *mdx* animal data and were measured subsequently as serum levels in these subjects when on drug.

In vivo, end-of-treatment muscle dystrophin expression (as assessed by immunofluorescence staining for the C-terminal portion of dystrophin, indicating full-length protein expression) appeared increased in the majority of subjects, with no clear dose-dependency or relationship to the type of mutation (UGA, UAG, UAA) or the site of the exon harboring the mutation. Serum creatine kinase reductions were observed in most patients during ataluren administration and trended back toward baseline within a month after discontinuation of drug. Adverse events were infrequent, generally mild, and were not usually considered ataluren-related. None of the subjects had clinically concerning laboratory abnormalities.

Based on these proof-of-concept data, a randomized, double-blind, placebo-controlled dose-ranging Phase 2b trial was designed to evaluate the safety and efficacy of 48 weeks of ataluren therapy in ambulatory patients 5 years of age with nonsense mutation Duchenne/Becker muscular dystrophy [clinicaltrials.gov identifier NCT00592553]. The study enrolled 174 participants at 37 sites. Outcome measures in this study have included the 6-minute walk distance (as the primary outcome measure), other measures of muscle function and strength, and muscle dystrophin expression in pre and mid-treatment biopsies. The study has completed accrual and therapy and initial results have been released by PTC Therapeutics. There was a very high rate of drug compliance and no significant safety concerns were identified over the 48 weeks of therapy. No significant difference in the 6-minute walk distance was demonstrated in the treated groups (40 mg/kg/day and 80 mg/kg/day) compared with the placebo group. Further analysis examining patient subgroups, muscle dystrophin expression, exon location, and type of nonsense mutation is pending.

Future Directions

Drugs that target the type of mutation rather than the disease offer the prospect of personalized medicine. Evolving therapeutic strategies such as exon skipping and nonsense mutation suppression support the concept that all boys with Duchenne/Becker muscular dystrophy should be fully genotyped. Once a mutation-specific drug is shown to be clinically effective, a further challenge will be that of determining the optimal age to initiate such therapy. Use of drugs such as gentamicin or ataluren, which addresses the underlying cause of the disease, may offer benefits to patients throughout the course of the disease. However, because Duchenne muscular dystrophy is already established at birth, with high creatine kinase levels and dystrophic muscle, it may become particularly important to initiate this type of therapy at the time of diagnosis, prior to the development of intractable disease manifestations. Assessment of ataluren safety and pharmacokinetics in younger patients (ie, those < 5 years of age) may be appropriate. In addition, ataluren is being evaluated for therapeutic potential in other genetic disorders among those patients whose

disease is caused by a nonsense mutation. To this end, ataluren is currently being investigated for use in patients with hemophilia A and B [clinicaltrials.gov identifier NCT00947193].

Conclusion

Novel strategies designed to induce ribosomal readthrough of premature termination mutations can produce full-length functional protein necessary for cellular function. In the case of Duchenne/Becker muscular dystrophy, the small-molecule drugs gentamicin and ataluren have achieved convincing proof-of-concept in vitro and in vivo, with production of a full-length dystrophin protein that localizes correctly to the sarcolemma. Recent clinical trials of gentamicin and ataluren have not demonstrated clinical efficacy, making the path toward regulatory approval for Duchenne/Becker muscular dystrophy a challenging one. Gentamicin has particular safety issues to address in long-term administration and studies to date have not been designed specifically to capture clinical efficacy. Ataluren has the benefit of being a potent nonsense mutation suppressor that is orally administered and shows a favorable safety and tolerability profile in initial human testing. Despite eliciting a favorable pharmacodynamic response to drug, demonstrating clinical benefit remains problematic. Lessons learned from the recent ataluren efficacy study will prove useful in the design of future clinical trials in Duchenne/Becker muscular dystrophy. Large-scale international long-term placebo-controlled studies in boys with Duchenne/Becker muscular dystrophy are feasible. What remains to be defined are the age and stage of disease when intervention in Duchenne/Becker muscular dystrophy will have a clinically meaningful benefit, the minimal amount of full-length dystrophin expression necessary to achieve this, the duration of a study necessary to demonstrate this, how to capture the effect with an appropriate outcome measure, and how to monitor for and potentially mitigate an adverse immune response.

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Table 1

Comparison of Gentamicin and Ataluren (PTC124) as Drugs That Promote Nonsense Mutation Suppression

Characteristic	Drug	
	Gentamicin	Ataluren (PTC124)
Ribosomal subunit where drug binds	40S	60S
In vitro readthrough potency	Low	High
Route of delivery	Intravenous or intramuscular	Oral
Toxicity profile	Risk of nephrotoxicity and ototoxicity with narrow therapeutic window	Excellent preliminary safety and tolerability profile at doses exceeding those planned for clinical use
Limitations	Batch variability in potency	Not currently available outside of clinical trials