Degenerative Neurological and Neuromuscular Disease

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HYPOTHESIS

A Hypothesized Therapeutic Role of (Z)-Endoxifen in Duchenne Muscular Dystrophy (DMD)

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Video abstract

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Abstract: Duchenne Muscular Dystrophy (DMD) is an inherited, X-linked disorder that is progressive, debilitating, and ultimately fatal. The current therapeutic landscape offers no cures, but does include palliative treatments that delay disease progression, and there is progress on genetic therapies that have the promise to be curative. There is much room for new therapies, and foundational work with the estrogen receptor modulator tamoxifen suggests the potential of a unique spectrum of therapeutic benefit from endoxifen, a metabolite of tamoxifen. Here we describe the potential for this new DMD therapy in the context of the overall DMD therapeutic landscape.

Keywords: Endoxifen, Duchenne Muscular Dystrophy, DMD carrier associated pathologies, repurposed therapy, estrogen, protein kinase C

Introduction

Duchenne Muscular Dystrophy (DMD) is a rare, recessive X-linked muscle disorder caused by mutations in the dystrophin gene (*DMD*).[1] These mutations cause a lack of functional dystrophin, a protein localized to the cytoskeletal network of skeletal and cardiac muscle. The lack of functional dystrophin leads to use-dependent muscle wasting and weakness, motor impairment, and dilated cardiomyopathy. Affected individuals experience progressive increases in muscle atrophy, loss of mobility, respiratory and cardiac insufficiencies, and severely shortened life expectancy.

Although other types of dystrophies exist, DMD is the most common. Global birth prevalence of DMD in 2020 ranged from 7.7 to 23.6 per 100,000 males.[1,2] Symptoms of DMD are first noticed between the ages of 1 to 3 years, with loss of ambulation occurring between 8 and 14 years, ventilation becoming necessary a few years later (18 to 20 years), and cardiomyopathy presenting by the early 20s. The median survival of patients with DMD is highly variable and ranges from approximately 24 to 41 years.[1–3] Prior to the development and introduction of assisted ventilation and palliative treatments, respiratory failure was the leading cause of death. Cardiomyopathy is now the leading cause of death in DMD patients.[2–6]

The economic costs of DMD are substantial: annual cost estimates based on inflation-adjusted data from 2014 project a total burden of illness per patient in the US of approximately $159,519 per year in 2024.[3,7] Direct medical costs increase with progression of the disease, as do indirect costs such as loss of productivity for families of DMD patients.[7] In addition, the psychological costs to patients are high, with a range of symptoms having different impacts during the progression of DMD.[8]

Received: 19 September 2024
Accepted: 27 February 2025
Published: 15 March 2025

The monogenic nature of dystrophies such as DMD make them attractive candidates for genetic therapies, yet these treatments are challenging because of the nature of the dystrophin gene.[9] It is one of the largest genes in the human genome (2200 kilobases [kb]), with a complex intron/exon structure and a coding region of 2.7 kb.[9–11] This extremely large structure means that all the multiple strategies employed in development of curative genetic approaches – mini genes, gene editing, exon skipping, and stem-cell based treatments – face significant logistic challenges.[9–14] It is therefore important to continue to improve upon those treatments that, although not curative, can ameliorate the impact of the dystrophin mutations and prolong the life and health of DMD patients.

Materials and Methods

Extensive and thorough literature search was conducted to support the information presented in the manuscript. Article types included relevant book chapters, clinical trials (clinicaltrial.gov), meta-analyses, randomized controlled trials, reviews, editorials and systemic reviews. The search strategy prioritized relevance to DMD mechanism of action, and current and potential therapeutic solutions. Searches also were focused on endocrine therapies with emphasis on tamoxifen/endoxifen and other selective estrogen receptor modulators and on selective estrogen degraders. Non-confidential data from (Z)-endoxifen's investigator brochure were also used in relevant sections.

Therapeutic Landscape

Current approved therapies for DMD include traditional glucocorticoids, vamorolone[15] (Agamree, a recently approved glucocorticoid), givinostat[16] (Duvyzat, a histone deacetylase inhibitor), and delandistrogene moxeparvovec-rokl[17] (Elevidys), a micro-dystrophin gene therapy. Therapeutics currently under development[18] include multiple genetic approaches, stem cell therapies, anti-inflammatories, and tamoxifen and its active metabolite endoxifen.

Approved Therapies

Glucocorticoids

Glucocorticoids such as prednisone or deflazacort represent the current standard of care for DMD patients.[19,20] Generally, these drugs act by modulating tissue-specific gene expression, inflammatory response pathways, and metabolic activity via their interaction with the cell receptor for the endogenous glucocorticoid cortisol. Glucocorticoids are also known to interact with other nuclear receptors, including the androgen receptor, which plays a crucial role in skeletal muscle regulation.[19] This may contribute to both improved muscle strength and prolongation of ambulation, the major benefits of glucocorticoid treatment in DMD patients.[21,22]

Glucocorticoids have also been shown to activate aldosterone (mineralocorticoid) receptors, an effect that may contribute to adverse effects such as osteoporosis, growth reduction, and cataract formation.[23] Aldosterone receptor antagonists have cardioprotective effects and have been explored as potential therapeutics for DMD.[23] Synthetic glucocorticoids vary in their pharmacokinetics and pharmacodynamics, and so they yield a variable spectrum of effects including their impact on mineralocorticoid signaling.

A recent meta-analysis[24] comparing prednisone/prednisolone with deflazacort in DMD patients showed that while deflazacort was generally better at slowing disease progression, it also exhibited greater degree of adverse effects such as decreases in bone density and growth rates, and cataract development. Despite their benefits, glucocorticoid use contributes to DMD sequelae including obesity, osteoporosis, and stunted growth. Balancing benefits with these significant limitations remains an important aspect of improving DMD therapy.

Vamorolone (Agamree) is a glucocorticoid approved by the US Food and Drug Administration (FDA) for use in DMD patients in 2023.[15] It was developed with the goal of retaining muscle-building and anti-inflammatory effects of glucocorticoids while minimizing adverse effects such as growth inhibition and osteoporosis. Results from initial short-term studies suggest that Vamorolone can provide improvement in motor outcomes while minimizing the adverse effects on growth rate and bone density observed with traditional glucocorticoids.

Givinostat (Duvyzat)[16] is a histone deacetylase inhibitor approved by the FDA for use in DMD patients in 2024. Histone deacetylase inhibitors modulate gene expression similarly to glucocorticoids, but their precise mechanism of action in DMD is not known. Studies in animal models suggest that deacetylase inhibitors might act to stabilize

mitochondrial disruption and the oxidative stress observed in DMD. In a Phase 3 trial (NCT02851797), Givinostat significantly slowed the progressive loss of motor function measured in a stair climb protocol.[16] Of note, in the completed Phase 3 study and in the ongoing long-term follow-up trial (NCT03373968), participants receive glucocorticoid therapy concurrently with Givinostat.

Gene Therapies

Gene-based therapies are promising, with the potential to provide long-lasting therapeutic benefit with a single treatment by repairing/replacing mutations in the dystrophin gene. Mutations in the dystrophin gene that lead to DMD can be the result of gene deletions, truncations, or insertions.[10,25] Multiple approaches are in development to address these genetic errors including gene editing, gene addition, or exon skipping.[10] For example, estimates project that 60% of DMD patients could receive clinical benefits from CRISPR/Cas9 gene editing.[11] However, the issues surrounding excessive immune responses caused by viral vectors and Cas9 remain to be solved before the effective clinical application of this method. Conventional gene therapy work such as AAVs provide a shorter but yet functional version of the dystrophin gene. Limitations in this approach arise when the mutated copy of DMD is still present which causes conflict and competition for binding the many natural partners of dystrophin. Additionally, potential off-target effects and other side effects necessitate further research to ensure the safety of these approaches before widespread clinical adoption.[26]

Gene addition strategies use genetic methods to deliver a mini-dystrophin protein that serves some of the functions of the native protein.[27] Multiple attempts to use this strategy are underway, and one of these, Elevidys, received accelerated approval from the US FDA in 2023[17] and expanded approval in 2024.[28] Early results indicate that continuous production of the partially functional dystrophin protein may result in improvements in motor function, including time to rise from the floor, 10-meter walk/run, and time to ascend four steps.

Other efforts to use the mini-dystrophin strategy have been less successful, including fordadistrogene movaparvovec, a mini-dystrophin based gene replacement program that was recently terminated after failure to meet endpoints and adverse events including the death of one study participant.[29] Gene editing and exon skipping programs are also in clinical development.[10,11,25]

Experimental Therapies

Most of the other treatments, including approved agents discussed above and others described here, target the downstream pathological mechanisms that result from the loss of dystrophin. Currently, major downstream targets are:

1. Muscle damage is a key pathogenic process in DMD. Dysregulation of cytosolic calcium and nitric oxide, increases in oxidative stress, and mitochondrial dysfunction drive this damage. Homeostatic restoration of these processes is a current therapeutic approach under investigation.[1,30]
2. Cellular communication network factor 2 (CCN2) plays a key role in fibroblast regulation and fibrosis. Clinical severity of DMD is positively correlated with CCN2 and transforming growth factor beta (TGF-β) levels in skeletal muscle, making them attractive therapeutic targets.[31]
3. Muscle atrophy resulting from dysregulation of skeletal muscle maintenance is a hallmark of DMD. Beta 2-agonists and urocortins have been identified as key modulators of the process of skeletal muscle maintenance and are being pursued as therapeutic targets.[32]
4. Bone loss is a common symptom of DMD arising from multiple causes, such as decreased physical activity, dystrophin-deficient induced cytokines, and long-term treatment with glucocorticoids. Therefore, treatments that preserve or restore bone health have the potential to increase the quality of life for patients with DMD.[33]
5. Fibrosis is a response to chronic tissue damage and immune response. It is a major cause of mortality in patients with DMD. Reduction or reversal of fibrosis is actively being investigated to ameliorate symptoms of DMD.[31]
6. Inflammation plays a critical role in many of the above processes, as well as in overall DMD pathology. Key therapeutic targets under investigation for reducing inflammation include the NF-κB pathway, protein kinase C (PKC), p65-dependent pathways, and IGF1 and Nrf2 pathways.[18–20,34]

Here, we focus on the properties and physiological actions of one experimental therapy for DMD: tamoxifen and its metabolites.[35] Currently used as a cancer treatment, tamoxifen is a selective estrogen receptor (ER) modulator that is being tested as a potential palliative treatment for DMD.[25] Tamoxifen interactions with different ER isoforms result in an anti-estrogen effect in the breast, and an estrogen-like effect in other tissues. This, in combination with a host of demonstrated physiological actions on other cellular pathways, has led to repurposing of tamoxifen, and its metabolites, beyond its use as breast cancer therapeutic.[35,36]

Tamoxifen Pharmacology

Tamoxifen is a lipophilic pro-drug which is extensively metabolized in the liver into its active metabolites (4-hydroxytamoxifen [4HT], N-desmethyltamoxifen [NDMT], and [Z]-endoxifen). A key player in this pathway is the cytochrome P450 (CYP) family of enzymes, including CYP2D6 and CYP3A4, which are involved in the metabolism of tamoxifen into 4HT and NDMT, as well as the conversion of NDM-tamoxifen into endoxifen (Figure 1, see Jayaraman et al[37], Sanchez-Spitman et al[38] and Lim et al).[39] It is important to note that CYP2D6 polymorphisms only explain interpatient variability of metabolite concentrations to a limited extent, suggesting that other key factors impact the

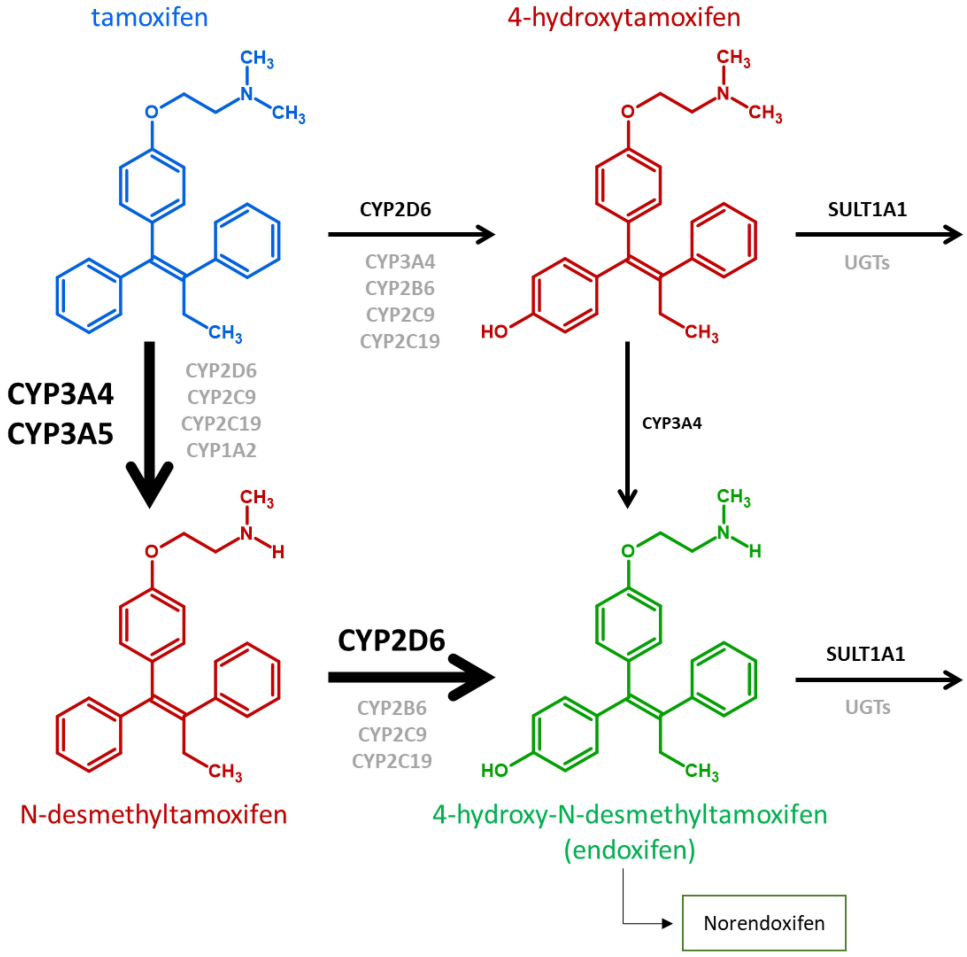

Figure 1 Metabolism of Endoxifen. Tamoxifen (blue structure) is a prodrug that relies on hepatic metabolization for becoming active. In the liver, cytochrome P450 enzymes convert tamoxifen into 4-hydroxytamoxifen (4-HT) and N-desmethyltamoxifen (NDMT). These intermediate metabolites (red structures) are produced in variable amounts by patients. Although 4-HT and NDMT show higher affinity for estrogen receptors than the prodrug, they are thought to cause most of the side effects related to the use of tamoxifen by breast cancer patients. Endoxifen (in green), the most active metabolite, is mainly produced from NDMT by CYP2D6, and is eliminated after further modification by glucuronosyltransferases and sulfotransferases. The main metabolic enzymes are shown in black, and their relative contribution is illustrated by the size of the arrows. Additional enzymes are shown in gray. Norendoxifen, a metabolite of Endoxifen, is shown. See Endocrinol, 162 (12), Dec 2021, bqab191, https://doi.org/10.1210/endocr/bqab191;[37] Expert Rev Clin Pharmacol, 12, 523–536 (2019). https://doi.org/10.1080/17512433.2019.1610390.[38] Br J Clin Pharmacol, 81(6):1142–1152 (2016), https://doi.org/10.1111/bcp.12886.[39]

metabolism of tamoxifen, including factors influencing the subsequent metabolism of 4HT and endoxifen by uridine glucuronosyltransferase and sulfotransferase 1A (see Figure 1).

Most of our knowledge regarding the mechanisms of action of tamoxifen and related compounds on cellular targets have been acquired in cell cultures and are essentially focused on breast cancer and reproductive organs. Tamoxifen is a known modulator of cellular calcium signaling and a reactive oxygen species (ROS) scavenger.[40] While the exact mechanism(s) behind tamoxifen's modulation of calcium cellular signaling have yet to be elucidated, this regulation may be mediated via ERs.[41] Similarly, tamoxifen has been shown to induce autophagy in a G protein-coupled estrogen receptor (GPER)-dependent manner in Jurkat cells.[41] However, the relevance of these proposed mechanisms of action is matter of caution because (i) drug concentrations applied to cell cultures often exceed by several logs of magnitude the clinically relevant exposure; (ii) the expression of estrogen receptors, nuclear cofactors, and other targets in cell cultures may be materially different from the in vivo situation; (iii) most cell lines are unable to convert tamoxifen into active metabolites. Preclinical studies in animal models and in clinical setups in humans help to identify the actual effects and their related mechanisms of action: whether this is through ERs or other targets, tamoxifen has been reported to decrease low-density lipoproteins, high-density lipoproteins, total cholesterol, and triglyceride levels in men and postmenopausal women, which suggests it may have a clinically relevant cardio protective effect. In vitro treatment with tamoxifen supports this and shows improvements in the contractile function and survival of stem cells induced to become cardiac muscle cells in a cellular model of DMD.[42]

Tamoxifen Studies in DMD

Input of the Preclinical Development of Tamoxifen for DMD Into the Mechanisms of Action

Early studies suggesting that tamoxifen may have therapeutic value in DMD came from work with a mouse DMD model, where tamoxifen stabilized ERβ expression, enhanced muscle protein expression and contractile tissue quality, and improved wire test scores, a mouse correlate of muscular function.[36]

It is robustly established that tamoxifen acts as an anti-estrogen in breast cancer but as a pro-estrogen on many other tissues and organs, including uterus and bone.[36,43] Similarly, there is evidence that point to pro-estrogenic actions in skeletal muscles and in the heart, both in healthy and dystrophic contexts. Unpublished data (by OMD, LAN, LS) in aromatase-deficient mice demonstrate that the lack of estrogens precipitates structural damage and loss of function in dystrophic muscles. Similarly, dystrophic mice lacking ERα or ERβ or both receptors, exclusively in skeletal muscle, developed a more severe disease.

Published and unpublished data (by OMD, LAN, LS) strongly suggest that most effects of tamoxifen in dystrophic mice are mediated through ERs.[44] In short: (i) phenotypic rescue is achieved with nanomolar concentrations of tamoxifen and metabolites in the plasma and muscles, suggesting actions through high affinity targets; (ii) tamoxifen-mediated improvements of motor function are reversed by co-administration of fulvestrant, a full inhibitor of both ERα and ERβ; and (iii) kinetics of functional improvements are consistent with muscle adaptation via regulation of gene transcription, driving progressive shifts in muscle protein isoforms. Some effects, however, might be independent of ERs and gene transcription.[45–47] Indeed, it has been demonstrated that acute exposure of muscles, cells, or mitochondria to tamoxifen increased membrane fluidity and compliance to a variety of noxious stresses. Accumulation of tamoxifen and its metabolites (all being highly lipophilic compounds), in biological membranes may result in low micromolar concentration locally in membranes, compatible with changes reported by others on phospholipid dynamics, alteration of the properties of receptors, channels and pumps, and scavenging of reactive oxygen species. We believe that such immediate biophysical action independent of ERs may contribute to the protection afforded to tamoxifen, its metabolites, and drugs structurally related to estrogens. Moreover, dystrophic mice treated with fulvestrant or cotreated with tamoxifen and fulvestrant (thus deprived of ERs), showed no phenotypic improvement, except for CK leakage from myofibres into the bloodstream (a marker of muscle damage), which was abolished, presumably due to accumulation of the drugs into muscle membranes.

Clinical Studies With Tamoxifen for DMD

The preclinical findings mentioned above, and other results led to a single-arm monocentric Phase I trial (NCT02835079)

which indicated that treatment with up to 20 mg/day of tamoxifen resulted in increased retention of muscle and respiratory function when compared with age- and performance-matched historical controls on glucocorticoids only. Mild and transient gynecomastia was the only noteworthy adverse effect reported in this trial.[48]

In a subsequent multicenter, randomized, double-blind, placebo-controlled, Phase 3 trial (NCT03354039) tamoxifen was safe and well-tolerated in DMD participants.[49] After 48 weeks of treatment, slower disease progression (loss of motor function over time) was indicated in the tamoxifen group. However, no significant difference was reported between groups for the primary efficacy outcome (change in participants' ambulation) or any of the secondary efficacy outcomes. Post-hoc analyses comparing the tamoxifen treatment group with historical control data did not show any clinically significant differences between the treatment and control. However, this trial was terminated prematurely due to the COVID-19 pandemic before the planned number of participants could be recruited, thus limiting the statistical interpretation of this study. Additionally, this trial also did not investigate the impacts of tamoxifen on respiratory function or cardiovascular function, all of which are known to be impacted by tamoxifen. Furthermore, post-hoc power analysis of this study indicated that 80% power would be achieved at 461 participants/group and 90% power at 617 participants/group using a 5% significance level with 2-tailed test.

In a further post-hoc analysis,[49] available echocardiographic data of 14 ambulant patients recruited at one study center were retrieved and compared before and after treatment. While there were no statistically significant findings, measures of both left ventricular end-diastolic diameter and left ventricular fractional shortening showed changes associated with the cardiomyopathy of DMD, and these trended toward a preservation of cardiac structure in the tamoxifen-treated group.

Recently published data from the evaluation of the safety and efficacy of tamoxifen in non-ambulatory patients with DMD from the same phase 3 trial mentioned above (NCT03354039), suggest that although tamoxifen was well tolerated it did not change efficacy between placebo (n=6) and treated groups (n= 8).[50]

Overall, tamoxifen has shown promise as a therapeutic agent for the reduction of DMD symptoms demonstrating anti-fibrotic and muscle-protective effects.[8,25] Given that the Phase 3 study (NCT0335403) was prematurely ended and subsequently determined to be underpowered, continued investigation into tamoxifen and its metabolites appears to be warranted. It is important to note that most patients in the trial opted for continuing on tamoxifen treatment in an open label extension phase of the clinical trial.

Endoxifen as a Possible DMD Therapy

Like many therapeutics, tamoxifen is metabolized in vivo to generate related compounds with their own unique pharmacology.[51] Analysis of the clinical work on tamoxifen in DMD provides a rationale for use of endoxifen as a therapy for DMD. Endoxifen is an active secondary metabolite of tamoxifen and has an ER affinity that is 30- to 100-fold greater than that of tamoxifen. Variability of tamoxifen metabolism, due to both CYP2D6 polymorphisms and to potential interference of CYP2D6 activity by other drugs, can impact the therapeutic bio-availability of the active metabolites.[52] Use of endoxifen circumvents these issues and is likely to provide more controlled circulating endoxifen levels. Of particular relevance to DMD, circulating plasma levels of endoxifen are thought to be approximately 10-times lower than tissue levels.[53] The potential for a more consistent, sustained pro-estrogenic effect exerted on tissues such as uterus, bone, and muscles serves as the basis for use of endoxifen rather than tamoxifen in DMD.

Clinical results indicate that endoxifen, like tamoxifen, reduces cholesterol and triglyceride levels. Endoxifen is also known to inhibit PKC allosterically and has been shown to impact Akt signaling.[54,55] Moreover, in vitro endoxifen exhibits up to a 4-fold higher potency for inhibition of PKC activity (and thus Akt signaling) than tamoxifen, suggesting that endoxifen, unlike tamoxifen, may have a therapeutic benefit for conditions where a role for PKC activity has been established, such as bipolar affective disorder (BPAD) and DMD. Understanding potential clinical applications for endoxifen can start with an examination of known physiological effects, as in the case of PKC.

Key Pathways Impacted by Endoxifen

Although the mechanisms of action for endoxifen are unknown, studies indicate they are concentration dependent and different from those of tamoxifen's other important metabolite, 4HT.[56] For instance, as shown in Figure 2, unlike 4HT, endoxifen targets ERα for proteasomal degradation and is also thought to cause the formation of ERβ homodimers and

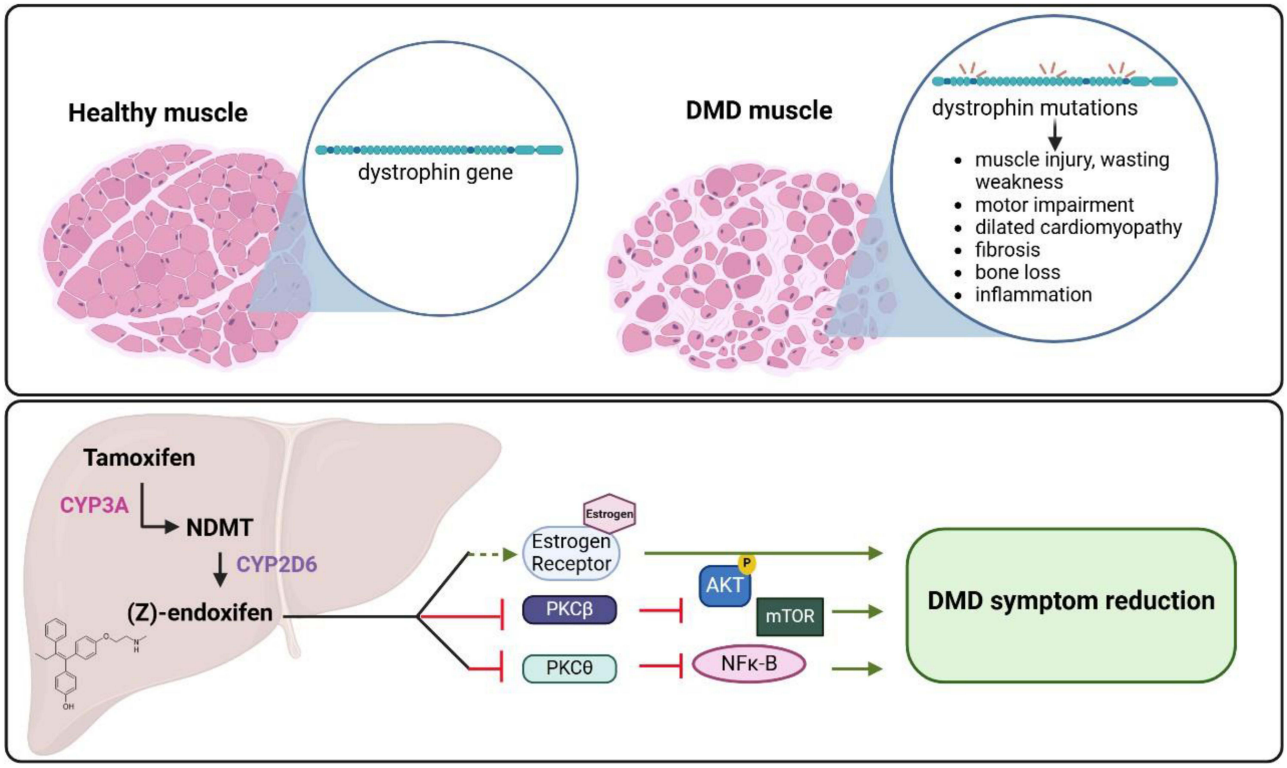

Figure 2 Proposed Mechanisms of Action of Endoxifen in Duchenne Muscular Dystrophy. The proposed mechanism of action of endoxifen in DMD relies on the bidirectional effects it has on estrogen signaling as well as estrogen independent effects. Unlike the anti-estrogen effects seen in breast cancer, in muscle, endoxifen/tamoxifen activates estrogen receptor signaling playing a protective role in DMD progression. Non estrogen dependent effects are centered of its inhibition of PKCs resulting in blockage of AKT/mTOR and NFκ-B signaling (red lines). Inhibition of these pro-inflammatory pathways is hypothesized to prevent DMD disease progression. Abbreviations: DMD, Duchenne Muscular Dystrophy; NDMT, N-desmethyltamoxifen; PKC, protein kinase C; AKT, protein kinase B; mTOR, target of rapamycin NF κB, Nuclear factor kappa-chain-enhancer of activated B cells. Created in BioRender. Hammer, (**S**) (2025) https://BioRender.com/d78n986.

ERα/ERβ heterodimers.[57] These endoxifen-induced dimerization events have been shown to stabilize ERβ, sensitizing cells to the anti-estrogenic effects of endoxifen in breast cancer cells. Additionally, endoxifen, but not 4HT, is able to block estrogen-induced changes in gene expression at clinically relevant levels.[56] In silico modeling and preclinical data also suggest that endoxifen has actions that are independent of ERs. It remains to be established whether these molecular targets and subsequent actions established in breast cancer cells also apply to myofibres in vivo. It is very likely that upon binding to ERs in muscles, endoxifen would exert pro-estrogenic actions, as tamoxifen and other SERMs do.PKC-β and Akt are suspected to be responsible for mediating endoxifen effects on protein phosphorylation. Endoxifen inhibits PKC-β1 kinase activity more potently than other PKC isoforms. Inhibition of PKC-β1 has been shown to lead to degradation which in turn attenuates phosphorylation of Akt.[54] However, endoxifen inhibition of PKC-θ is also of clinical relevance given its extensive regulatory role in skeletal muscle homeostasis and chronic inflammatory response. The role of endoxifen-mediated PKC signaling in DMD remains mostly unknown. Moreover, genetic ablation of PKC-θ in mdx mice prevents muscle wasting and improves muscle generation.[58] Other studies have also shown positive effects with pharmacologic inhibition of PKC-θ in terms of preventing force drop associated with the DMD phenotype.[59,60]

Estrogen Receptors

Endoxifen action in the context of DMD is primarily mediated via its effects on the two types of ERs that mediate cellular estrogen action. Nuclear ERs (ERα and ERβ) and membrane ERs (GPER/GPR30) are the 2 main classes of ERs.[61,62] Endoxifen modulates ER activity in the regulation, maintenance, and function of cardiac and skeletal muscle.[63] Both ERα and ERβ are expressed in the cardiac and skeletal muscles of men and women, with ERα predominating in adipose tissue and ERβ predominating in skeletal muscle.[64] In men, mRNA expression of ERα and ERβ has been shown to increase with endurance training, suggesting ERs are involved in the adaptation of skeletal muscle to endurance

training.[65] In mice, ERα and ERβ have been found to be 4.3 and 3.5 times more abundant in dystrophic muscles, respectively.[66]

Clinically, estrogen deficiency is associated with loss of skeletal muscle mass and strength in postmenopausal women. The impacts of estrogen deficiency on skeletal muscle mass and strength in men are less clear, as studies have shown that ERs activate the proliferation of muscle satellite cells and maintain the muscle satellite cell number in female but not male mice.[66] However, while ERs are thought to have minimal impact on skeletal muscle mass, strength, and contractile function in healthy men, they may still be important in muscle recovery after injury and in dystrophic patients as they are thought to be involved in the regulation of inflammation and fibrosis.[67] This is especially relevant in DMD where chronic inflammation and insufficient muscle repair mechanisms result in progressive muscle degeneration.

ERs are also known to be key regulators of other multifaceted pathways. Notably, GPER is involved in regulation of the secreted phosphoprotein 1 (Spp1) gene.[68,69] This gene encodes osteopontin, a protein which regulates muscle inflammation and regeneration. ERα is involved in the regulation of mitochondrial function and metabolism, and ERβ is involved in the regulation of PI3K, Akt, and glutathione peroxidase.[70] The protective role of estrogen and ERs in cardiac health has also been well documented and ER signaling, specifically ERβ, is known to be involved in the regulation of cardiac fibrosis and inflammation.[71]

Calcium Signaling

Disruptions in muscle calcium homeostasis and increased intracellular calcium levels are central pathophysiological components of DMD. Calcium ions are pivotal in muscle contraction, signaling, and overall health. Perturbations in calcium signaling pathways have been shown to result in elevated intracellular levels of calcium and ROS, like those seen in patients with DMD. Disruptions in calcium homeostasis are also known to impact mitochondrial function resulting in reduced adenosine triphosphate (ATP) production and loss of membrane potential.[72] Subsequently, this alteration results in cellular damage, necrosis, and contraction-induced muscle damage. Over time, this leads to fibrosis and increased adipose tissue, further perpetuating the decreases in muscular function observed in patients with DMD. Therapies that can stabilize calcium levels within muscle cells have the potential to mitigate some of the symptoms of DMD. While the impacts of endoxifen on intracellular calcium in muscle cells are unknown, similar SERMS have demonstrated a dose-dependent protective effect against calcium influx in U2OS cells.[73]

Mitochondrial Dysfunction

Calcium homeostasis in both skeletal and cardiac muscle is linked with mitochondrial function, where a significant cellular energy expenditure is devoted to maintenance of transmembrane ionic and electrical balance. Evidence from both animal models and patients with DMD implicates mitochondrial dysfunction in DMD pathogenesis, linking it with energy dyshomeostasis, increased ROS production, and reduced muscle cell membrane repair. Increased cytochrome b-245 beta chain (Cyb-beta) levels have been implicated in DMD pathology. In a mouse model, ROS induces expression of the Src proto-oncogene, a non-receptor tyrosine kinase that stimulates Cyb-beta ROS generation during stretched contractions, creating a positive feedback loop that generates more ROS.[74]

The Src activation via Cyb-beta is thought to stimulate the PI3k/Akt/mTOR pathway, impairing autophagy and inhibiting lysosome formation.[74] In vivo results support this as treatment of *mdx* mice with simvastatin (a cholesterol-reducing agent) reduced Cyb-beta protein levels, which correlated with a partial restoration of dystrophic parameters.[75] While the actions of endoxifen on mitochondria have not been fully elucidated, studies to date suggest that, unlike tamoxifen, endoxifen may have a protective effect by preventing membrane depolarization in response to oxidative stress.

Protein Kinase C (PKC)

The PKC family of proteins has been implicated in DMD pathogenesis as well as in the broader spectrum of muscular diseases, indicating their essential role in muscle physiology and pathophysiology. With respect to DMD, PKC-θ is of particular interest as it is thought to be involved in skeletal muscle regulation and chronic inflammatory responses.[58] In *mdx* mice, genetic ablation of *Prkcq* (the PKC-θ gene) resulted in reduced inflammation and improved muscle healing

and regeneration, which was paired with reductions in pro-inflammatory genes and pro-fibrotic markers, including a reduction in NF-κB activation. Studies using a PKC-θ inhibitor were similar to those in the genetic ablation model: treatment led to reduced inflammatory pathway activation and immune cell infiltration, which was associated with a significant reduction in muscle damage. Inhibition of PKC-θ also resulted in maintained muscle generation, preserved muscle integrity, and improvements in muscle activity- recovery performance.[76] Inhibition of PKC-θ has also been found to reduce dystrophic heart inflammation and improve the dystrophic heart phenotype and function of DMD cardiomyopathy in *mdx* mice.[76] Together these data show that PKC-θ significantly contributes to driving DMD pathology and that targeting PKC-θ may be an attractive therapeutic approach for treatment of this disease.

PKC-β, another PKC family member, and known target of endoxifen, is also known to be involved in NF-κB activation although its effect on DMD pathogenesis, if any, is unknown. Although the role of PKC-β in DMD progression is an active area of research, several studies have shown that activation of PKC-β leads to muscle damage in DMD by promoting inflammatory processes, increasing oxidative stress and modifying calcium signaling within muscle fibers. Thus, it is hypothesized that inhibition of PKC-β signaling has the potential to reduce inflammation cytokine production and consequently reduce immune cell infiltration into damaged muscle tissue.[77,78] Importantly, future studies are warranted to fully understand the specific role that PKC-β plays in DMD progression.

Protein Kinase B (Akt)

Protein kinase B, also known as Akt, is a serine threonine kinase, crucial for cellular growth, proliferation, survival, and autophagy. Akt is a key kinase in the multifaceted PI3K/Akt/mTOR pathway, which helps regulate numerous processes. Critically, activation of the PI3K/Akt/mTOR pathway has been shown to be a key modulator of skeletal muscle hypertrophy. Overexpression of Akt in *mdx* mice resulted in improved muscle function, improvement of histopathological measures, and enhancement of endogenous compensatory mechanisms.[79] However, activation of the PI3K/Akt pathway also stimulates the proliferation of fibro-adipogenic progenitors (FAPs), which are muscle-resident stem cells. FAPs are believed to differentiate into fibrogenic cells in response to TGF-β signaling and are a determinant source of fibro-adipogenic depositions, which are a hallmark of DMD.

This finding suggests that inhibition of Akt phosphorylation would paradoxically lower fibro-adipogenic depositions but also decrease endogenous compensatory mechanisms and overall muscle function. Thus, further study into modulation of the PI3K/Akt pathway is needed to determine its impacts on inflammation, cell survival, and muscle regeneration rates in the context of DMD.

Bone Mineralization

Both tamoxifen and endoxifen have also demonstrated positive effects on skeletal bone. In mice, endoxifen treatment led to significantly higher bone mineral density and bone mineral content throughout the skeleton relative to control animals, and induced the expression of osteoblast, osteoclast, and osteocyte marker genes.[80,81] These positive effects clearly warrant further study as preventing bone loss and/or promoting bone density would benefit DMD patients.

Inflammation

Muscle damage stimulates cytokine expression resulting in infiltration of muscle cells primarily by macrophages, neutrophils, and T cells which further drives the dystrophy seen in DMD. One such inflammatory pathway involved in this process involves activation of the NF-κB transcriptome of pro-inflammatory cytokines, chemokines, and adhesion molecules. This contributes to the recruitment of immune cells to the muscle tissue and exacerbation of muscle damage. Based on this, innovative compounds such as edasalonexent have been developed to inhibit NF-κB.[82] However, clinical trials testing NF-κB inhibition via edasalonexent although well tolerated and safe, failed to improve primary and secondary functional endpoints tested.[83] Additional subgroup analysis demonstrated that DMD progression could be slowed down if treatment started prior to 6 years of age.[82] Another potential inflammatory target linked to DMD pathology is the c-Jun N-terminal kinase (JNK) pathway. JNK plays a significant role in mediating the inflammatory response of DMD, with activation of JNK leading to increased apoptosis, inflammation, and muscle pathology.[84,85] TGF-β is also a key inflammatory regulator. High TGF-β activity is associated with chronic muscle damage and inflammation and is linked to the progression of fibrosis

and impaired muscle regeneration. In Wistar rats, antibody inhibition of TGF-β1 resulted in a reduction of fibrosis development along with significant improvement in skeletal muscle generation.[86] However, little success was seen through attempts to inhibit TGF-β, specifically myostatin, in the clinical setting due to an inability to improve specific muscle quality in DMD while increasing muscle mass. Signaling of TGF-β also involves the Spp1 gene product osteopontin. Clinical data have found that expression of osteopontin is a predictive factor for DMD severity. In *mdx* mice, osteopontin has been shown to influence macrophage polarization and promotes the fibrosis that drives DMD pathology.[87] It has also been shown to influence regulation of TGF-β signaling through induction of matrix metalloprotein 9.[88] Overall, it is thought that increases in osteopontin promotes muscle fibrosis upstream of TGF-β.

Cholesterol Metabolism

Elevated levels of cholesterol have been found in muscle biopsies of patients with DMD. Increased plasma lipid levels have also been linked with decreased cardiac function, exacerbation of DMD muscle pathology, and increased myofiber damage and fibro-fatty replacement.[89]

Tools to explore metabolic dysregulation in DMD include assessment of circulating micro RNAs (miRNA) or activity of transcription factors associated with lipid biosynthesis. miRNAs have the potential to be diagnostic tools and their plasma levels can provide clues to the over metabolic state of the patient based upon their potential to alter activity of regulatory transcription factors. In one study, transcription factors involved in cholesterol biosynthesis were specifically upregulated in gastrocnemius and diaphragm muscle of *mdx* mice.[89] Network analysis revealed one of these transcription factors (SREBF1) was central in signaling pathways mediating muscle hypertrophy and inflammatory responses as well as its impact on cholesterol. In vivo results support this hypothesis, as treatment of *mdx* mice with simvastatin has been reported to partially restore dystrophic parameters, suggesting cholesterol homeostasis may play a role in DMD pathogenesis.[89] Interestingly, ERα helps regulate SREBF1, which suggests that endoxifen could influence regulation of this pathway.[90]

Endoxifen Clinical Studies

The only clinical studies of endoxifen to date have been limited to BPAD and oncology.

A Phase 2, double-blind, active-controlled trial evaluating endoxifen for use in Type I BPAD found endoxifen to be safe and well-tolerated. It also found endoxifen to have similar efficacy to but numerically fewer side effects than divalproex (valproate), an anti-seizure medication that is also used for BPAD.[91] This is noteworthy because both endoxifen and divalproex act, at least in part, via their inhibition of PKC.

In comparison of tamoxifen and endoxifen for refractory metastatic breast cancer, endoxifen was found to be safe and well-tolerated in doses up to 80 mg, while tamoxifen can only be safely administered in doses up to 20 mg.[92] As endoxifen does not require liver metabolism by CYP2D, it can reach similar therapeutic concentrations at much lower doses than tamoxifen and provide consistent dosing between patients. Of note, gender differences in tamoxifen metabolism have been reported; male patients given tamoxifen for breast cancer reached significantly lower endoxifen concentration levels than female patients.[38] In addition, tamoxifen use has also been linked with adverse reactions such as mood changes, hot flashes, and thromboembolic events;[93] none of these have been reported with endoxifen use.

Endoxifen Therapeutic Context and Rationale

DMD remains a formidable medical issue due to its severe progressive muscular degeneration and the absence of a cure. The current therapeutic landscape, dominated by glucocorticoids, offers palliative benefits but also presents substantial adverse effects and limited efficacy. Adjuvant therapy with endoxifen may provide an improved risk-benefit profile when compared to current therapeutic options. While both gene and cell therapies are promising, and offer the potential of curative therapies, there are currently many barriers to their widespread adoption, including the costs of greater than $1.0 MM US for these treatments. While it is too early to estimate the cost of endoxifen for DMD, using a small molecule approach provides significant opportunities to achieve a fair price for treatment. In this context, given both the previous successes and limitations of tamoxifen and the known differences between tamoxifen and endoxifen, exploring endoxifen as an adjuvant treatment for DMD is not only innovative, but necessary.

Discussion

Mechanisms of Action

It has been suggested that the enhancement of estrogenic signaling in muscle by ER modulators might be a therapeutic approach to treat DMD. This review robustly outlines endoxifen mechanisms, particularly its interaction with ERs that are pivotal in regulation of both muscle maintenance and repair, and in potential non-ER pathways. Estrogen receptors, especially ERβ, are abundant in dystrophic muscles and are known to influence muscle cell survival and regeneration. Modulation of these receptors may help mitigate the inflammatory and fibrotic progression seen in DMD. This hypothesis is supported by ongoing preclinical studies, with initial results indicating that endoxifen can modulate key cellular pathways implicated in DMD pathology via ERs. Further investigation on endoxifen potential to bidirectionally modulate estrogen signaling, using in silico modeling, is also underway.

The role of endoxifen in regulating calcium homeostasis and reducing oxidative stress provides additional mechanisms that could reduce muscle deterioration and increase the resilience and repair of muscle cells. Evidence also suggests that the reduction of plasma lipid levels by tamoxifen or endoxifen use may result in a clinically relevant reduction in cardiovascular risk and reduce levels of fibrofatty replacement.[94] Finally, the modulation of PKC and Akt signaling pathways by endoxifen at clinically relevant levels underscores its multifaceted mechanistic profile,[54] influencing muscle fiber stability, immune and inflammatory responses, and FAP proliferation. This multi-pathway intervention is critical, given the complex interplay between the multiple pathways implicated in DMD pathogenesis.

Symptomatic (manifesting) DMD carriers have also been reported with various DMD carrier associated pathologies (D-CAPs). Estimates indicate that approximately 2.5–19% of DMD carriers have skeletal muscle symptoms and 7.3–16.7% develop dilated cardiomyopathy.[1] Research on this population is extremely limited, in part because of difficulties in diagnosis of carrier status. The above estimates of the percentages of carriers who manifest skeletal muscle symptoms and who develop dilated cardiomyopathy show that D-CAPs represent an important healthcare need.

There is preliminary evidence that the downstream pathological mechanisms described above are therapeutic targets for DMD and D-CAPs that are each potentially modulated or effected by endoxifen's multiple mechanisms of action in a manner that would be beneficial to patients. This evidence, as described above, is both in vitro and in vivo. There is now a substantial body of clinical evidence developed in indications other than DMD that supports the safety profile of endoxifen as a therapeutic agent.[92,95,96] While there have been many difficulties in developing therapies for DMD, these failed therapies were in large part curative therapies, directly targeting the lack of functional dystrophin. Curative therapies have safety, technical, and financial challenges to meet. The role of endoxifen would be reviewed solely with respect to targets downstream from dystrophin, and the goal of endoxifen as a therapy is palliative, with the aim of slowing disease progression, rather than curative. Given the safety profile of endoxifen, its use should not have an adverse effect on the DMD and D-CAPs patients who may benefit from endoxifen. Endoxifen may provide a material mitigation of symptoms. Mitigation of symptoms in and of itself can have a profound and positive effect on DMD and D-CAPS patients, and provide them with time during which curative therapies may be developed.

Future Directions and Research Needs

This review highlights endoxifen potential as a repurposed therapeutic, given the current state of research and clinical needs in the DMD landscape and suggests the necessity of clinical trials to examine endoxifen efficacy and safety as an adjuvant therapy in humans with DMD and who are DMD carriers. Comparative exploration of tamoxifen versus endoxifen effects on cardiomyopathy and respiratory function in patients with DMD and those who are DMD carriers could provide further insights into their potential as adjuvant therapeutics. Prior to initiating any clinical trials, artificial intelligence assisted in silico work, along with targeted cell and in vivo preclinical work, should be completed to efficiently validate and prioritize specific target pathways and endoxifen activity with respect to those pathways. These subsequent clinical studies should be designed to not only corroborate the findings from cell and animal models and in silico work, but also to explore the pharmacokinetics, optimal dosing, and long-term impacts of endoxifen treatment. The development of endoxifen does not diminish the need to continue to attempt to find curative and other palliative therapies

that will benefit DMD patients and carriers. Data generated from endoxifen clinical trials will be useful in the development of future curative and palliative therapies for this population.

Abbreviations

Akt, Protein kinase B; COVID-19, Coronavirus disease 19; DMD, Duchenne Muscular Dystrophy; IGF1, insulin-like growth factor 1; mTOR, mammalian target of rapamycin; NF-κB, Nuclear factor kappa-chain-enhancer of activated B cells; Nrf2, Nuclear factor erythroid 2-related factor 2; PKC, protein kinase C; PI3K, Phosphoinositide 3-kinase; p65, component of NF-κB signaling pathway; Spp1, Secreted phosphoprotein 1 gene, codes for osteopontin.

Data Sharing Statement

Data sharing is not applicable to this article as no data sets were generated or analyzed.

Acknowledgments

The authors would like to thank Josedarling Desane for the creation of Figure 1 and Figure 2 was created in BioRender. Created in BioRender. Hammer, S. (2025) https://BioRender.com/d78n986

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

Mr H. Lawrence Remmel holds shares in Atossa Therapeutics, Inc.; he is the lead independent director of Atossa Therapeutics, Inc. & conducts research partially sponsored by Atossa Therapeutics, Inc. Dr. Sandra S. Hammer receives a salary, bonus and stock from Atossa Therapeutics Inc. Dr Steven Quay reports personal fees from Atossa Therapeutics Inc, outside the submitted work. In addition, Dr Steven Quay has multiple patents licensed to Atossa Therapeutics, Inc and he is the Founder and CEO of Atossa Therapeutics, Inc. He receives a salary, bonus, and stock options from Atossa. He is also the inventor of multiple patents and patent applications related to endoxifen and its clinical use. The authors report no other conflicts of interest in this work.

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