P2X7 purinoceptor as a therapeutic target in muscular dystrophies

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Abstract

Mutations in the dystrophin and sarcoglycans genes result in muscular dystrophies causing severe disability and premature death and where no effective treatment is available. New therapeutic approaches targeting secondary disease mechanisms have a strong translational potential. Dystrophic muscle damage triggers release of ATP whilst loss of ecto-ATPase activity of sarcoglycan further elevates extracellular ATP (eATP) levels. Such high eATP activates P2X7 purinoceptors on immune cells; these contribute to chronic inflammatory and immune responses that exacerbate the dystrophic pathology. Dystrophin mutations coincide with a significant P2X7 upregulation in Duchenne muscular dystrophy (DMD) muscle and alter receptor signalling in mouse dystrophic myoblasts and myofibres. P2X7 overexpression combined with the eATP-rich environment lead to cell dysfunction and death and ultimately to ineffective regeneration. P2X7 is therefore a therapeutic target for reducing damaging inflammation and supporting the repair of dystrophic muscles. Accordingly, genetic ablation and pharmacological inhibition of the eATP-P2X7 axis alleviated dystrophic phenotypes in mouse models of dystrophinopathy and sarcoglycanopathy. Thus, P2X7 inhibitors are good candidates for rapid re-purposing for the treatment of these highly debilitating diseases. Such therapy is not constrained by causative mutations, so it would be suitable for all patients. Moreover, it appears effective in alleviating both muscle and non-muscle symptoms.

Highlights

Inflammation is a pathological hallmark of muscular dystrophies
Dystrophin and/or sarcoglycans loss alter eATP degradation and P2X7 signalling in muscles
Treatment of this purinergic abnormality reduces damaging inflammation and supports dystrophic muscle repair
P2X7 inhibitors are good candidates for rapid re-purposing as therapeutics in several muscular dystrophies

Muscular dystrophies (MD) are a group of inherited diseases of a varying severity. The most common, Duchenne muscular dystrophy (DMD), causes disability and death of young men due to progressive muscle degeneration aggravated by sterile inflammation. DMD is also associated with a loss of bone mineral density, an independent disease manifestation contributing to impaired ambulation, and with a cognitive impairment [1••]. Sarcoglycanopathies (LGMD2C-F) caused by mutations in the γ,α, β and δ sarcoglycan genes are heterogeneous for onset and severity [2••]. No treatment offers any long-term improvement for any of these diseases.

Purinergic signalling in healthy and diseased muscle

Skeletal muscle (SM) is responsible for movement and posture maintenance but also thermogenesis and metabolic homeostasis. All these functions require ATP and, at 5–10 mM range, its cytosolic concentration within the SM cell [3] is particularly high.

In response to stimuli or muscle activity, muscle cells release small amounts of ATP [4]. This extracellular ATP (eATP) becomes a signal acting *via* specific ionotropic (P2X) or metabotropic (P2Y) purinergic receptors, evoking increased intracellular Ca^{2+} levels and a range of downstream effects (reviewed in [5,6]).

Damage or death of muscle cells result in large amounts of eATP being released, reaching millimolar concentrations in the injury area. This eATP becomes an important danger/damage associated molecular patter (DAMP) signal, triggering inflammatory responses. The critical role in this process is played by the P2X7, responding to much higher (> 1mM) eATP concentrations than other purinoceptors and being expressed by virtually all the immune cell types [7•]. Importantly, acute muscle inflammation is essential for the removal of dead and dying cells and ultimately regeneration [8]. However, the continuous, high level eATP-mediated stimulation accompanying a chronic muscle damage and one that is typical for MD, can exacerbate disease symptoms.

The mechanism of this purinergic phenotype in MD is complex: eATP concentrations are regulated by extracellular hydrolysing enzymes (nucleotidases) [9]. Importantly, 25% of eATP degradation in muscles has been attributed to α-sarcoglycan, the muscle-specific ATP hydrolase [10••,11] that also is a member of the dystrophin-associated protein (DAP) complex. Thus, *α*-sarcoglycan is lost from the sarcolemma in DMD and also in limb-girdle muscular dystrophies (LGMD2C-F). Given that ectoATPases cannot cope with eATP in

excess of 100 μM [12], the balance between ATP release and its degradation in DMD and sarcoglycanopathies is altered even further, with eATP levels being increased dramatically. By over-activating purinoceptors on both muscle and immune cell, high eATP contributes to abnormal intracellular Ca^{2+} homeostasis in the former (see below). It also triggers the damaging chronic inflammatory and immune responses, which are found in dystrophic muscles [13].

Inflammation and immune responses in muscular dystrophy

Several studies demonstrated that chronic muscle inflammation plays a crucial role in the pathogenesis of DMD. Specifically, anti-cytokine therapies or treatments inducing depletion of CD4, CD8 lymphocytes, neutrophils, dendritic cells or macrophages (that are all found to infiltrate necrotic muscle) significantly improved the dystrophic phenotype *in vivo* [13, 19-27]. Muscles of the mdx mouse, the most commonly used model of DMD, contain 20 times more macrophages and 7 times more dendritic cells than is found in healthy individuals [28].

Interestingly, transmission of pathology from mdx to healthy mice by adoptive transfer of primed immune cells [23] and the presence of specific cytotoxic T-cell receptor gene rearrangements in DMD [19] also suggest breakdown in the peripheral tolerance to self-molecules. Moreover, dystrophin is immunogenic: The presence of alloreactive T-cells against dystrophin epitopes has been found in DMD patients [29••] and exon skipping and suppression of stop codons used to re-express dystrophin in DMD patients [30] can prime or recall dystrophin-specific T-cell responses. Such problems have been documented in both experimental and clinical trials [31].

Therefore, suppression of inflammatory/immune responses can not only reduce the direct dystrophic muscle damage evoked by infiltrating cells but is also pre-requisite for successful molecular therapies. Given that the mechanism underlying the dystrophic inflammation involves “danger signals” activating P2X7 expressed by all the muscle-infiltrating immune cells, this purinoceptor emerges as the key player.

P2X7 expression in dystrophic muscle cells

Furthermore, alongside P2X7 expression and activation in inflammatory cells, studies showed a dramatic up-regulation of this purinoceptor in DMD patients’ muscle biopsy

samples [32] and also in muscle and isolated muscle cells from the mouse model of DMD [33-35]. P2X7 overexpression was particularly evident on mdx fast skeletal myofibres, where it was linked to enhanced sarcolemma permeability [35•]. Crucially, fast muscle fibres are preferentially affected in DMD [1].

Whilst P2X7 overexpression has not been documented for muscle cells from DMD patients, significant functional abnormalities of this receptor in DMD lymphoblasts have been described [36•]. This might indicate a DMD-evoked purinergic abnormality being common across species and also cell types.

When exposed to eATP, dystrophic mdx myoblasts respond with increased cytosolic Ca^{2+} influx and IL-1β release [34,37]. Interestingly, P2X7 purinoceptor upregulation and NLRP3 inflammasome activation were also detected in the dysferlin knockout, a mouse model of yet another muscular dystrophy (LGMD2B). These findings suggest that dystrophic muscle cells are not just targets for but can also actively contribute to the aforementioned inflammatory process through purinergic signaling [37].

Moreover, high concentrations of eATP have been shown to activate abnormal Ca^{2+} influx into dystrophic muscle cells and alter their migration [38]. Treatment with apyrase, an eATP degrading enzyme, have reduced intracellular Ca^{2+} levels in *mdx* fibers [39]. While both P2Y and P2X components were noticeable [33], the ERK phosphorylation and the large pore opening indicated involvement of P2X7 purinoceptors [34]. This large pore opening, dye permeability and, in some circumstances, cell death via cell-specific and even unique mechanisms, e.g. pyroptosis and autosis are consequences of P2X7 activation under appropriate conditions [40]. Indeed, activation of the large pore was found triggering autophagic death [41] and increased release of active MMP-2 matrix metalloprotease from dystrophic muscle cells [38]. Specific P2X7 antagonists reduced these effects, confirming that this receptor contributes significantly to the deregulated homeostasis in dystrophic muscles.

In conclusion, large amounts of eATP released from dead and damaged dystrophic muscle cells, combined with reduced degradation of eATP due to loss of ecto-ATPase activity of α-sarcoglycan result in an environment consistent with over-activation of ATP receptors. The property of P2X7 making it activated by higher eATP, and the increased expression of P2X7 in dystrophic cells result in a strong and prolonged activation of this receptor in dystrophic fast myofibres and myoblasts. The consequences include increased sarcolemma permeability

[35•] contributing to the death of dystrophic myofibres [1] and the reduced myogenic potential of dystrophic muscles and therefore inefficient regeneration.

Finally, P2X7 activation contributes to severe and damaging sterile inflammation in a mechanism akin to that in other inflammatory diseases [40, 42] but also by inducing NLRP3 inflammasome activation and IL-1β release from dystrophic muscle cells themselves [37].

In view of the multiple mechanisms by which P2X7 contribute to the pathology (***Figure 1***), this purinoceptor emerges as an attractive therapeutic target, where its inhibition could reduce unwanted inflammatory responses but also reduce damage to myofibres and support the regenerative potential of dystrophic myoblasts.

P2X7 blockade as an approach to treat muscular dystrophies

Indeed, we and others have shown the therapeutic impact of both genetic ablation and pharmacological blockade of P2X7 in mdx mice *in vivo*. The mdx mouse is the animal model most widely used in pre-clinical studies of novel therapeutic approaches.

Using molecular and histological methods we demonstrated that ablation of P2X7 in mdx (mdx/P2X7$^{-/-}$) mice resulted in a profound attenuation of dystrophic symptoms [43••]. There were improvements in muscle structure, decreased inflammatory and pro-fibrotic molecular signatures. Comparisons of immune cell populations showed that pan-macrophage marker levels were lower and the F4/80 to CD163 (pro-inflammatory to pro-regenerative) ratio was significantly reduced in mdx/P2X7$^{-/-}$ compared to mdx muscle. While the CD4 and CD8 lymphocyte numbers were not affected, a significant increase in Foxp3 and IL-12α expression in mdx/P2X7$^{-/-}$ muscles indicated a shift towards Treg cells [43••]. Thus, P2X7 ablation ameliorated tissue inflammation and promoted T_{reg} cell functions, known to suppress dystrophic muscle damage [44•]. These improvements were evident both at the peak of disease severity but also at 20 months in leg, diaphragm and cardiac muscles (the heart outcome being important because long-surviving patients die of cardiac failure). Notably, functional analyses showed increased muscle strength *ex vivo* and also *in vivo*. Furthermore, in addition to the alleviation of muscle disease and decreased inflammation, reduced non-muscle symptoms including cognitive and bone improvements, were evident in mdx/P2X7$^{-/-}$ mice [43••,45]. The cognitive recovery is important given that severe impairment affects one-third of patients, further reducing the quality of life of sufferers and their families. It is currently not known whether this effect was due to P2X7 ablation in specific brain cells or

because of reduced levels of inflammatory mediators reaching the dystrophic brain through its leaky blood-brain barrier [46].

Bone deformities contribute significantly to the loss of ambulation in DMD patients. P2X7 plays significant roles in bone cells. Its ablation did not exacerbate but corrected the bone phenotype and the reduced inflammatory signature in mdx/P2X7$^{-/-}$ muscles may again translate into the reduced bone loss [43••,47].

These wide-ranging improvements reflected the impact of P2X7 activation on multiple disease processes (***Figure 1***). While the mechanism(s) require further studies, such a clinically-relevant therapeutic strategy that modifies both muscle and non-muscle symptoms represents a significant progress.

Therapeutic potential of the pharmacological inhibition of P2X7 in dystrophies

Pharmacological P2X7 inhibitors are available and improvements were observed even following short-term treatment of mdx mice with broad-spectrum compounds such as CBB and ox-ATP [32,43]. The therapeutic effect of ox-ATP also included reduced tissue inflammation and increased T_{reg} cells [32]. Other studies reported suramin treatment reducing mdx muscle damage [48,49]. Furthermore, in α-sarcoglycan null mice, blockade of the eATP/P2X purinergic pathway also delayed the progression of muscle disease and reduced inflammation [50•]. These data suggest that pharmacological purinergic antagonism might be therapeutic in DMD and also LGMD2C-F. However, ox-ATP and suramin used in those studies are non-specific. These drugs have not been optimized for potency and selectivity at the P2X7 receptor or cleared for use in humans. Yet, all the current data identify this receptor as the main player in the dystrophic pathology and so the specific P2X7 blockade emerges as an attractive target for translational approaches in muscular dystrophy.

More selective P2X7 inhibitors have been developed and some of these (e.g. AZD9056 and CE-224,535) used in phase 2 clinical trials in inflammatory diseases [51-53]. Such drugs, if re-purposed for DMD, would offer the most specific treatment effect. However, none of these compounds have been approved as medicines and none tested in children. On the other hand, Zidovudnine (azidothymidine, AZT), one of the mainstay nucleoside reverse transcriptase inhibitors, is a potent P2X7 blocker [54] binding the same allosteric site as other P2X7 drugs [55,56••]. A short-term AZT treatment in mdx mice attenuated the key disease parameters causing reduced sarcolemma permeability, decreased inflammation in leg and heart muscles

and producing increased muscle strength. Recovery was evident without any detectable side effects [56]. Given these results, AZT with its established pharmacological profile also in the pediatric population, is a candidate for rapid re-purposing for the treatment of this highly debilitating and invariably lethal disease.

Conclusions

Dystrophic muscle environment is consistent with elevates levels of eATP leading to damaging P2X7 purinoceptor activity in dystrophic muscles and also triggering chronic inflammation and immune responses that exacerbate the dystrophic pathology. Studies in mouse models of MD demonstrated that P2X7 antagonists are good candidates for rapid re-purposing for the treatment of these highly debilitating diseases. P2X7 therapy is not constrained by causative mutations in both DMD and LGMDs. It could therefore be used in all patients irrespective of their genetic defect and may be effective in alleviating both muscle and non-muscle abnormalities of DMD. Finally, specific targeting of inflammatory/immune functions via P2X7 inhibition could not only reduce the dystrophic muscle damage but also prevent immunization by dystrophin re-expression through molecular therapies.

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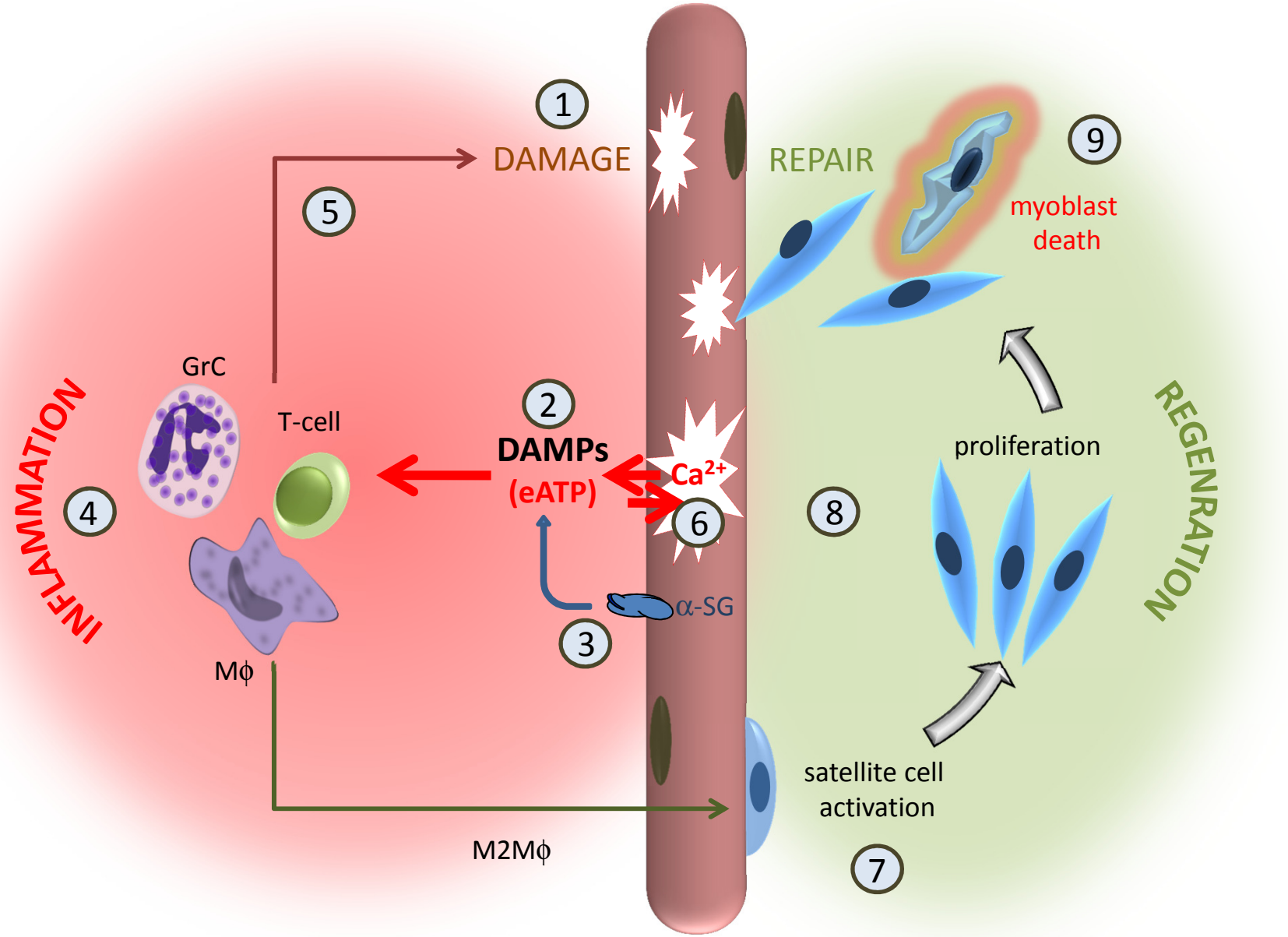
Figure Legend

Fig 1. The involvement of P2X7 purinoceptors in the dystrophic pathology. Loss of dystrophin and/or sarcoglycans affects myofibers (1). Dying muscle release large quantities of DAMPs, including eATP (2) whereas deficiency of α-sarcoglycan (3) leads to reduced eATP hydrolysis. Resulting greatly elevated levels of eATP and DAMPs trigger chronic inflammation (4). Infiltrating cells including T-lymphocytes, granulocytes (GrC), dendritic cells and macrophages (Mϕ) cause further damage to myofibers (5), while chronically elevated levels of inflammatory mediators affect brain and bone functions (not shown). The intracellular Ca^{2+} build-up *via* permeable sarcolemma and abnormalities of Ca^{2+} signalling pathways exacerbate myofibre injury (6). Inflammation induces muscle regeneration by activating satellite cells (7), subsequent myoblast proliferation, migration to damaged sites and repair (8). However, chronic inflammation and high eATPe levels reduce repair of dystrophic muscle by promoting the M1 over the pro-regenerative M2 macrophages (M2Mϕ) and by affecting myogenic cell functions. Moreover, high eATP levels combined with P2X7 over-expression contribute to dystrophic myoblast death (9) and thus reduce muscle regeneration further still.


1
DAMAGE
REPAIR
9
myoblast
death
5
GrC
T-cell
2
DAMPs
(eATP)
Ca2+
6
proliferation
INFLAMMATION
4
8
REGENRATION
α-SG
3
Mϕ
satellite cell
activation
M2Mϕ
7