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Maximizing the Efficiency of Prednisolone in Duchenne Muscular Dystrophy through reformulation

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Abstract

Duchenne muscular dystrophy (DMD) is a genetic disease in which skeletal muscle membranes lack the protein dystrophin, decreasing membrane stability and causing progressive muscle degeneration. DMD has no cure, but is commonly treated with glucocorticoids such as prednisolone to reduce inflammatory symptoms and prolong ambulation. However, chronic use of prednisolone yields systemic side effects, thus we aim to reduce the dose needed and preferentially target this drug to muscle tissue. To this end, we have focused on two strategies for improved prednisolone formulation: 1.) combination with thiamine (vitamin B1) to synergistically improve anti-inflammatory properties and 2.) entrapment within liposomes to allow a concentrated payload ultimately targeting muscle cells. Thiamine, in its diphosphate form, is a cofactor to enzymes in the TCA cycle and pentose-phosphate pathway. Mechanistically, thiamine supplementation should improve muscle weakness and inflammatory symptoms beyond prednisolone alone. Our approach used DMD and iHSKM cells treated with LPS to induce inflammation and rescued with prednisolone alone and in combination with thiamine. IL-6 protein expression by Western blot and mRNA expression by real-time PCR will be monitored, along with reactive oxygen species measurements in live cells. Further, we have prepared a liposomal prednisolone formulation co-entrapping fluorescent dye for understanding the uptake mechanism in these cells using fluorescence microscopy.

About Duchenne Muscular Dystrophy

Duchenne Muscular Dystrophy (DMD) is a progressive, X-linked disease caused by a mutation in the dystrophin gene.[1] It affects 1 in 3,000 newborn boys with signs of the disease occurring around 2 to 5 years old.[1] Boys afflicted with this disease have delayed development of motor skills with the average age of death being 20 years old.[1,2]

The loss of the dystrophin protein causes inflammatory cell reactions and the replacement of muscle fibers with fibrosis.[3]

Current Treatments

Two corticosteroids are commonly used to treat DMD: Deflazacort and prednisone.

The main action of corticosteroids is to reduce inflammation. They have been shown to improve pulmonary function, delay the onset of cardiomyopathy which delays heart failure, and allows the patient to have independent ambulation for longer.[5] Prednisone is a steroid used to treat many different diseases, including DMD, arthritis, and lupus. Deflazacort is a derivative of prednisone with similar anti-inflammatory activity, but less side effects with prolonged use.[4,5] DMD requires that prednisone be taken in high doses for a prolonged period of time. Because of this, some longer term side effects are weight gain, hypertension, hyperactivity, and cataracts.

Prednisone Mechanism of Action

The inflammatory pathway is initiated by activation of Nuclear factor-κB (NF-κB) which induces the expression of inflammatory cytokines such as interleukin-6 (IL-6).[6] Glucocorticoids bind to glucocorticoid receptors in the cytoplasm which are then transported to the nucleus to promoter regions known as glucocorticoid response elements (GRE) where gene expression is stimulated or repressed.[7] Reactive Oxygen Species (ROS) are produced by cells involved in the host-defense response.[8]

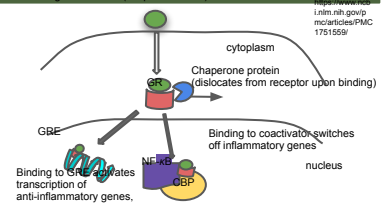

Figure 1: Two mechanisms shown, GR bind to GRE and activate anti-inflammatory genes. GR can also interact with coactivator molecules, such as CBP(CREB-binding protein) , which are activated by NF-κB, and switch off pro-inflammatory genes

Thiamine Mechanism of Action

Thiamine is an essential water-soluble B-vitamin. Thiamine is absorbed in the small intestine and is then phosphorylated to its biologically active form, thiamine diphosphate. Thiamine diphosphate is an essential coenzyme for enzymes needed in carbohydrate metabolism. Benfotiamine is a lipophilic derivative of thiamine which ensures improved oral bioavailability.[9] It has been shown that benfotiamine prevents the activation of NF-κB, which prevents oxidative stress.[9]

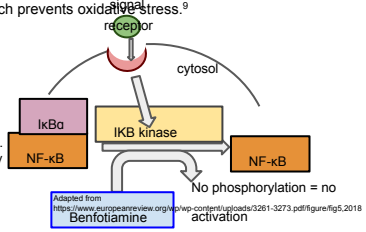

Figure 2.
Benfotiamine inhibits the binding of NF-kB. This has been shown in LPS-induced inflammation. NF-kB is activated when it becomes phosphorylated by IKB kinase. This activation is prevented by benfotiamine and causes the degradation of IkBα. This decreases the migration of inflammatory cells.[10]

Liposome Preparation and Analysis

- DPPC(1,2-dipalmitoyl-sn-glycero-3-phosphocholine) PEG-DSPE (1,2-distearoyl-phosphatidylethanolamine-methyl- polyethyleneglycol conjugate-1800), cholesterol, and lissamine rhodamine-DPPE was combined in a molar ratio of 1.84, 0.14, 1, and .086 respectively.[11]
- Liposomes were formed using the reverse evaporation method.[12]
- Prednisolone sodium phosphate and sulforhodamine B dye were dissolved in the aqueous phase.
- Liposomes were extruded sequentially through 400 nm and 100 nm Nucleopore® membranes.
- Liposomes were purified using size-exclusion chromatography followed by dialysis.
- The phospholipid concentration of the liposomes was determined using the Bartlett method.[13]
- The encapsulation efficiency of prednisolone was estimated through the determination of the dye concentration in the liposomes and was found to be 1.89%.

Expected Results

Prednisolone reduces inflammation in DMD, but causes undesirable side-effects when used chronically at therapeutic levels. Our investigations hope to supplement prednisolone with thiamine or a thiamine derivative to decrease the dosage of prednisolone and thus reduce its side effects. We also hope to deliver the drug efficiently via liposomes.

ROS and IL-6 are both inflammatory markers. These components are mediated through the NF-kB pathway. Prednisolone acts on the NF-kB pathway, as does benfotiamine. Our hypothesis is that if both of these components inhibit NF-kB, lowering the concentration of prednisolone and adding a higher concentration of benfotiamine should result in the same decreased inflammation with fewer side effects.

In our experiments, we used lipopolysaccharide (LPS) in cultured healthy and DMD skeletal muscle cells to induce an inflammatory response and began testing the anti-inflammatory action of prednisolone sodium phosphate, a hydrophilic salt of prednisolone, alone and in combination with thiamine hydrochloride.The lowest expression of IL-6 and ROS will help us determine what the optimal levels of prednisolone and thiamine are.

Liposomes can be formulated with different lipid compositions to yield optimal stability and delivery of drugs. Our liposomes were made with DPPC, cholesterol, PEG-DSPE, and lissamine rhodamine DPPE.[12] Cholesterol contributes fluidity and stability-a fluid liposomal membrane helps mediate fusion rather than endocytosis by the cell membrane.[14] The liposomes were PEGylated using a poly-ethylene glycol-lipid conjugate which lowers direct fusion into cell membranes and prolongs the physiological circulation time ('Stealth' liposomes) allowing for a longer duration of therapeutic action.[15] Our experiment will test the optimal level of prednisolone and thiamine in the liposomes. The lowest expression of IL-6 RNA indicates the optimal liposomal composition, fusion, and drug-thiamine level.

We would expect that the prednisolone liposomes supplemented with thiamine will lower the expression of RNA IL-6 to a greater degree than with the liposomes that carry prednisolone alone. By co-entrapping thiamine and prednisolone, we ultimately hope to provide simultaneous delivery and optimal pharmacokinetics. Our present work was planned in cell culture to optimize concentrations and study the mechanism of the combination formulations, but future work would be carried out in *mdx* mice (a common mouse model of DMD). There, we would be able to monitor pharmacokinetics and overall physiological impact on the inflammatory response.

Experimental Design:

Detection of ROS
- Pre-treat DMD and iHSKM skeletal muscle cells with prednisolone sodium phosphate, prednisolone sp + thiamine, or media only
- Induce ROS by adding LPS to the cells
- Measure ROS using an ROS detection assay kit

Detection of IL-6 in skeletal muscle cells
- Pre-treat DMD and iHSKM skeletal muscle cells with prednisolone sodium phosphate, prednisolone sp + thiamine, or media only
- Treat cells with different concentrations of LPS
- Run RT-PCR/Western Blot to determine levels of IL-6 mRNA/protein expression

Detection of IL-6 in skeletal muscle cells post-Liposomal Treatment
- Pre-treat half of DMD and iHSKM cells with determined amount of LPS to induce inflammation
- Treat all cells with free prednisolone sp, liposomal prednisolone sp, liposomal prednisolone sp+thiamine, or media only
- Run RT-PCR to determine levels of IL-6 mRNA in induced and non-induced cells

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