Research Proposal to the Blazeman Foundation for ALS

Muscle Fiber Type Switch as a Potential Therapeutic Target for ALS

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INTRODUCTION

Amyotrophic Lateral Sclerosis (ALS) is the most common neurodegenerative disorder affecting motor neurons, and is characterized by the loss of cortical, spinal, and bulbar motor neurons which leads to progressive motor dysfunction, disability and death. Although, motor neuron death is a prominent feature of the disease, preventing motor neuron death has only a modest impact on the timing of disease progression and life-span [1-3] and it does not prevent muscle denervation [4]. Indeed, muscle denervation is most likely the cause of muscle weakness, one of the first signs of pathogenesis in ALS. From physiological studies, large motor neurons associated with fast fatigable (FF) motor units and that innervate fast-type muscle fibers (type IIa, IIx and IIb) are the most vulnerable in ALS [5, 6]. However, these findings do not explain the incidence of ALS in the endurance athlete's population characterized for a higher percentage of slow-type fibers (glucolitic/oxidative and oxidative). Muscle fibers are classified as type I (oxidative/slow) or type II (glycolitic/fast). Type I fibers are rich in mitochondria and use principally oxidative metabolism for energy production, which provides a stable and long-lasting supply of ATP, thus are fatigue-resistant. Type II fibers comprise IIa, IIx, and IIb. Type IIb fibers have the lowest level of mitochondrial content and oxidative enzymes, rely on glycolitic metabolism as an energy source, and are susceptible to fatigue. Type IIa and IIx lie between type I and IIb [7, 8]. Some of the physiological adaptations to endurance exercise are: increased mitochondria size and content, increased vasculature, and higher proportion of slow type fibers. Importantly, in a study on the effects of cessation of training in subjects who had been training for 6-20 years, citrate synthase, β-hydroxyacyl-CoA dehydrogenase, and malate dehydrogenase declined significantly over 12 weeks period but were still 40% above control values in mixed muscle. Single muscle fiber analysis showed that elevation of mitochondrial enzymes levels above control values was much more marked in fast-twitch fibers than in slow-twitch fibers after 12 weeks of inactivity [9]. This may suggest the possibility that many years of endurance training may result in long-lasting adaptation, such as changes in the firing frequency of the nerve innervating the slow-type fiber or in the recruitment pattern of these fibers [10]. Nonetheless, the number of endurance sportsmen afflicted with ALS suggests that exercise benefits which positively act on vulnerable targets (mitochondria, vasculature, neuromuscular junction and fiber type) do not reduce the risk of ALS. Exercise induced-injury could be an explanation for the lack of exercise benefit; studies done in humans who performed 2h of cycle ergometer

exercise (60-65% VO^2_{max}) had an increased level of interleukin-6 (IL-6) immediately after exercise and peaked 3 hours later, as well as increased tumoral necrotic factor (TNF-α) [11]. TNF-α triggers motor neuron death in vitro [12] and transgenic mice over expressing TNF-α undergo extensive neurodegeneration [13]. In addition, transgenic mice over-expressing IL-6 develop severe neurologic disease characterized by tremor, ataxia and seizure. These findings suggest that IL-6 may have a direct pathogenic role in neurodegenerative diseases [14]. In a human study, serum IL-6 levels were detected in 73%of ALS patients (8 out of 11) whereas only 9% (1 out of 11) were detected in control subjects, and Il-6 levels are correlated directly with the duration of the disease in ALS patients [15].

An ideal therapeutic approach would be to induce the positive effects of exercise, (increase mitochondria number and size, switch fiber type towards a more resistant phenotype, prevent atrophy, and increase vasculature) but without any of the possible negative effects (exercise induced-injury). In sedentary mice, AICAR (AMPK agonist) administration has been shown to induce a muscle fiber type switch from fast-to-slow, which results in an increase in endurance of about 44% [16]. There was also an up-regulation of 32 genes linked to oxidative metabolism. This article had a great impact in the media as a potential exercise mimetic drug; however, exercise and muscle researchers criticized the fact that by activating AMPK, AICAR does not induce all the same benefits of physical exercise. Nonetheless the same researchers also maintained that agents like AICAR could be especially important for patients who are unable to exercise because of severe musculoskeletal or cardiovascular conditions [17]. According to the website, ALS-TDI is conducting a study with a different AMPK activator, metformin; however, the results of this are not available. AICAR has been shown to have long-term effects in muscle and is a more potent activator of AMPK than metformin [18, 19].

AICAR: Mechanism of action:

AICAR enters into the cell and is metabolized to the monophosphorylated nucleotide, ZMP, an AMP analog that activates AMPK. AMPK is a fuel-sensing serine/threonin kinase that is activated under conditions of energetic demands, such as exercise, to restore energy balance [20]. Chronic administration of AICAR activates AMPK increasing the expression of genes implicated in oxidative metabolism and mitochondrial biogenesis. These effects are in part, mediated by stimulation of peroxisome proliferator-activated receptor delta (PPARδ), a nuclear receptor super-family of transcriptional regulators [16, 21]. In addition, AICAR administration induces a 20% increase in PGC-1α levels 24 hours after administration in both fast and slow type muscle; and a 24% increase in the expression of SIRT1 in fast type muscle but not slow type [22]. The expression of PGC-1α in muscle increases chronically with exercise [23, 24], an overexpression of PGC-1α results in a substantial increase in muscle

mitochondrial content, resistance to fatigue [25, 26], and more importantly, reduces the effects of denervation, dietary fasting, and even activated FoxO3 on rapid fiber atrophy [27]. However, AICAR treatment for a period of 4 weeks at 500mg/kg/day failed to block loss of muscle mass upon experimental denervation, although the levels of PGC-1α were increased [28]. Atrophy following experimental denervation may occur at a faster rate than denervation in neurodegenerative diseases. In fact, we found that denervation in ALS mice occurs as early as 25 days post-natal while the mice do not show the first clinical signs of the disease until 90-100 days post-natal [4]. An explanation from the authors of the original paper [28] is that protection against muscle wasting may require a larger increase in PGC-1α. It seems likely that the level reached was not enough to compensate for the rapid decline after experimental atrophy; and these trends may not correlate with the progression of ALS or, alternatively, the levels of PGC-1α may increase after longer treatment.

The effect of AICAR treatment is not specific for skeletal muscle; activation of AMPK by AICAR may affect other tissues, including the brain, as it has been shown that AICAR crosses the blood-brain barrier [29]. Astrocytosis and microgliosis are found in the central nervous system of ALS-patients as well as in animal models of ALS; administration of AICAR to cultured rat primary astrocytes, microglia, and peritoneal macrophages inhibited the LPS-induced production of TNFα, IL-1b, and IL-6, as well as inhibited the expression of iNOS at mRNA and protein levels [30].

In addition to all the possible therapeutic effects that AICAR may induce, AICAR has also been used previously as a drug for treating Lesch-Nyhan syndrome at relatively high doses (100 mg/kg body weight) with no side effects [31]. The safety, tolerance, and pharmacokinetics of intravenous doses of 10-100 mg/kg of AICAR in healthy men have been previously reported [30, 32].

HYPOTHESIS

We hypothesize that activation of AMPK through AICAR administration in ALS mice will increase PGC-1α levels. PGC-1α will induce:

a) Switch in muscle fiber phenotype from vulnerable (fast-type fiber) to a more resistant phenotype (slow-type fiber).
b) Increase the mitochondria content in muscle fiber and motor neurons.

As a result we expect to see an increase in muscle performance and delay in the onset and/or progression of the disease based on:

1. Delayed denervation and/or increased re-innervation by healthy motor units in the fibers innervated by large motor neurons (FF motor units) in the SOD1^{G93A} mice.
2. Increased mitochondrial turnover that will improve the energetic state of the cells –muscular, neuronal, and non-neuronal.

EXPERIMENTAL DESIGN

A large number of successful pharmaceutical interventions in ALS mouse models have been reported, none of which could be prospectively translated into human therapeutics. It is now clear that this failure may, at least in part, be related more to methodological pitfalls than to problems inherent in the models themselves [33]. Thus, we will use the guidelines regarding animal number and confirmation of $SOD1^{G93A}$ approved by ALS/MND in a meeting in 2006 and published in 2007 [33]; in combination with the guidelines for preclinical testing and colony management from "The Jackson Laboratory" [34].

Animals: For all studies, B6.Cg-Tg($SOD1^{G93A}$)1Gur/J mice will be used in the experiments as a model of ALS; as a control in Aim1 we will use littermates negative for $SOD1^{G93A}$ determined by PCR.

Aim1: Determination of the dose response:

There are several reports in the literature indicating different effective doses of AICAR [16, 28, 35]. Additionally, personal communication with other investigators also indicates that the source of AICAR is critical, as one company's purity is less than another. The first critical experiment in this study will be to determine an effective dose of AICAR in the $SOD1^{G93A}$ mouse model of ALS. AICAR induces changes in the fiber type through activation of PGC1-α [16], which is also implicated in the protection of skeletal muscle from atrophy [27], and mitochondria biogenesis [36]. Mice will be treated with different doses of AICAR (Sigma; Cat#9978; 100, 250, 500 and 1000 mg AICAR) at 30 days postnatal (P30) for 4 weeks. AICAR will be freshly prepared everyday before injection, and prior to the injection will remain on ice as suggested by Dr. Vihang A. Narkar. Following treatment, animals will be euthanized and the Tibialis Anterior (TA) and soleus muscle, and spinal cord will be isolated. PGC-1α levels will be determined by Western Blot analysis. Mitochondrial activity (Malate dehydrogenase and succinate dehydrogenase), and mitochondrial marker (Cytochrome C) will be determined as previously described by Winder.,et al [36]

This experiment will determine the dosage of AICAR that induces the maximum level of PGC1-α expression and mitochondrial biogenesis. As stated in our hypothesis we expect that both, higher levels of PGC1-α and mitochondrial content will have a therapeutic effect in the $SOD1^{G93A}$ mouse model of ALS. Thus, it is extremely important to determine this dosage in order to develop the following aims. If we are successful in Aim 1, we will move on to Aim 2 as described below.

Aim2: Effect of AICAR on preventing muscle denervation:

We have determined that denervation of the TA muscle begins between days 14 and 30 [4, 37]. By day 60, a high percentage of the FF/type IIb fibers in the TA are denervated

[6, 38]. For this study we will determine if AICAR can prevent, or delay this early denervation. Twenty mice will be used and the extent of muscle denervation, muscle fiber type composition, and number of motor neurons in the lumbar spinal cord will be determined. Animals will be divided in groups of 5 animals as shown in the table below and will be administered AICAR daily beginning at 30 days post-natal (P30) and will continue until day 60.

Genotype	**Daily Intra-peritoneal injection AICAR/saline**	**Daily Intra-peritoneal injection saline**
SOD1^{G93A}	5 male/5 female mice	5 male/ 5 female mice

The guidelines for preclinical animal research in ALS/MND indicate that an n=5-6 animals is mandatory for positive outcomes [33].

Histology: Muscle and spinal cord will be removed for further correlation between histological and biochemical changes with survival, and SOD1 expression levels. The principal information we want to obtain from these tissues are:

a) Muscle:
    a. Cross sections from fresh-frozen muscle will used to determine the level of atrophy (due to decrease in cross section area), as well as the percentage of different muscle fiber type. Both ATPase and SDH staining will be used to determine the percentage of different fiber types.
    b. Extent of denervation, immunohistochemistry methods will be used to stain the presynaptic axons (NF-L) and presynaptic terminal (VChaT), as well as the postsynaptic side of the neuromuscular junction using a-BTX. Innervation and denervation will be determined using previously published criteria [4]. Results from AICAR-treated mice will be compared to saline-treated controls.
b) Spinal cord:
    a. A cresyl-violet stain and a method for motor neuron counting developed in our lab will be used to determine number of motor neurons in the thoracic and lumbar spinal cord.
    b. Immunohistochemistry techniques will be used to determine the level of reaction from microglia (Iba-1 antibody) and astrocytes (GFAP antibody).

Molecular level: To correlate these findings with the direct effects of AICAR, western-blotting will be used to determine the levels of protein that are directly or indirectly activated by AICAR as mentioned above (AMPK phosphorylated, total AMPK, PGC-1α, PPARδ).

Many changes that are attributed to AICAR that could be beneficial in ALS such as: 1) switching muscle fiber type from a vulnerable (fast-fiber type) to a more resistant-to-waste phenotype (slow-fiber type), and 2) preventing atrophy which would favor the re-innervations process from collateral axons. In addition, an enhanced energetic state in motor neurons and muscle due to the increase of mitochondrial content, and an attenuated inflammatory response induced by AICAR administration may delay the denervation process in the fast-type muscle. Preserving the interaction between the motor neuron and the muscle fiber is highly important. The finding that muscle-derived GDNF, and NOT glial-cell-line-derivated GDNF, acts as a neuroprotective in the $SOD1^{G93A}$ mouse model stresses this importance [39]. Improving the metabolic state in motor neuron and muscle fiber could be a mechanism to stabilize this interaction, maintaining the anterograde/retrograde transport of neurotrofic factor between them.

A practical problem with this project is the cost of AICAR ($157.00/25mg), and fortunately, we have been able to obtain a discount of 50% from Sigma (see quotation). At the conclusion of this study we will have 1) determined an optimal dose for treatment, and 2) demonstated efficacy in the $SOD1^{G93A}$ mouse model of ALS. Positive results from this study, together with the reported safety, tolerance, and pharmacokinetics of intravenous doses of 10-100 mg/kg of AICAR in healthy men [30, 32] lay the critical foundation for moving AICAR into Phase 2 and 3 clinical trials in ALS. WFUSM ALS Clinic, together with NEALS is in a prime position to lead these studies.

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Below is the budget for the initial dose-response (Aim 1) and primary efficacy (Aim 2) studies outlined in the proposal. Funds from the Blazeman Foundation for ALS will be used to obtain AICAR, pay for animal care and housing and contribute to the costs of critical reagents to determine the effective dose of AICAR. The Blazeman Foundation for ALS does not fund salary, fringe benefits or indirect costs. The Blazeman Foundation for ALS will be acknowledged for its support of this project in any formal or informal presentations of these data, on WFUBMC's ALS Center website, in a press release and in any resulting publications. Additionally, the investigators acknowledge that funding for Aim 2 will be contingent on positive results following successful completion of Aim 1.

Budget Request for Aim 1: Dose-response curve

Duration: **4 months**

Antibodies, Reagents, Histological supplies	$3,600
Mice: 4 cages for 28 days ($0.85/day)	$95
AICAR-A9978-1G $3140.00 (Sigma)* Average mouse weight at P40: 15g Total days: 28 days Total mice: 12 control mice Dosages: a) 100mg/kg/day → 126 mg total for the 3 mice and 28 days b) 250mg/kg/day → 315 mg total for the 3 mice and 28 days c) 500mg/kg/day → 630 mg total for the 3 mice and 28 days d) 1000mg/kg/day →1260 mg total for the 3 mice and 28 days Total amount needed for all mice and the 28 days = 2331mg ~ 2.5g	$7,536
TOTAL	$11,231

*Price from Quotation # 21295569

Budget Request for Aim 2: Effect of AICAR on preventing muscle denervation

Duration: **8 months**

Mice: 12 cages for 30 days ($0.85/day)	$306
AICAR-A9978-1G $3140.00 (Sigma)* Average mouse weight: 18g Total days: 30 days Total mice: 20 mice (5 male $SOD1^{G93A}$; 5 female $SOD1^{G93A}$ + 10 untreated controls) Estimated dose[#]: 500mg/kg/day We need 270 mg/mice for the whole study→2.7g total for the 10 mice	$10,362
TOTAL	$10,668

*Price from Quotation # 21295569; # Estimated dose from literature, real doses will depend on dose-curve study. Price based on ordering sufficient drug for 12 animals in case we loose an animal during the study due to fighting or other unrelated reason.

CONSIDERATIONS

It has not escaped our attention that the cost of this project is high, which is mainly due to the price of AICAR from Sigma. As seen in the quotation attached to the proposal we have negotiated a generous 50% reduction in price from Sigma. We are also aware that Toronto Research Chemical (TRC) sells AICAR at a more affordable price ($120.00/1g) compare to Sigma ($3140.00/1g). However, in our preliminary data obtained with AICAR from TRC, in a 250mg/kg/day dose, we failed to induce the changes reported in the original article by Narkar et al [16]. Although in our pilot study we only used a single-dose (250mg/kg/day) and a very small number of mice, data obtained from Dr. Goldberg's lab using higher doses of AICAR (500mg/kg/day) from TRC, also failed [28]. As mentioned above, an explanation given in the article was that the levels of PGC-1α induced by 500mg/kg/day were too low. These findings raised our concern about the purity of AICAR from TRC. Thus, we propose to use AICAR from Sigma instead of Toronto Research Chemical (TRC).

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Signature Date

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