THE EUROPEAN SOCIETY OF MEDICINE
Medical Research Archives, Volume 12 Issue 7

RESEARCH ARTICLE

Amyotrophic Lateral Sclerosis is a spectrum of diseases that needs a broad treatment approach

Siobhan P Ellison DVM PhD[1]

[1] Neurodegenerative Disease Research Inc Reddick Florida USA

OPEN ACCESS

PUBLISHED
31 July 2024

CITATION
Ellison, PE., 2024. Amyotrophic Lateral Sclerosis is a spectrum of diseases that needs a broad treatment approach. Medical Research Archives, [online] 12(7). https://doi.org/10.18103/mra.v12i7.5448

DOI
https://doi.org/10.18103/mra.v12i7.5448

ISSN
2375-1924

ABSTRACT

Amyotrophic lateral sclerosis consists of multiple diseases that lead to a common endpoint that initiates motor neuron loss. The difficulty in developing treatments is that both the presentation and the course of the disease differs for patients and there aren't methods to stratify patients in clinical trials that are expected to benefit from a drug, thus most treatments that were positive in animal studies fail in Phase 2 human studies. It is possible harnessing innate immune responses involved with the cell danger responses may be a starting point to stack therapies in patients. A major step in regulating the cell danger response is creating a retro-inverso thymopentin that can increase innate immune targets to regulate T cell responses.

Recent work on thymopentin bring a major contribution to the ALS field and we present FC-12738 designed to regulate the cell danger response by natural innate immune pathways.

Keywords: amyotrophic lateral sclerosis, thymopentin, thymopoietin, FC-12738, cell danger response

Introduction

Amyotrophic lateral sclerosis is a rare disease with a critical need for treatments.[1] Although some positive results have been indicated in numerous early studies the lack of effectiveness in larger studies prevents moving treatments to patients. In this editorial we propose a treatment to modulate natural immune responses, specifically inflammation, that will allow the exploration of potential synergistic drugs that could change the course of disease in ALS patients. We provide references to our work and other works that support a step to developing a multi-treatment approach to this devastating disease.

Discussion

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease accompanied by significant neuroinflammatory changes leading to neuronal death, although the cause is unknown it is generally accepted that genetics and environmental factors play a part in the development of both familial and sporadic ALS.[2] There are multiple pathways in ALS that lead to initiating motor neuron loss and possibly explain the disappointing results obtained with treatments targeting a single pathological process.[2 3] Genin *et. al.* hypothesize that mitochondrial damage in ALS was a consequence and an exacerbating factor of the disease. [4] The ALS Association reports on their website that patients differ in their clinical presentation as well as the course of their disease however, there is a final common pathway resulting in progressive muscle weakness and paralysis experienced in all patients. We believe that immune modulation, particularly modulating neuroinflammation is an attractive goal to target the course of ALS.

Mitochondrial dynamics and metabolic pathways may control the fate of T cells in the periphery.[5] A crucial role of regulatory T cells (Tregs) in ALS is recognized.[5,6] Tregs are a T cell subpopulation with immunomodulatory properties that are controlled by the hormone thymopoietin. In ALS patients Tregs are progressively reduced in number, suppressive function decreases and Tregs levels correlate with the rate of disease progression and patient survival.[6] A Phase 1 ALS study that expanded and infused autologous Tregs combined with subcutaneous interleukin (IL)-2 in ALS patients was well tolerated, Treg suppressive function increased and disease progression stabilized.[7]

Regulating T cells is a complicated process with important considerations when employing Tregs as therapeutic tools.[8] Thymopentin (T5), a pentapeptide exhibiting identical physiological function as thymopoietin II, a natural hormone, promotes T cell phenotype differentiation and modulates the immune system.[8, 9] Thymopentin has had a large footprint in the literature for many years and has been applied clinically to boost immunity and treat immunocompromised patients. Recently, T5 was investigated and found to remarkably improve the micro-inflammation status in patients with end stage renal disease undergoing maintenance hemodialysis and to improve their immune function and quality of life.[10] A known limitation of T5 is the short half-life of the molecule.

Increasing target engagement of T5 receptors is a long desired goal and we agree with Povoleri *et. al.* that natural pathways that restore balance to the inflammatory responses resulting in regulating Tregs may be useful in ALS treatment.[8] The advantage of modulating the immune system with an molecule that mimics an innate hormone is all the natural related regulatory pathways will be operational. It is hoped that engaging thymic hormone receptors may provide a base that will facilitate utility of other therapies that have shown promise in ALS but were not found to have clinically significant outcomes.[11]

It is generally known that there are initial and adaptive responses that predictably play a role in the pathogenesis of ALS. The presence of T cells at the site of pathology can occur as part of a harmful processes or as a response to damage caused by disease.[12] Danger signals are molecules released from tissue injury and part of the cell danger response (CDR).[13] Energy and metabolic resources are provided by mitochondrial and metabolic transformations that drive the three phases of the CDR and create the phases of the healing cycle.[13] An important point Dr. Naviaux makes is that each phase requires a different mitochondrial phenotype and without different mitochondria there can be no healing. Mitochondria provoke systemic responses to injury and in the brain, microglia respond to the damage. The rise and fall of extracellular ATP (eATP) signaling is a key driver of the mitochondrial and metabolic reprogramming required to progress through the healing cycle.

Our unpublished early work investigated biospecimens obtained from a cohort of ALS patients and suggested sustained activation of purinergic signaling pathways, perhaps with desensitization effects.[14] In Dr. Kokai's analysis ALS patient derived adipose stem cells were grown *in vitro*, the cells were exposed to extracellular ATP and annexin V (apoptosis) and membrane permeability (necrosis) were measured in real time. Robert Naviaux explained that purinergic signaling is the combined effects of extracellular purines (adenine, guanine, hypoxanthine, xanthine, theophylline, theobromine, caffeine, uric acid and isoguanine), purine receptor expression and internalization, and ectoenzyme conversion of pro-inflammatory purines to anti-inflammatory metabolites.[13] High purine concentrations are generally indicative of stress and concentrations of purines signal through ionic P2X receptors to increase intracellular Ca2+, rapidly inducing downstream effects. Upon activation, P2X7, a purine receptor modulated by lipids that is highly expressed in the spinal cord, triggers cellular apoptosis and necrosis. Another example of detrimental effects due to mMol purine concentrations is inhibition of nNos expression upon activation of the colocalized purinergic receptors P2X1 and P2X2.[13] Kokai hypothesizes that systemic desensitization in ALS patients may make it harder for peripheral cells to respond briskly to environmental stress, slowing cell death and replacement.[12]

It is well known that a number of ALS-related genetic mutations are associated with defects in cell adaptation to stress (SOD, TDP43, C9orf72). If ALS patients incur sustained activation of purinergic signaling, as clinical

measurements indicate, do peripheral and/or disease-specific cells become habituated and desensitized to purinergic signaling? Dr. Kokai posed that cells that are resistant to death by eATP and other CDR triggers accumulate damage and become dysfunctional before actually dying. Early cell death of infected or damaged cells protects neighboring cells from infection and stress. Determining ALS-related resistance to beneficial apoptosis opens a new avenue for therapeutic intervention and is a current topic of investigation.

Thymalfasin and levamisole HCl, that are already in use in other indications to affect purinergic signaling, were investigated.[15,16,17] Thymalfasin (Zadaxin®) is a synthetic analog of thymosin α1, which is a 28 amino acid hormone regulates several aspects of dysregulated innate immunity. Zadaxin® is used in humans as a treatment for hepatitis B, hepatitis C, and some types of cancer. Levamisole HCl is also an immune modulating drug that currently is not in use in the United States in humans (Ergamisol®) but is widely used in veterinary applications (NeuroQuel® for horses; LevaMed® pig dewormer). Levamisole HCl is used therapeutically outside the U.S. in humans and there are multiple reports that it has been used as an adulterating agent in illicit street drugs in the United States.[18] Levamisole was used in an ALS clinical trial in which the drug was administered once a week for 6 months, but no benefit was observed in that study.[15]

A 14 week dosing study used Zadaxin® and NeuroQuel® in the SOD1^{G93A} and Prp-TDP43^{A315T} mouse models of ALS to determine the effects on disease.[16] NeuroQuel® was given orally once daily for two weeks and then every other day for 6 weeks for a total of 8 weeks of treatment. Zadaxin® was given subcutaneously twice a week for a total of 8 weeks. Outcome measurements included efficacy assessment on the neuromuscular phenotypes, and pathological analyses of ubiquitin load and neuro-inflammatory markers in spinal motor neurons. Neither of these drug treatments produced significant extensions in survival of the mice; however, there were changes in brain ubiquitin load that suggest the drugs could be beneficial as additions to other therapies. There were several observations that indicated increasing the target engagement of Zadaxin® would be useful.

Thymalfasin was originally isolated from thymosin fraction 5, a bovine thymus extract containing a number of immunologically active peptides.[17,19,20] Although the mechanism of action of thymalfasin is not completely understood modulation is thought to be related to its immunomodulating activities, centered primarily around enhancing T-cell function. Thymopentin is a soluble pentapeptide hormone with pleiotropic effects and the minimal sequence that reproduces the biological activities of thymopoietin.[21] Thymopentin is a pentapeptide agonist for the toll-like receptor 2 (TLR2) that constitutes an essential active element of thymopoietin.[22]

Thymopentin is an all-natural, all-L pentapeptide, that is subject to proteolytic cleavage and a short *in vivo* half-life of 30 seconds.[21] As predicted, reversing of the orientation of the peptide bonds taken together with inversion of stereochemistry from the natural L- to the unnatural D- amino acids resulted in a high degree of topochemical equivalence between the parent peptide and its isomeric replacement, called the retro-inverso approach, while increasing metabolic stability against proteolysis.[22] The full retro-inverso analog of T5, RI-T5, is the all-D amino acid sequence (D-Tyr)-(D-Val)-(D-Asp)-(D-Lys)-(D-Arg).

Initially we determined that FC-12738 (RI-T5) had a greatly increased plasma stability with a half-life of 120 minutes.[22] The influence of proinflammatory cytokines on the immunoregulatory function of immune cells from healthy donors was assessed using mitogen-stimulated PBMC responses and then used to determine the effect of T5 and FC-12738 on cytokine levels after stimulation by Concanavalin A (ConA) (unpublished). In unpublished data, FC-12738 was similar to T5 lowering the expression of TNFα and IL6, both inflammatory cytokines *in vitro*.

The pharmacological properties of FC-12738 were evaluated in *in vivo* studies.[22] Based on the absorption, distribution, metabolism, and excretion studies, FC-12738 demonstrated rapid absorption and high bioavailability following subcutaneous administration in Sprague-Dawley rats, beagle dogs and humans. The compound showed low permeability across the Caco-2 cell monolayer and was a poor or non-substrate for efflux transporters. Although it was detected in the brain, the levels were much lower than visceral organs in animal studies. The low toxicity of FC-12738 and its immune modulating properties are supportive of its further investigation in Phase I clinical trials for conditions in which TP5 has been implicated as a possible therapeutic, including ALS (FC-US-001). Evaluation of unpublished pre-clinical data (Hesperos Inc, Orlando, FL) determined the dose of FC-12738 in people would be 10 µG given sub-cutaneously.

Hesperos Inc is the "human-on-a-chip company" that evaluated FC-12738 to ameliorate ALS phenotypes on the neuromuscular junction systems (NMJ). The system measures the ability to rescue deficits in NMJ formation, fidelity and fatigue index in diseased SOD1 (E100G) and TDP43(G298S) motor neurons compared to healthy NMJs. The data indicated that, for both TDP43 and SOD1 systems, functionality of the NMJ is maintained or restored with the degeneration of the NMJ halted. In the context of ALS, this system is unable to extrapolate to predict the effects on progressive degeneration and treatment over many years. However, efficacy was demonstrated in this system recapitulating the basic function of the NMJ in a progressive degenerative model, providing evidence that FC-12738 can prolong and extend the life of ALS NMJs transiently. Modulating the innate immune system at multiple points using a thymopoietin-reactive molecule was useful in animal models where mice develop ALS, although survival was not increased.[16]

The drug FC-12738 engages some TP5 receptors with increased bioavailability thereby decreasing pro-inflammatory immune responses while concurrently up-regulating anti-inflammatory responses and may prove clinically beneficial in ALS patients. Pre-clinical data

demonstrated many favorable pharmacokinetic properties, although it is possible that further dose optimization may be required to improve CNS penetrance.[19]

FC-US-001, a Phase 1, randomized, double-blind, placebo-controlled, first-in-human, single-ascending-dose study to evaluate the safety, tolerability, pharmacokinetics, and pharmacodynamics of FC-12738 in healthy adult participants and patients with ALS is being conducted. It is anticipated that pro-inflammatory cytokines will be influenced with treatment, NMJ loss may slow and a positive response in lipid metabolism may be achieved (data not shown). McCluskey *et. al.* reported lipid dysregulation is well recognized in motor neuron diseases including ALS.[23] Several lipid markers have been shown to occur in plasma prior to neurodegeneration and predict prognosis in ALS including sphingolipids, sphingomyelin and its metabolite ceramide.[23] Normalizing lipid dysregulation may be achieved with FC-12738 but this has not yet been shown in humans. The safety profile of FC-12738 from animal, human-on-a-chip and Normal subjects supports moving into clinical trials in ALS patients. Data from Phase 2 studies will add more to our understanding of the pathways active in ALS to guide the field toward effective treatments.

Based on current understanding of ALS and recent failures to find successful treatments, we hypothesize a single treatment approach will likely fail. An anticipated advantage of FC-12738 is targeting dysregulated mitochondrial metabolism, decreasing pro-inflammatory cytokines while increasing anti-inflammatory cytokines thus harnessing cell danger response pathways as we observed in *in vitro* and *in vivo* models. Our goal in developing FC-12738 is to correct these dysregulated innate immune pathways important in ALS. If FC-12738 achieves this goal, the stage may be set to stack available and future therapies, guided by personalized medicine, for the changing landscape in each ALS patient.

Conflicts: Dr. Siobhan Ellison is CEO and chief science officer at Neurodegenerative Research Inc. (NDR Inc) a not-for-profit organization that funds primary research. Dr. Ellison owns Pathogenes Inc. an equine research company that owns a patent on FC-12738.

Funding: All work discussed in this study was funded by NDR Inc. with generous donations from Provenance Initiatives Inc.

Acknowledgements: NDR Inc. thanks the many scientists who made this work possible, particularly the extensive time and work of Dr. Robert Naviaux (University of California San Diago School of Medicine), Dr. David Borchelt (University of Florida Dept. Neuroscience College of Medicine), and Dr. Lauren Kokai (University of Pittsburgh McGowan Institute for Regenerative Medicine), as well as other researchers on the NDR Inc. team. We thank Richard H Helms, February 1949-May 2024, a person with ALS who made it his goal to change the landscape of ALS for future patients.

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