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Bedside to bench to bedside research: Estrogen receptor beta ligand as a candidate neuroprotective treatment for multiple sclerosis

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ABSTRACT

Protective effects of pregnancy during MS have led to clinical trials of estriol, the pregnancy estrogen, in MS. Since estriol binds to estrogen receptor (ER) beta, ER beta ligand could represent a "next generation estriol" treatment. Here, ER beta ligand treatment was protective in EAE in both sexes and across genetic backgrounds. Neuroprotection was shown in spinal cord, sparing myelin and axons, and in brain, sparing neurons and synapses. Longitudinal *in vivo* MRIs showed decreased brain atrophy in cerebral cortex gray matter and cerebellum during EAE. Investigation of ER beta ligand as a neuroprotective treatment for MS is warranted.

1. Introduction

Multiple sclerosis (MS) is a putative autoimmune disease targeting the central nervous system (CNS) leading to neurodegeneration. Currently approved treatments for MS were designed to modulate peripheral immune responses to decrease CNS inflammation. These treatments reduce relapse rates by about half compared to placebo treatment in clinical trials, with only modest effects on slowing permanent disability accumulation. Many MS treatments are relatively safe and well tolerated. More aggressive anti-inflammatory treatments reduce relapse rates further, but are associated with toxicities related in part to immunosuppression. Rather than escalating to more aggressive anti-inflammatory treatments for better long term disability outcomes, an alternative would be to combine relatively safe anti-inflammatory treatments with a neuroprotective treatment in patients with relapsing remitting MS (Voskuhl, 2016). Such neuroprotective treatments may also benefit progressive MS patients.

"Bedside to Bench to Bedside" research is a way to capitalize on a known clinical observation, mechanistically dissect it at the laboratory bench, then translate basic findings back to the clinic in the form of a novel clinical trial (Voskuhl and Gold, 2012). This approach has a clinical observation as its foundation. In contrast, "Bench to Bedside" research is based on a molecule or pathway thought to be involved in a disease mechanism, with trials designed to block this molecule. The latter approach carries risk that the molecule or pathway of interest may not ultimately be physiologically significant in humans with disease, since most biological processes involve redundant mechanisms and compensatory pathways. Blocking one molecule or pathway may not have a significant effect in complex diseases in humans. This is one reason why pharmaceutical company success rates are low compared to the number of lead candidates initially passing *in vitro* screening and *in vivo* preclinical models. The "Bedside to Bench to Bedside" approach mitigates this risk since it starts with a clinical observation known to be physiologically relevant. Mechanisms underlying clinical observations may involve several molecules and complementary pathways. A multifaceted approach may indeed be what is required to impact complex diseases. The "Bedside to Bench to Bedside" approach in drug development is a conceptual shift, since the "Bench to Bedside" approach has focused on the molecule first, with questions of physiologic relevance coming later.

A major clinical observation in MS is that pregnancy is protective (Confavreux et al., 1998). Relapses are decreased by over 70% in the last trimester. Hormones and other factors change during pregnancy, each warranting consideration for mediating this protection. Estriol is an estrogen of pregnancy, distinct from estradiol of ovulatory cycles. It is made by the fetal placental unit and rises progressively during pregnancy to reach high levels in the last trimester (Lindberg et al., 1974). Estriol was given to mice with experimental autoimmune

Abbreviations: MS, multiple sclerosis; EAE, experimental autoimmune encephalomyelitis; ER, estrogen receptor; MBP, myelin basic protein; NF200, neurofilament 200; PSD-95, post synaptic density-95; WM, white matter; GM, gray matter; CNS, central nervous system.

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encephalomyelitis (EAE) at doses to induce a level in the blood physiologic with mouse pregnancy, and disease protection was observed (Kim et al., 1999). This disease protection was found in both female and male mice, in both relapsing and progressive EAE models, and when administered either before or after EAE onset, reviewed in (Spence and Voskuhl, 2012). Estriol is a relatively weak estrogen, acting on estrogen receptor (ER) alpha and ER beta, with higher affinity for ER beta (Katzenellenbogen, 1984; Kuiper et al., 1997). ER alpha ligand treatment during EAE was protective early in EAE (Morales et al., 2006), was shown to be anti-inflammatory during peripheral immune responses (Lelu et al., 2011; Morales et al., 2006), and neuroprotective by binding to astrocytes to reduce CCL2 and immune infiltration in the CNS (Kim et al., 2014; Spence et al., 2011). ER beta ligand treatment was protective later during EAE (Tiwari-Woodruff et al., 2007), did not alter peripheral immune responses (Tiwari-Woodruff et al., 2007), and did not target astrocytes (Spence et al., 2013). Instead ER beta ligand and estriol each had protective effects on microglia and dendritic cells (Drew and Chavis, 2000; Du et al., 2011; Papenfuss et al., 2011; Saijo et al., 2011). Also, ER beta ligand was shown to act on oligodendrocytes to increase remyelination (Crawford et al., 2010; Khalaj et al., 2013). In EAE (Ziehn et al., 2012; Ziehn et al., 2010) and non-EAE (Kramar et al., 2009; Liu et al., 2008) ovariectomized mice, estriol and estrogen receptor beta ligand treatment improves cognitive behavioral testing and hippocampal synaptic plasticity, respectively. Coming full circle, these latter preclinical data are consistent with another clinical observation in humans, namely cognitive dysfunction occurs in healthy women after surgical ovariectomy (Sherwin, 1988).

Two trials have been completed, and one is ongoing (www.clinicaltrials.gov), with oral estriol treatment of 8 mg per day in women with MS to induce an estriol level in blood that recapitulates pregnancy. The first pilot clinical trial used the biomarker of enhancing lesions on monthly MRIs as the primary outcome measure. There was an over 70% reduction in enhancing lesions with estriol treatment compared to pretreatment in a single arm crossover design (Sicotte et al., 2002). The second trial was a Phase 2b, placebo-controlled, multicenter trial with relapse rate reduction as the primary outcome measure (Voskuhl et al., 2016). Relapses were reduced by a third to a half more in the estriol plus glatiramer acetate group compared to the glatiramer acetate plus placebo. Exploratory analyses showed that higher estriol blood levels correlated with reduced relapses and with reduced enhancing lesion positive scans. Higher estriol blood levels also correlated with improved cognitive testing performance. Trends for protective effects on cerebral cortical gray matter atrophy by MRI were also observed, particularly in patients who were enhancing lesion negative, suggesting neuroprotective effects (Voskuhl et al., 2016).

Oral estriol is taken at doses of 1–2 mg per day in Europe and Asia to alleviate menopausal symptoms. Estriol has been considered the safest of the estrogens for decades, likely due to its preferential binding to ER beta over ER alpha, since toxicities related to breast and uterus are mediated by ER alpha, not ER beta (Head, 1998; Lauritzen, 1987; Takahashi et al., 2000). While safety was shown in the two completed MS clinical trials (Sicotte et al., 2002; Voskuhl et al., 2016), the search for a next generation estriol has begun that entails the use of selective estrogen receptor modifiers (SERMs). An ideal candidate would be an ER beta ligand. Here, we will investigate neuroprotective effects of ER beta ligand treatment in EAE, using a ligand that previously showed promise in other neurodegenerative disease models of Alzheimer's disease (George et al., 2013) and Parkinson's disease (McFarland et al., 2013). Beneficial effects in EAE will be shown that go beyond preservation of spinal cord white matter myelin and axons. We will show reduction of atrophy of cerebral cortex and cerebellum by *in vivo* longitudinal MRIs and preservation of neurons and synapses in gray matter by neuropathology.

2. Materials and methods

2.1. Animals

C57BL/6 and NOD mice, 8 weeks of age, were purchased from Jackson Laboratories (Bar Harbor, ME). Animals were maintained under environmentally controlled conditions in a 12-hour light/dark cycle with access to food and water *ad libitum*. All procedures were done in accordance with the guidelines of the National Institutes of Health and the Chancellor's Animal Research Committee of the University of California, Los Angeles Office for the Protection of Research Subjects.

2.2. Reagents/treatments

ER beta ligand (AC186) was provided by Acadia Pharmaceuticals (McFarland et al., 2013). It was dissolved in Miglyol 812 N liquid oil (Sasol North America) or sesame oil (Sigma Aldrich) at 15 mg/ml for delivery of a 30 mg/kg treatment dose which was administered subcutaneously every other day, achieving a final dose of 15 mg/kg/day. The generic ER beta ligand, diarylpropionitrile (DPN, Tocris Biosciences), was dissolved in 10% molecular-grade ethanol and diluted with 90% Miglyol 812 N liquid oil (Sasol North America) to achieve a final dose of 8 mg/kg per day, as described (Tiwari-Woodruff et al., 2007).

2.3. EAE

C57BL/6 and NOD mice were injected subcutaneously with Myelin Oligodendrocyte Glycoprotein (MOG), amino acids 35–55 (200 μg/animal, American Peptides), emulsified in complete Freund's adjuvant (CFA) and supplemented with *Mycobacterium tuberculosis H37ra* (300 μg/animal, Difco Laboratories), over two sites drained by left inguinal and auxiliary lymph nodes in a total volume of 0.1 ml/mouse. One week later, a booster immunization was delivered over contra lateral lymph nodes. Pertussis toxin (500 ng/mouse) (List Biological Laboratories, Inc.) was injected intraperitoneally on days 0 and 2. Animals were monitored daily for EAE signs based on a standard EAE 0–5 scale scoring system: 0—healthy, 1—complete loss of tail tonicity, 2—loss of righting reflex, 3—partial paralysis, 4—complete paralysis of one or both hind limbs, and 5—moribund. Treatments with ER beta ligand or vehicle were initiated at the first clear signs of clinical disease (EAE grade 2 at day 13–15) and continued to the endpoint of the experiment, as described (Wisdom et al., 2013).

2.4. Rotarod testing

Motor behavior was tested up to two times per week for each mouse using a rotarod apparatus (Med Associates Inc., St. Albans, VT). Briefly, animals were placed on a rotating horizontal cylinder for a maximum of 200 s. The amount of time the mouse remained walking on the cylinder, without falling, was recorded. Each mouse was tested on a speed of 3–30 rpm and given three trials for any given day. The three trials were averaged to report a single value for an individual mouse, and then averages were calculated for all animals within a given treatment group, as described (Du et al., 2014).

2.5. Histological preparation

Mice were exposed to a lethal dose of isoflurane and perfused transcardially with ice-cold 1× PBS for 8–15 min, followed by 10% formalin for 10–15 min. Spinal cords and brains were dissected and submerged in 10% formalin overnight at 4 °C, followed by 30% sucrose in PBS for 24 h. Tissues were embedded in 75% gelatin/15% sucrose solution for cryostat sectioning then post-fixed overnight in 10% formalin and cryoprotected in 30% sucrose. The embedded tissues were stored in −80 °C after flash frozen in dry ice. 40 μm thick free-floating spinal