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Screening Remyelinating Drugs Using Primary Cell Culture Models

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by

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Acknowledgments

To Dr. Seema Tiwari-Woodruff: Thank you so much for supporting me and mentoring me.

To Dr. Hamblin and Dr. Garcia-Castro: thank you for being part of my committee.

To the Tiwari-Woodruff lab: Thank you all for allowing me to learn from you.

Dedication

To my Family

ABSTRACT OF THE THESIS

Screening Remyelinating Drugs Using Primary Cell Culture Models

by

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Master of Science, Graduate Program in Biomedical Sciences
University of California, Riverside, June 2025
Dr. Tiwari-Woodruff. Chairperson

Multiple sclerosis (MS) is an inflammatory autoimmune disease of the central nervous system (CNS). Demyelination is a hallmark symptom of MS and leads to a decrease in neuronal, motor and cognitive function which significantly decreases quality of life. Current MS treatments are exclusively immunosuppressant and immunomodulatory with the goal of halting disease progression. For this reason, current treatments fail to address the loss of function associated with MS as well as the individual response to therapeutics. Recent studies have identified Estrogen as having ameliorating effects in MS, specifically Estrogen Receptor Beta (ERβ). ERβ ligands are potential therapeutics as they activate Estrogen's neuroprotective and proliferating effects without the negative side effects exerted by ERα activation. Our lab identified two novel ERβ ligands, CAD-102 and CAD-110, as potential remyelinating therapeutics in MS. Previously, our lab has established a reliable Primary Mouse Oligodendrocyte (OL) culture model to assess efficacy of myelinating compounds in differentiating OL progenitor differentiation to mature OLs. While OL differentiation is important, efficacy of myelination compounds must be confirmed using in vivo mouse models. Here I

describe two reliable methods to assess the OPC differentiation and axon myelination efficacy of various compounds *in vitro* using primary mouse OLs in the presence of primary cortical mouse neurons as well as to assess differentiation efficacy in primary iPSC derived human OPCs. Using this method, I established CAD-102 and CAD-110's superior OPC differentiation to OLs and axon myelination using primary OL-Neuron co-cultures when compared to known remyelinating agents like Bazedoxifene, Clemastine, LY500307, and ErB-041 as well as superior differentiation in primary iPSC derived human OPCs culture. These results establish the use of these culture models to screen superior remyelination agents.

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Chapter 1: Introduction

Multiple Sclerosis Overview

Multiple Sclerosis (MS) is an inflammatory autoimmune disease of the central nervous system that leads to fine motor movement malfunction due to chronic demyelination and neurodegeneration. MS is a widespread disease as it affects around 900,000 people in the US alone and a total of 2.8 million people worldwide. Incidence of MS is rising in every corner of the world with someone being diagnosed every 5 minute (Walton et al., 2020). The average age of incidence is 32 years old with women being affected at twice the rate when compared to men and in some countries, this difference increases to four times as much. While disease phenotypes may vary from person to person, quality and expectancy of life greatly decreases compared to the general population (Walton et al., 2020). The cause of MS is still widely debated with many environmental, genetic, and lifestyle factors being implicated in its development (Ramagopalan, Dobson, Meier, & Giovannoni, 2010). Some environmental factors include exposure to tobacco, mononucleosis infection, and Vitamin D deficiency. Nearly every MS patient has been infected by the Epstein-Barr virus (EBV), which is the cause of mononucleosis. Patients with higher levels of anti-EBV antibodies have been found to have a higher risk of developing MS when compared to patients with lower levels of anti-EBV antibodies (Ramagopalan et al., 2010). Interestingly, anti-EBV antibody levels seem to increase years before initial neurological MS symptoms appear (Ramagopalan et al., 2010). Vitamin D deficiency has been identified as a risk factor as lack of sun exposure is associated with increased risk of developing MS while high serum concentrations of 25-

hydroxycholecalciferol, the primary form of vitamin D that circulates in the body, is found to be protective. Smoking, both first and second-hand, has also been found to increase the risk of MS (Ascherio, Munger, & Simon, 2010).

Pathology

Pathology of MS begins with the breaking of the Blood Brain Barrier (BBB) which leads to immune cell infiltration giving rise to widespread demyelination, inflammation, gliosis and neurodegeneration (Dendrou, Fugger, & Friese, 2015). The presence of T cells around lesions in the brain, a hallmark of MS, is possibly due to autoreactive lymphocytes initiating aggressive responses to CNS antigens (Dendrou et al., 2015). This event leads to demyelination, which may initially present itself as partial blindness, or loss of fine motor function in some patients. While the reason for BBB infiltration is unclear, it is believed that overactivity of matrix metalloproteinases (MMPs) is the reason for the degradation of the basement membrane under epithelial cells. MMPs are expressed by immune cells such as monocytes, macrophages, T cells, as well as cells of the CNS like microglia, astrocytes and OLs (Dhib-Jalbut, 2007). Activity of MMPs is inhibited by Tissue Inhibitor of MMP (TIMPs) and increased levels of MMP have been associated with increased damage and infiltration of the BBB (Cabral-Pacheco et al., 2020). High levels of MMPs are commonly found in blood vessels with active lesions, as well as in endothelial cells (Cabral-Pacheco et al., 2020).

Demyelination in the CNS

Demyelination is a common symptom in MS incidences post-BBB infiltration. Myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG) proteins are thought to be the predominant targeted antigens (Dendrou et al., 2015). Blood from MS patients frequently contain T-cell clones that are specific to a MBP peptide that includes amino acids p85-99 (Dhib-Jalbut, 2007) which is the immuno-dominant epitope of myelin basic protein and binds to the major histocompatibility complex haplotype HLA-DR2 (Kant, Pasi, & Surolia, 2015) which further supports this point. Demyelination is a hallmark symptom of MS and due to its abundance and importance in the human brain, these symptoms significantly degrade quality of life. The importance of Myelin in the brain cannot be understated as 20% of the total mass of the human brain is Myelin (Snaidero & Simons, 2014) and carries an especially important role in the central nervous system (CNS) as well as the peripheral nervous system (PNS). Myelin allows for efficient rapid conduction called saltatory conduction to take place across large distances, which is important for everyday body functions (Snaidero & Simons, 2014). The cells responsible for producing myelin in the CNS are OLs, and Schwann cells in the PNS. These cells are critical for the maintenance and repair of myelin sheathes (Snaidero & Simons, 2014). OLs have also been implicated in mediating axonal metabolism as axons are vulnerable to bioenergetic failure (Chamberlain et al., 2021). Using extracellular vesicles found in non-compacted myelin, OLs deliver SIRT2, a NAD-dependent deacetylase, to axons which are important for axonal energy enhancement (Chamberlain et al., 2021). OLs are also neuroprotective under glycemia as mature OLs can deliver lactate which is converted

into pyruvate by axons to aid in mitochondrial oxidative phosphorylation(Chamberlain et al., 2021).

Oligodendrocyte

For my thesis study, only OLs will be discussed as MS pathology primarily targets the CNS. First discovered by Robertson in 1899 and classified by Rio Hortega in 1921, OLs have been the focus for many neurological studies. While the development of OLs is not fully known, studies have shown that majority of OLs are produced during Embryogenesis (Jakovcevski, Filipovic, Mo, Rakic, & Zecevic, 2009). OL precursor cells first appear in the ganglion eminence at 9 gestation weeks and spread to the cortex during the following weeks (Jakovcevski et al., 2009). Mature myelinating OLs are first seen at 18 gestation weeks in the intermediate zone, the precursor to white matter (Jakovcevski et al., 2009). It is possible that OLs have many zones of origin as they are present in both the Ganglionic Eminences and cortical subventricular zones at mid-gestation (Jakovcevski et al., 2009). OLs undergo many stages of development, starting as early OL progenitor cells(OPCs) to OL precursor cells(OPCs) to Mature OLs (Jakovcevski et al., 2009). OL Progenitor cells are still mitotic and retain the ability to proliferate with stem cell like features as they can also produce astrocytes and neurons (Yang, Lewis, & Miller, 2011) and are identifiable by their expression of platelet derived growth factor alpha (Jakovcevski et al., 2009). These cells will then turn to OL Precursor cells (OPCs) as they lose the ability to proliferate and begin expressing sulfatide which then turns to glucocerebroside, which can be identified by the markers O4 and O1 respectively

(Jakovcevski & Zecevic, 2005). OL precursor cells have the highest density in the subplate at gestation weeks 17-23, which may indicate the subplate may play a role in OL Precursor cell maturation (Jakovcevski & Zecevic, 2005). OL precursor cells will then migrate to white matter regions where they develop into Mature Myelinating OLs, characterized by their expression of Myelin Basic Protein, Proteolipid Protein, and Myelin OL glycoprotein, and will myelinate multiple axons at a time(Jakovcevski & Zecevic, 2005). Not all OPCs mature to Myelinating OLs (Yang et al., 2011) as humans tend to have the highest volume of myelin at age 17 with a stable decrease during aging (Yeung et al., 2014). The remaining OPCs, which are referred to as Adults OPCs, tend to proliferate more slowly and respond to different mitogens (Yang et al., 2011). Adult OPCs may play a role as a reservoir of OPCs to replace mature OLs in adult hood as remyelination can occur in a healthy brain but in pathological diseases like Multiple Sclerosis, Adult OPCs fail to remyelinate axons (Yang et al., 2011) and clinical symptoms continue to progress.

Current State of Therapeutics in MS

While MS research has advanced in recent years, current treatments are extremely limited focusing only on immunosuppressants and immunomodulating drugs. Currently approved FDA treatments include treatments which include injectable IFNB products and Copaxone, which are immunomodulating and require long-term self-injections, and injectable humanized anti-bodies like daclizumab, natalizumab, and alemtuzumab

(Gholamzad et al., 2019). While effective in slowing disease progression, these medications have a constant need for self-injections which can lead to infections, flu-symptoms and increased liver enzymes (Gholamzad et al., 2019). Oral treatments for Multiple Sclerosis include Dimethyl Fumarate (DMF), Teriflunomide, and most notably Fingolimod (Gholamzad et al., 2019). While these oral drugs delay disease progression without the need for injections, they include serious side effects. Most notably is Fingolimod which had its clinical trials ended due to a patient death (Gholamzad et al., 2019). New drugs in clinical trials include but are not limited to Monoclonal antibodies, which deplete pre-B cells and mature B Cells, Laquinimod, which prevents BBB infiltration and stem cell transplantation, which is used in patients with negative responses to all treatment available (Gholamzad et al., 2019). While these new treatments have a significant reduction in disease activity and progression, they fail to address the increasing demyelination lesions as well as the loss of function in many of the lesions all while having undesirable side effects like cardiovascular complications with Laquinimod and Progressive Multifocal Leukoencephalopathy due to anti-CD20 agents (Gholamzad et al., 2019). To address these gaps in treatment, our lab has been studying the efficacy of Estrogen Receptor Beta (ERβ) ligands as remyelination therapy in MS.

Estrogen

In humans, Estrogen (E2) is an important hormone as it is critical during development and is neuroprotective, as women after menopause tend to suffer from stroke at higher rates than men (Maioli, Leander, Nilsson, & Nalvarte, 2021). There are three Estrogen

receptor subtypes, Estrogen Receptor Alpha (ERα), Estrogen Receptor Beta (ERβ) and G-protein coupled receptor 1 (GPER1). ERα and ERβ are classified as part of the activated nuclear receptor family of transcription factors, meaning they are in the cell nucleus or cytoplasm and bind to Estrogen response element (ERE) regulatory sites on DNA through its interactions with other proteins which activate or repress the transcription of target genes, particularly proliferation, differentiation and survival genes (Maioli et al., 2021). ERs can also bind to other transcription factors independently of E2 activation. In addition, ERA and ERβ have various splice variants with ERβ variants being studied the most (Maioli et al., 2021). GPER1 is a membrane bound G-protein coupled receptor with no transcriptional activity that helps modulate E2's rapid non-genomic actions (Maioli et al., 2021). E2 has been implicated in having neuroprotective properties (Maioli et al., 2021). Previous studies have shown that both receptors have neuroprotective properties with ERα having toxic effects and ERβ having none, making ERβ a potential target for therapeutics (Moore et al., 2014).

Estrogen Receptor Beta Ligands

In recent years, our lab collaborated with Dr. Katzenellenbogen and have been screening various ERβ ligands with an IndCl core using the EAE mouse model and *in vitro* studies resulting in an increase in OL differentiation and proliferation as well as amelioration of disease severity (Karim et al., 2019). Two novel ERβ ligands, CAD-102 and CAD-110, were identified of having the highest affinity for ERβ alongside having the most significant effects on OL differentiation and disease amelioration (Karim et al., 2019).

Human therapeutic studies of ERβ ligands are limited as we must first study efficacy, tolerance, and toxicity. For that, we have used primary cell culture models, animal models and more recently, iPSC models. Our *vivo* models have shown that CAD-102 and CAD-110 have immunomodulating and remyelinating effects in a EAE mouse model (Karim et al., 2019). Like *in vivo,* our *in vitro* studies showed a significant increase OL proliferation and differentiation (Karim et al., 2019). This study aims to establish a novel cell culture model to study myelination in primary OLs in the presence of primary cortical neurons. We use Primary OLs extracted from P0-P1 and culture them alongside cortical neurons extracted from the same litter. At 14 days, OPCs are isolated using the shaking method and added to the cortical neuron cultures which will proliferate, differentiate and myelinate axons in their surroundings. Here we compare novel ERβ analogues that have been previously confirmed to be successful *in vivo* (Tiwari-Woodruff & Voskuhl, 2009) and assess their differentiation capacity. Compounds that show superior OL differentiation will then be assessed using our novel co-culture myelination model for myelination efficacy and will be tested against known remyelination agents. Lastly, to show that these results translate to other models, we will use these ERβ ligands to assess differentiation capacity in primary iPSC derived human OPCs.

General Methodology

Animals

This study followed the protocol established by the American Veterinary Medicine in accordance with the National Institute of Health (NIH) and were approved by the Institutional Animal Care and Use Committee (IACUC) at UCR, Riverside. C57BL6 male mice were obtained from Jackson Laboratory and maintained in-house at the animal facility. Animals were held at five per cage with standard light/dark cycles.

Primary Cell Culture Extractions

C57BL6 P0-P1 pups were used for primary cell culture. Cortex is removed from P0-P1 pups and placed in MEM (Vendor)media complemented with 25 mM HEPES (HMEM). Meninges are removed before incubating in HMEM with Papain (Worthington)[160u] for 15 minutes. Brains are then washed with HMEM(Gibco) + Trypsin Inhibitor (Roche)[0.5mg/uL] and titrated to achieve single cell suspension. Cells are then counted with a hemocytometer and plated on coverslips in 12 well plates at 50k cells per well, and in T-75 flasks with 3 brains per flask. All glassware is coated with Poly-D-Lysine (Sigma)[1ug/mL].

OPC/OL Cultures

OPCs are isolated from glial cocultures grown and maintained in T-75 flasks with DMEM/F12 (Life Technologies) complemented with Insulin (Sigma) and N-Acetyl-L-Cysteine (Sigma). Once flasks reach ~80% confluency they are shaken at 200 RPM

overnight and OPCs are collected and plated at 50k cells per well on PDL coated coverslips in a 12-well plate. After plating, OPCs are maintained in DMEM Media complemented with NT3 [5uL/mL], CNTF [1ug/10uL], bFGF [0.1mg/mL], and PDGFAA [1ug/10uL] for 3 days. Media is then switched to DMEM media complemented with NT3 [5uL/mL], CNTF [1ug/10uL], bFGF [0.1mg/mL], and PDGFAA [1ug/10uL], Thyroxine (T4) [.8mg/100uL] and Tri-iodothyronine (T3) [.8mg/100uL] with our ERβ ligands and incubated for 5 days. Media is switched every two days. After 5 days, cultures are fixed with 10% formalin (vendor).

Neuronal-OL Co-Culture

Neuronal cultures are plated post-extraction at 50k cells per well on PDL coated coverslips in 12 well plates and maintained for 14 days in Neuronal Basal (NB) media complemented with 2% B27, GlutaMax [2mM], NT3[10ng/mL], NGF7 [50 ng/mL]. At 14 days, OPCs are isolated as previously described and added to each neuronal culture at 5k cells per well. Media is changed to NB media and DMEM media as previously described at a 1:1 ratio for 3 days. Media is then changed to NB Media and DMEM Media with T3/T4 as previously described at a 1:1 ratio with our ERβ ligands and incubated for 5 days. Cultures had media change done every 3 days. After treatment, cultures are fixed with 10% formalin (vendor)

iPSC derived Human OL cultures

iOligodendrocytes (SKU102) were purchased from Tempo Bioscience. Cells were thawed and passaged twice before being cryoprotected at -80C. Cells were plated at 20k cells per well on Matrigel [10 ul/mL] coated coverslips and maintained in Growth Media (SKU601) purchased from Tempo Bioscience for two days. Media was switched to Maturation Media (SKU602) purchased from Tempo Bioscience treated with remyelinating agents and complemented with T3 [200 ng/mL] and Insulin Growth Factor -1 (IGF-1) (Peptrotech) [10ng/mL]. Cultures were treated for 21 days. After treatment, cultures were fixed in 10% formalin for 15 minutes.

ERβ Ligand Compounds and other remyelinating agents

Novel ERB ligands were developed in collaboration with Dr. John Katzenellenbogen's laboratory at the University of Illinois Urbana-Champaign. Other remyelinating drugs were purchased.

Immunohistochemistry

Cells were permeabilized using 0.3% Triton X-100 and blocked using 20% normal goat serum (NGS). The following antibodies were used: mouse anti-myelin basic protein (MBP, 1:500, Biolegend 808401), and rabbit anti-Beta-tubulin-III (Sigma T2200, 1:500). Secondary staining was performed using polyclonal fluorophore-conjugated antibodies: goat anti-mouse Alexa Fluor cy3 and goat anti- rabbit Invitrogen 647 (Thermo fisher scientific). Nuclei were counter-stained with 4', 6-Diamidino-2-phenylindole (DAPI) for

10 minutes after incubation with secondary antibodies. Coverslips were mounted on glass slides using Fluoromount G mounting medium (Thermo Fisher Scientific) and allowed to dry overnight.

Quantification and Microscopy

Image analysis was conducted using an Olympus BX61 confocal microscope (Olympus America Inc., Center Valley, PA) with z-stack projections performed using slidebook6. Counts and analyses of the coverslip images were performed using ImageJ (NIH, Bethesda, MD). Colocalization was quantified using ImageJ plugin *Just another Colocalization Plugin* (JaCOP).

Statistics

Statistical analyses were performed using Prism® (GraphPad, La Jolla) program for Windows. There were 3 coverslips per treatment group. Graph values are expressed as mean $\pm$ standard error of the mean. For i*n vitro* studies, statistical analysis of mean values was conducted using one-way ANOVA with Bonferroni's correction. Differences were considered significant at the *$p<0.05$, **$p<0.01$, ***$p<0.001$, and ****$p<0.0001$ level

Results

ERβ ligands induce significant differentiation in primary OLs

To assess differentiation efficacy of ERβ Ligands, primary OL cultures were exposed to novel ERβ ligand CAD-102, CAD-110, CAD-109, and CAD-101 at two concentrations, [10 nM] and [100 nM] for 5 days. After 5 days, cells were fixed in 10% formalin. Immunohistochemistry (IHC) analysis was done using ImageJ (**Figure 1 i**). Myelin basic protein (MBP) positive cells were quantified, showing a significant increase of MBP+ cells in all ligand-treated cultures except for CAD-109. (**Figure 1 ii**). Interestingly, DAPI count was not affected across all cultures. CAD-102 and CAD-110 had the highest differentiation capacity when compared to CAD-101 and CAD-109. While differentiation at 100 nM was higher when compared to 10 nM, we decided to continue the study with our ERβ ligands at 10 nM as the response was significant with a lower concentration

ERB ligand differentiation efficacy was then compared with approved remyelinating agents in primary OL cultures. Cultures were exposed to CAD-102 [10nM], CAD-110 [10nM], ERB-041 [10], E2 [50 nM], Clemastine [500 nM], Bazedoxifene [500 nM] and LY500307 [30 nM] and were incubated for 5 days. IHC analysis was performed using ImageJ. (**Figure 2i**). Myelin basic protein (MBP) positive cells were counted which showed a significant differentiation effect across all remyelinating agents with CAD-102 and CAD-110 having the most significant differentiation effects while having no effect on total cell counts. (**Figure 2ii**).

ERB ligands induce significant myelination effects on Neuronal/OL Co-Cultures

Having identified novel ERB ligands CAD-102 and CAD-110 as the most effective OL differentiation ligands, we established their myelination efficacies in the presence of cortical neurons when compared to approved remyelination agents. Primary cortical neurons were dissected from P0-P1 pups and incubated for 14 days. OPCs are then isolated overnight using the shaking method and primary cortical neuron cultures are supplemented with 5k OPCs per well. OPCs allowed to proliferate for 3 days and treated with remyelinating agents for 5 days before being fixed and stained (**Figure 3i**). Cultures were stained using an anti-MBP as a marker for OLs and anti-B3-Tubilin for axons Colocalization analysis was performed using ImageJ plugin JaCOP (**Figure 3ii**). All remyelinating agents showed an increase in myelination when compared to vehicle with CAD-102 and CAD-110 having the most significant myelination effect (**Figure 3iii**).

Differentiation Efficacy of ERB ligands on Primary iPSC derived Human OPCs

To assess differentiation efficacy of superior ERB ligands on a human model, we used primary iPSC derived human OPCs purchased from Tempo Bioscience(SKU102). iPSC derived OPCs were plated at 20k cells per well on Matrigel [10 ul/mL] coated coverslips. Cultures were allowed to stabilize in Growth Media(SKU601) before being switched to Maturation Media(SKU602) supplemented with T3 and remyelinating agents. Cells were incubated for 21 days with media changes done every other day. After 21 days, cells were fixed using 10% formalin and ICC was preformed using anti-MBP as a marker for OLs. We observed a significant increase in MBP expression intensity in CAD-

102. The other myelination agents had an increase in MBP expression although not significant (**Figure 3**).

Discussion

There is a need for an *in vitro* myelination model since demyelination is difficult to study due to its common occurrence in the CNS. As a critical symptom necessary in MS pathology progression, it is important to identify compounds that can remyelinate demyelinated axons as this approach will address the loss of function followed by demyelination. As previously stated, current treatment approaches are immunomodulary or immunosuppressant. While effective in reducing disease progression, these medications often rely on constant self-injection with increased risk of infections and other undesirable side effects. This study establishes the use of Primary Mouse Neuronal and Oligodendrocyte co-cultures as a reliable myelination model to study remyelinating agents *in vitro*. This model allows for quick screening of differentiation and myelination efficacy in a brief period of time, allowing for faster identification of potential MS therapeutics. With our model, we were able to confirm previous results showing ERβ ligands CAD-102 and CAD-110 as potential MS therapeutics with increased remyelination potential *in vivo* (Tiwari-Woodruff & Voskuhl, 2009).

Immunohistochemistry (IHC) is commonly used to assess myelination *in vitro* and *in vivo* from tissue samples. Myelin Basic Protein (MBP) expression as an overall intensity percentage is used as a measurement of MBP presence and quantity. While effective, the results may be overrepresented as MBP is expressed in both the myelin

sheathes surrounding axons as well as in the cytoplasm of OLs. Compensating for the additional intensity from MBP cytoplasm expression is difficult to do. By pairing this model with ImageJ plugins that quantify colocalization like *Just another Colocalization Plugin* (JaCOP), this model becomes a powerful tool to accurately assess myelination as JaCOP quantifies and assesses whether two overlapping fluorescent points on different channels are colocalized (Bolte & Cordelieres, 2006). This allows for an accurate representation of myelination without having to compensate for cell bodies as only colocalized fluorescent points will be quantified.

Current myelination models dissect pups during embryogenesis, 18 days post conception being the most common timepoint. While neural stem cells and OPCs are abundant at this stage, it is resource intensive as not only are the pups sacrificed but a capable breeder is sacrificed as well. Our model can produce healthy cultures capable of robust myelination using P0-P1 pups without the need to sacrifice a breeder. This timepoint makes this model cost-effective as a breeder can produce multiple liters throughout her life and allows for inexpensive experiments capable of reproducing results.

Our model can produce results that are translatable to other models as we were able to see using primary derived iPSC human OLs. We coated coverslips with Poly-L-Ornithine (PLO) and Matrigel and compared Oligodendrocyte development under normal conditions. On PLO coated coverslips, OPCs began to divide on top of each other forming tumor like structures. For this reason, we decided to continue studies on Matrigel as cells were able to proliferate without forming these structures. OLs were fixed and

stained after 21 days where we showed an increase in MBP expression, further confirming previous studies as well as results obtained using our model. Our results are currently being submitted and are close to being published. A limitation of our model is the low branch morphology in mature OLs. While we do see high intensity in MBP expression, our cells are very bipolar, which is the morphology commonly seen in progenitors. Currently, our lab is optimizing this model with the use of Fibronectin, which is an extra cellular matrix to supplement the PLO coating. Additionally, we are supplementing the maturation media with IGF-1, an important mitogen in OL differentiation (Bibollet-Bahena & Almazan, 2009). Currently, the effects of these new modifications are under study, but preliminary data shows improved morphological features and more branched Oligodendrocytes.

In summary, this paper presents an OL/Neuronal co-culture that can be used as a dependable myelination model. We were able to confirm previous studies showing CAD-102 and CAD-110 as having superior differentiation ERβ ligands. Additionally, our model presented new colocalization data that further corroborated previous results in an accurate manner. This model is not limited to myelination as the media can be conditioned by supplementing the media with toxins that can mimic injury. Our model's findings were further confirmed through the use of iPSC derived human OPCs, further proving it to be a reliable model and our results are currently submitted for publishing. Future directions for our model will be to develop a human model of iPSC derived Neuron-OL cocultures to assess myelination potential in a human model as well as an organoid model to study myelination potential in a 3D model. These two models will be

useful in assessing and identifying remyelination potential in therapeutics currently on the market or novel therapeutics being developed. Its high efficiency and low cost make it an attractive model to identify safe and efficient remyelinating agents.

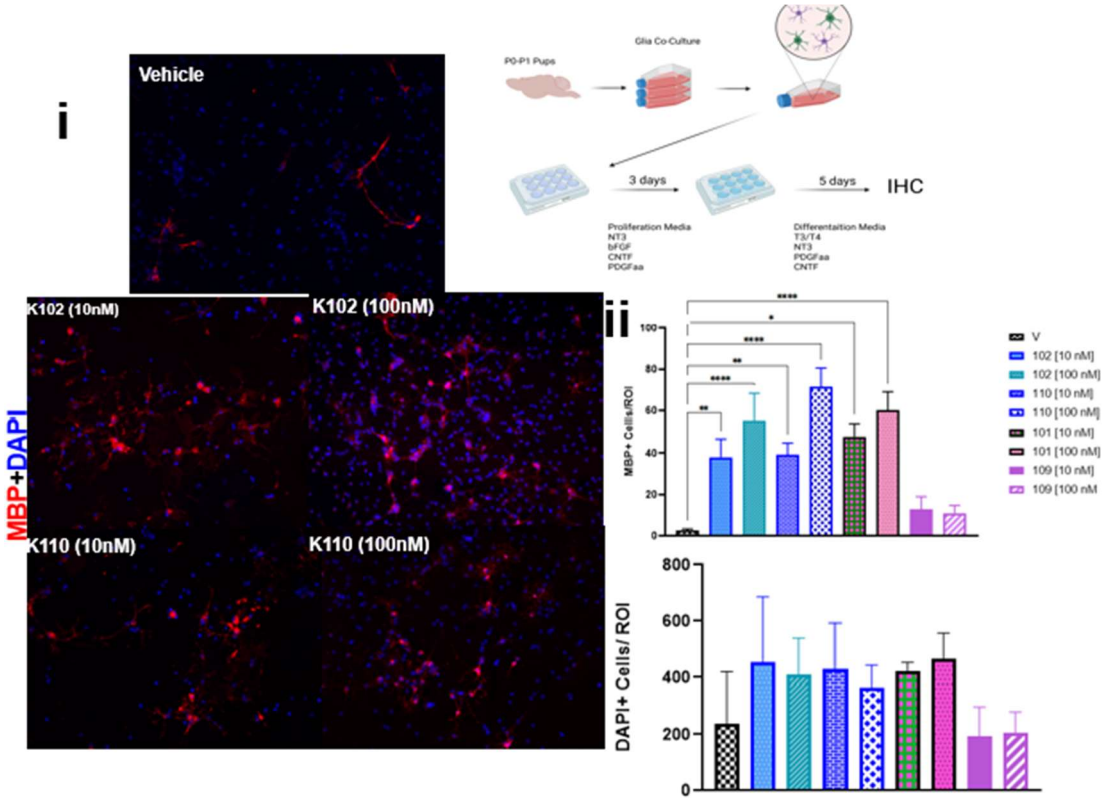

Figure 1. IndCl analogues effects on OL differentiation
(i) Representative images of primary OPCs/OLs protocol and images from wells containing differentiating media with 101,109, 102, and 110. OLs were immunocytostained with myelin basic protein (MBP; red) and co-stained with nuclear stain DAPI (blue). (ii)Quantified DAPI counts across all treatments. No significant differences across all treatments. Estrogen receptor β (ERβ) ligands except 109 promote significant differentiation of primary mouse OL progenitor cells at 10 nM and 100 nM. There were 3 wells/treatment groups with no significant differences in the total number of cells between groups. Statistical analysis performed by One-Way ANOVA with Bonferroni's multiple comparisons test.

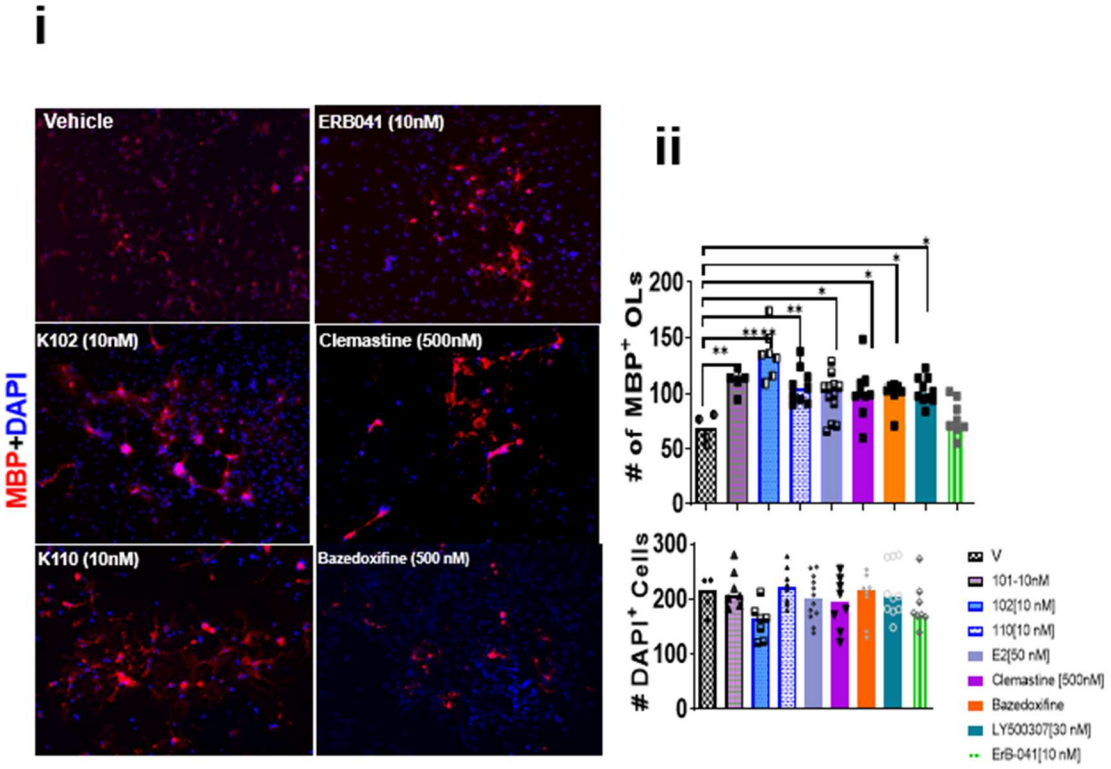

Figure 2. Comparing novel ERβ ligands to known remyelinating agents
(i) Representative images of OL differentiation in the presence of 10 nM of CAD-102, and CAD-110, alongside known remyelinating compounds, including ERB-041 (10 nM), Clemastine (500 nM), bazedoxifene (500 nM), LY500307 (30 nM), and E2 (50 nM). OLs were immunocytostained with myelin basic protein (MBP; red) and co-stained with nuclear stain DAPI (blue). **(ii)** Quantification of MBP+ cells per treatment and DAPI+ cells. All compounds increase MBP+ cells without affecting the total number of cells per group. There were 3 wells/treatment groups. Statistics were done using One-Way ANOVA with Dunnett's multiple comparisons test.

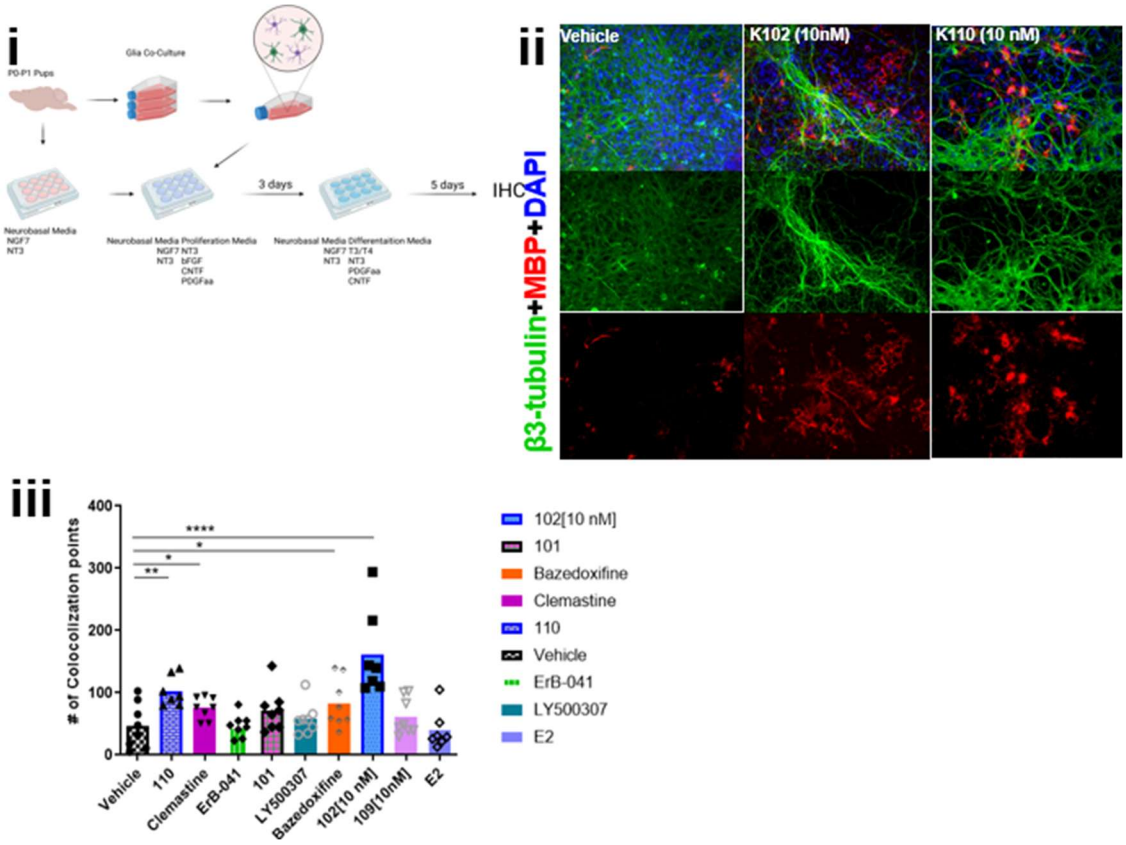

Figure 3. OL Neuron coculture Myelination efficacy comparison of IndCl analogues with known remyelinating agents

(i) The myelination potential of these compounds was evaluated using novel myelination model. Co-cultures were exposed to vehicle control, 10 nM of CAD-102, and CAD-110, or known remyelinating compounds, including ERβ-041 (10 nM), Clemastine (500 nM), bazedoxifene (500 nM), LY500307 (30 nM), and E2 (50 nM) (ii) Representative images stained of co-cultures immunocytostained with myelin basic protein (MBP; red), B3-Tubilin (B3-tubulin; green) and with nuclear stain DAPI (blue). (**iii**) Quantification of myelination showing a significant increase in cultures treated with CAD-102, CAD-110, Clemastine, and Bazedoxifene. There were 3 wells/treatment groups. Statistics were done using One-Way ANOVA with Dunnett's multiple comparisons test.

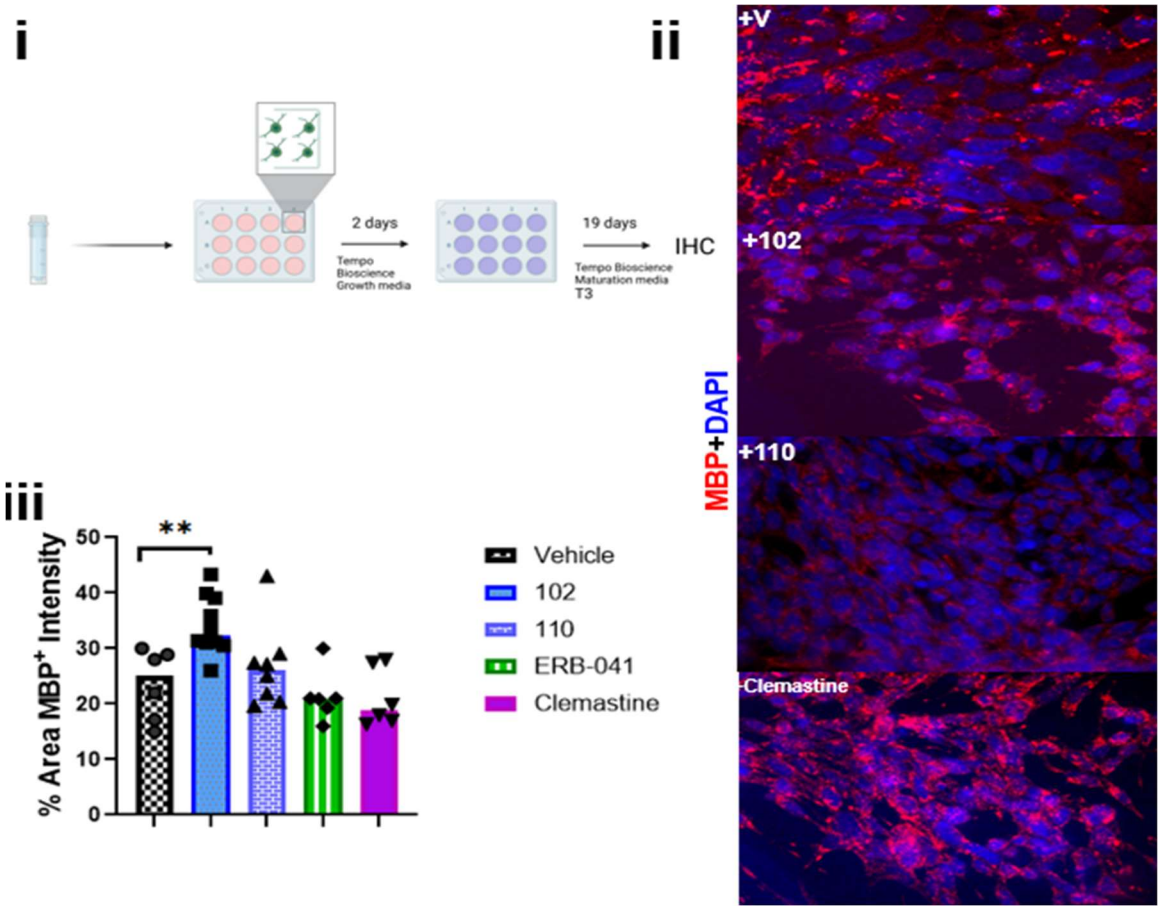

Figure 4. CAD-102 increases OL differentiation in human OLs
(i) Human iPSC model for assessing differentiation efficacy in human OPC's from Tempo Bioscience. Cells were treated with remyelinating drugs (CAD-102, CAD-110, ERB-041, Clemastine fumarate) in Maturation Media complemented with T3 [200 ng/mL]. Media was switched every two days. After 21 days, cells were fixed with 10% formalin and stained. **(ii)** Representative fluorescent microscopic images at 40x of OPCs/OLs containing maturation media(vehicle), CAD-102 [10 nM], CAD-110 [10 nM] and Clemastine [400 nM] stained with myelin basic protein (MBP; red) and co-stained with nuclear stain DAPI (blue)**. (iii)** Effects of treatment on the intensity of MBP+ OLs was quantified showing CAD-102 having a significant increase in the intensity of MBP+ OLs compared to vehicle-treated cells. No significant differences in total number of cells were observed between other groups. There were 3 wells/treatment groups. Statistics were done using One-Way ANOVA with Dunnett's multiple comparisons test.

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