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Review:

A Novel Multiple Sclerosis Model Utilizing Cuprizone and Rapamycin

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ABSTRACT

  Multiple Sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system leading to debilitating long-term neurologic damage, primarily due to axonal loss following unsuccessful remyelination and the limited ability to remyelinate in the adult human brain. This review summarizes the current knowledge regarding combined administration of Cuprizone (CPZ) and rapamycin as a novel animal model for MS. Utilization of CPZ induces non-immune mediated demyelination, therefore bypassing complexities of the immune system and allowing analysis of de-/remyelination. Furthermore, remyelination occurs simultaneously with demyelination due to ongoing oligodendrocyte progenitor cell (OPC) differentiation into mature oligodendrocytes (OLGs).

  The immunosuppressive agent, rapamycin inhibits the regulatory pathway Akt/mTOR, which primarily controls myelination. This inhibition prevents ongoing OPCs from differentiating into mature oligodendrocytes (OLGs), therefore preventing myelination. The dual CPZ/rapamycin model produces more complete demyelination and a slowed remyelination phase. The long-term relevance of this review is to assess distinct stages and contributors of de-/remyelination in MS to better assess possible therapeutics for patients diagnosed with demyelinating diseases, such as MS.

INTRODUCTION

  Multiple Sclerosis (MS) is an autoimmune demyelinating disease of the central nervous system (CNS). Myelin is a fatty substance composed of proteins and phospholipids that surround nerve fibers. Myelination of these nerve fibers are essential for facilitation of signal conduction and for supporting neuroglia in the axonal environment, two examples of which are, microglia

and astrocytes (Matsushima et al., 2001). Loss of myelin, also called demyelination, leads to debilitating neurological consequences ranging from motor related symptoms (loss of balance and loss of speech, loss of sight, muscle spasms, fatigue, weakness, bowel and bladder incontinence), and cognitive impairments (anxiety and memory loss) to in rare cases, seizures (Praet et al. 2014).

The etiology of MS is unknown and there is no cure for the disease. A recent study estimated that the number of people diagnosed with MS worldwide has increased from 2.1 million in 2008 to 2.3 million in 2013. In addition to having rising prevalence rates, MS is also classified as one of the world's most common neurologic disorders and one of the leading causes of non-traumatic neurologic disabilities in young adults (Browne and Chandraratna, 2014).

Current animal models of MS do not provide a complete de-/remyelination time frame in which to assess therapeutics, such as dietary supplementation. Based on the findings of this review, future therapeutic studies may utilize the CPZ/rapamycin model.

CURRENT MODELS FOR MULTIPLE SCLEROSIS

Animal models of demyelination have served as a primary resource in the ongoing effort to understand the molecular and cellular mechanisms responsible for demyelinating diseases such as MS. The two most commonly used models of demyelination are experimental autoimmune encephalomyelitis (EAE) and toxically induced demyelination via cuprizone.

Experimental autoimmune encephalomyelitis presents the organism with an immunization containing components from the CNS. These components may include myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), or proteolipid protein (PLP), all of which target myelin via antigen presentation and autoimmunity (Nathoo et al., 2014). The

EAE model has been used for many years and led to the development of many therapies; however, it's effects vary depending on animal strain, age, and duration of treatment. This variability makes it difficult to analyze mechanisms contributing towards de-/remyelination (Sachs et al., 2014). The EAE model is also characterized by varied lesion localization. These asynchronous lesions lack anatomical reproducibility between organisms making the model difficult to reproduce and quantify (Matsushima et al., 2001; Sachs et al., 2014). EAE induces an inflammatory immune response that leads to the compromise of the blood-brain-barrier (BBB) and infiltration of external T cells and other immune cells. Normally, these immune cells cannot pass the BBB. In the attempt to assess distinct stages of de-/remyelination, a more reliable model of demyelination would separate the two phases, in order to fully assess remyelination from completely demyelinated regions,

The second model induces demyelination toxically via cuprizone (CPZ) [oxalic acid bis(cyclohexylidene hydrazide)]. CPZ is a cooper-chelating agent added as a supplement to rodent chow that induces demyelination throughout the telencephalon. The majority of demyelination associated with CPZ occurs in the caudal regions of the corpus callosum (CC), hippocampus, and superior cerebellar peduncles (Nathoo et al., 2014).

In this model, acute demyelination is induced in 8-10 week old C57BL/6 mice via administration of CPZ-supplemented diet for 5-6 weeks. Commencement of 0.2% CPZ at this age provides synchronous, reproducible, and largely reversible patterns of demyelination while minimizing detrimental systemic effects, such as toxicity of the liver. If treatment is maintained for 14+ weeks detrimental effects are observed throughout the organism, such as disturbances to mitochondrial function in the brain and liver. Death of the mice is commonly reported at 16

weeks of treatment. This length of treatment is classified as "chronic demyelination" and is not completely reversible (Matsushima & Morell, 2001).

At three weeks of acute CPZ-induced demyelination myelin genes encoding mRNA for MBP, myelin associated glycoproteins (MAG), and ceramide galactosyltransferase (CAG) are quantifiably reduced (Bosio et al., 1996). An increase in microglia/macrophage levels and ongoing OPC differentiation coincide with a decrease in myelin mRNA translation. By 4 weeks of treatment, OPC and astrocyte levels surge (Praet et al., 2014). Meanwhile, CPZ continuously induces demyelination throughout the brain. After 5 weeks of treatment there is almost complete depletion of mature OLGs in the caudal regions of the CC and superior cerebellar peduncles. By 4-5 weeks a substantial amount of axons are demyelinated throughout the caudal regions of the CC and superior cerebellar peduncles (Matsushima et al., 2001; Thiessen et al., 2013). Remyelination starts in earnest immediately after CPZ removal, and within the first week of recovery numerous remyelinated axons appear in the medial CC. This suggests that remyelination ensues rapidly after CPZ removal, due to ongoing OPC proliferation into mature OLGs (Gudi et al., 2014).

Both the EAE and CPZ models can be used to model demyelination and to test the efficacy of certain drugs and treatment methods; however, the EAE model exhibits asynchronous lesions that are difficult to reproduce. Furthermore, the CPZ model exhibits varying demyelination based on mouse strain, age, and gender,, ongoing remyelination during CPZ treatment preventing complete demyelination and rapid axonal remyelination following CPZ cessation In the attempt to evaluate remyelinating therapeutics, rapid remyelination makes these assessments difficult to perform and interpret. Utilization of rapamycin, an Akt/mTOR inhibitor, in conjunction CPZ prevents remyelination during CPZ administration and exhibits more

complete demyelination compared to CPZ treatment alone. Inhibition of differentiating OPCs via rapamycin yields an elongated remyelination phase. While the CPZ and EAE animal models may mimic the demyelinating effects of MS, the combined treatment of CPZ/rapamycin presents a targeted model to assess remyelinating therapeutics by separating the de-/remyelination phases through Akt/mTOR inhibition, furthermore inhibiting OPC differentiation during the remyelination phase. (Sachs et al., 2014). Inhibition of ongoing remyelination during CPZ treatment via rapamycin would exhibit a significant amount of reproducible demyelination in specific regions of the CC (Tagge et al., 2016). Ample demyelination in these regions would allow for remyelinating therapeutics to be manipulated and quantified through in-vivo imaging techniques throughout the recovery phase without additional drug administration. Throughout the uninterrupted recovery phase, quantification of axonal remyelination would allow for targeted therapeutics to be assessed in specific regions that previously exhibited a considerable level of demyelination.

ROLE OF REGULATORY T CELLS IN MULTIPLE SCLEROSIS

Theories have attributed the origin of MS to genetic and environmental factors, with the most likely being immune dysregulation. The primary role of the immune system is to attack and eliminate foreign antigens that may be acquired genetically and/or environmentally. The thymus serves a pivotal role within the immune system by producing lymphocytes T-cells and B-cells. In a healthy individual the T-cells are non-reactive to self-cells, but if the thymus fails to eliminate the self-reactive T-cells an autoimmune disorder, such as MS may commence. Peripheral immunological tolerance is the primary mechanism that maintains a balance between immune reactions toward self and non-self antigens. Dysregulation of immune self-antigens is considered

key to understanding autoimmune disorders. Regulatory T cells (T_{REG}) are now understood to be a major component in CNS autoimmune inflammation. Deletion of T_{REG} cells leads to spontaneous autoimmune disease in mice, while an elevation prevents the progression of EAE induction. Maintenance of peripheral tolerance has been correlated with amplified T_{REG} cells and protects against EAE progression. T_{REG} cells are classified based on the cytokine secreted and surface phenotype (Zozulya et al., 2008). The most well understood type is nT_{REG} cells, which are characterized as $CD4^+CH25^+T_{REG}$ lymphocyte cells. These cells develop in the thymus and are responsible for the expression of interleukin 2 (IL-2) and transcription factor FOXP3 (Zozulya, Wiendl *et al.* 2007). FOXP3 regulatory T cells produce T_H3 regulatory cells which proceed to recruit anti-inflammatory cytokine TGF-B (McFarland et al. 2007). These studies found that an increase in T_{REG} levels is associated with preventing axonal damage characteristic with autoimmune diseases, therefore serving a protective role. Impaired $CD4^+CH25^+FOXP3^+T_{REG}$ cells may explain the intolerance for self-antigens and increased autoimmunity susceptibility. In support of this claim, Veken et al., (2014) found that impairment of FOXP3 was correlated with patients in the relapse-remitting phase of MS.

Later work by Dieckmann et al., (2007) found that T_{REG} can be identified and quantified in peripheral blood of healthy and diseased humans. This work exhibited that $CD4^+CH25^+T_{REG}$ cells isolated from MS patients are morphologically impaired, yielding impairments in the thymus (Zozulya, Wiendl et al., 2008). Impairment of $CD4^+CH25^+T_{REG}$ cells transforms the thymus's efficiency in eliminating self-reactive T-cells. This loss of function could be a primary route in the initiation of MS self-reactivity.

CUPRIZONE INDUCED DEMYELINATION

Demyelination induced via CPZ administration is attributed to the formation of megamitochondria in mature OLGs following a 3 week 0.2% CPZ supplemented diet (Praet et al., 2014). However, CPZ exhibits no effect on microglia, astrocytes, and OPCs (Praet et al., 2014; Matsushima et al., 2001). CPZ administration inhibits Complex IV activity and uncouples oxidative phosphorylation. While complexes I, II, and III are uninhibited by the treatment, the effect on Complex IV is significant. As the levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS) increase, oxidative stress leads to the formation of the megamitochondria, making the OLGs vulnerable to degradation (Acs et al., 2013). In addition to an increased vulnerability, ROS free radicals can then amplify the damage via lipid peroxidation by oxidizing the OLGs high concentration of intracellular Fe^{3+} into Fe^{2+}. The remaining Fe^{2+} then proceeds to initiate the degradation of lipid membranes (Praet et. al. 2014).

Increased concentrations of ROS and RNS will, in addition to causing the uncoupling of the electron transport chain, disrupt the myelin sheath via endoplasmic reticulum (ER) stress. The stress on the ER reduces mRNA transcription and translation in order to reduce an accumulation of misfolded proteins. Immediately following this down-regulation, myelin protein-related mRNA production is quantifiably reduced during CPZ treatment (Arnett et al., 2003). While undergoing CPZ treatment the misfolded proteins that are released from the ER accumulate and initiate an unfolded protein response (UPR), which leads to an efflux of calcium and subsequent OLG death. The mitochondrial enlargement caused by increased ROS/RNS levels may be viewed as a protective measure to reduce oxidative stress (Wakabayashi et al., 2002). This claim is supported by observations that after CPZ treatment termination the mitochondria in mature OLGs regain normal morphology and function (Praet et al., 2014).

While CPZ induced demyelination is in effect, oligodendrocyte progenitor cells are continuously attempting to remyelinate by producing OLGs that may proliferate and repair axons. Meanwhile the OLGs that are synthesized are vulnerable to protein misfolding and cell death. For studies that are attempting to understand potential therapeutics, it is crucial to exclusively produce demyelination with no underlying attempts of remyelination. A CPZ/rapamycin model would yield complete demyelination with no underlying remyelination and would exclude any OPC's attempt to differentiate into mature OLGs during demyelination. Such a model has yet to be universally approved by the scientific community; however recent studies have found that intraperitoneal injection of rapamycin can successfully inhibit production of OPCs when given in conjunction with CPZ treatment. This combination of treatments can allow for complete demyelination following a longer remyelination period (Sachs, Bercury, et al. 2014).

Current literature indicates that healthy OPCs are continuously proliferating in an attempt to differentiate into OLGs throughout CPZ administration. Utilization of CPZ limits differentiation of OLGs, but does not prevent ongoing remyelination during the demyelination phase of the study. Oxidative stress during treatment also reduces ATP production and the degree of irregularity within the CPZ model yields a broad mixture of cell responses. A recent 2014 study by Sachs et. al (2014) reported promising results using CPZ in conjunction with intraperitoneal injections (IP) of rapamycin in which the treatment plan yielded greater demyelination and slowed remyelination. Rapamycin inhibits astrocytes, which is a signaling factor for microglia, furthermore inhibiting active myelination via inhibition of Akt/mTOR signaling pathway. Therefore combined administration of CPZ/rapamycin exhibits significantly greater demyelination compared to CPZ treatment alone.

PREVIOUS APPLICATIONS OF RAPAMYCIN

The mammalian target of rapamycin (mTOR) was discovered in the 1970's in a soil sample obtained from the Polynesian island of Rapa Nui, which exhibited novel antifungal activity. It was named rapamycin in order to not only allude to its place of discovery, but its remarkable characteristics as well. Now, a widely utilized immunosuppressant, it has a variety of uses, including post-transplantation therapy, prevention of restenosis following angioplasty, and as a potential treatment for certain cancers.

The physical target of rapamycin is the mTOR pathway that functions in response to hormonal and nutrient cues as a controller of cell growth and metabolism. There are two main complexes mediated through mTOR. mTORC1 (complex 1) is bound by rapamycin through FK506-binding protein and directly decreases activity. Contrariwise, rapamycin indirectly inhibits mTORC2 (complex 2). Although, this form of inhibition takes place during chronic exposure and is primarily thought to contribute to metabolic control (Johnson et al., 2013).

mTORC1 can be controlled by a plethora of cues. Activation of the pathway is mediated by insulin-like growth factor-1 (IGF-1) and other growth factors such as Akt and PI(3)K signaling. Repression is mediated by AMP-activated kinase (sensor for cellular energy), dietary restrictions, and prolonged hypoxia (Johnson et al., 2013). mTORC1 activation increases protein synthesis through two substrates: ribosomal protein S6 kinase (S6Ks) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) (Johnson et al., 2013). Activation of mTORC1 leads to the phosphorylation of S6Ks and 4E-BP1, ultimately leading to mTOR dependent gene transcription, protein synthesis/lipid biosynthesis, and cell growth (Kim, et al., 2008; Harrison et al., 2009). This pathway also inhibits the degradation of autophagy. With age, this repression of autophagy is increasingly inhibited resulting in an accumulation of damaged protein aggregates

and degeneration of the mitochondria, both of which can contribute to age related cellular dysfunctions (Johnson et al., 2013; Neff et al., 2013).

Previous studies have additionally indicated that mTOR signaling regulates the aging process and are associated with an extension of lifespan (Fang et al., 2013; Johnson et al., 2013). Deletion of S6K in yeast (*S. cerevisiae*), mutations in mTOR pathway and mutations in mTORC1 pathway of fruitfly (*Drosophila melanogaster*) result in an increase in lifespan. Inhibition of mTOR and mTORC1 components can be mediated through rapamycin. Previous studies have confirmed that introduction of rapamycin in multiple organisms extends their lifespan, including yeast, nematodes, fruitflies, and mice (Johnson et al., 2013).

Similar effects of longevity are observed with dietary restrictions. This comes as no surprise because the mTOR path functions in response to nutrient and growth cues. Inhibition of mTOR via rapamycin or dietary restrictions would reduce cellular growth and reduce cues for aging. These findings have been confirmed in other studies as well. For example, Colman et al., (2009) found that dietary restriction reduces the likelihood of developing cancer, cardiovascular disease, diabetes, and brain atrophy in the rhesus macaque monkey. This study suggests that rapamycin supplementation would have similar beneficial effects on age-related decline.

IGF-1 promotes the mTOR pathway via Akt/PI3K signaling (Narayanan et al., 2009), therefore inhibition of mTOR via rapamycin would reduce IGF-1 and would reduce age-related pathologies. A reduction of IGF-1 would promote the normally inhibited autophagy, further removing damaged organelles and macromolecules from the cytoplasm, therefore promoting longevity (Johnson et al., 2013).

RAPAMYCIN PARADOX

Many studies have pointed towards the remarkable benefits of rapamycin, so why has this immunosuppressant agent not been utilized clinically to reduce aging in humans? Other studies have highlighted the altered metabolic effects of rapamycin. Fang, et al., (2013) demonstrated detrimental metabolic effects of rapamycin following IP supplementation to C57BL/6 mice, therefore exhibiting a paradox of the improved lifespan theory. Acute rapamycin treatment inhibits mTORC1, but longer treatment induces a chronic and systemic reaction that effects mTORC2 (Sarbassoc et al., 2006). Detrimental effects included insulin resistance, hyperlipidemia, and glucose intolerance. Interestingly, these negative effects were only observed 2 weeks into the treatment. The organism's pancreatic volume was also reduced and the liver was enlarged after 2 weeks of treatment, but after 20 weeks organ sizes were back to normal. In conjunction with this, 2 weeks of treatment was characterized by a 2.5 fold increase in insulin, induced glucose intolerance and insulin resistance. Yet, around 6 weeks there was a transition phase in which the mice exhibited an improved metabolic state. After 20 weeks of a prolonged treatment, the mice proceeded from being insulin resistant to having enhanced insulin sensitivity.

Along with the improved insulin sensitivity, there was also an increase in metabolism and in efficiency of oxygen consumption. All of these observed changes suggest that rapamycin initially prompts a negative effect, yet after 6 weeks a clear transition takes place and by 20 weeks of treatment, it supports the potential effects of longevity (Fang et al., 2013).

Rapamycin's beneficial effects are exhibited following a significant period of treatment. Therefore utilization of the immunosuppressant characteristics could be beneficial to a MS model if utilized within the 6 week (0.2% CPZ) treatment timeframe. Another important consideration is the effect rapamycin has on inflammation. Inflammation is associated with a

large variety of age-related disorders and hyperactivation of the mTOR pathway is associated with inflammation (Johnson et al., 2013). In assessment of remyelinating therapeutics, it would be beneficial to produce ample demyelination unaccompanied by ongoing remyelination, followed by recovery from CPZ/rapamycin administration by allowing remyelination. While CPZ lacks the inflammatory response associated with MS, addition of rapamycin yields a model that is ideal for assessing remyelination therapeutics by exhibiting the most complete and reproducible demyelination. Ample demyelination would allow full assessment and quantification of therapeutics that can alter and promote remyelination.

In summary, our present understanding of rapamycin is that it exhibits a plethora of reversible negative systemic effects after 2 weeks of treatment. After 6 weeks of treatment, a metabolic shift takes place towards an improved state from baseline. Finally, by 20 weeks of treatment, several of the initially detrimental effects transition towards progressively improved states from baseline. In the context of CPZ treatment, the time frame under investigation is the first 6 weeks, during which time rapamycin effectively prevents remyelination attempts in the presence of an acute demyelinating event. Therefore the dual CPZ/rapamycin provides a novel model of reversible demyelination that can be used to develop new therapies to promote remyelination.

RAPAMYCIN: INHIBITOR OF ACTIVE REMYELINATION

A major signaling pathway regulating myelination is the Akt/mTOR pathway. Chronic inhibition of this pathway in young wild type (WT) mice leads to reduction of myelination (Narayanan et al., 2009). The reduction in myelination that immediately follows inhibition is not recorded in WT mice after six to twelve weeks of age, demonstrating that in normal mice

myelination is no longer controlled by this major signaling pathway around this age. This suggests that remyelination is regulated by the Akt/mTOR pathway in adult mice (Narayanan et al., 2009).

It has been suggested that Akt signaling is a regulator of the amount of myelin generated per oligodendrocyte and has little control of the number of OLGs and OPCs (Flores et al., 2009). Hence, it is reasonable to hypothesize that with the use of rapamycin there would be a significant reduction in the translation of myelin mRNAs such as, MBP, MOG, and myelin associated glycoprotein (MAG). Inhibition of mTOR signaling by rapamycin has been effective in the inhibition of Akt/mTOR in studies using mouse models of MS *in vivo* (Narayanan et al., 2009).

More importantly, inhibitors to mTOR signaling have been previously used in many cancer therapies and neurological disorders (Narayanan, 2009). As discussed earlier, recent studies have suggested that the use of CPZ/rapamycin yields greater demyelination due to rapamycin's ability to inhibit the ongoing OPC's attempt to remyelinate during the demyelination phase (Sachs et al., 2014). The combined use of both components allows for more complete investigation of the remyelination process and analysis of clinically reflective therapeutics from the elongated remyelination phase. Remyelination in adult mice is controlled by the Akt/mTOR pathway and continuous expression allows for maintenance of OLGs (Flores *et al*. 2000). This pathway of activation is exhibited in Figure 1 below. OLGs are also more sensitive to rapamycin during active myelination (Narayanan et al., 2009). Long-term rapamycin treatment alone promotes longevity by inhibiting the mTOR pathway, but short-term inhibition of this pathway also inhibits OPCs from differentiating into OLGs. It would be reasonable to hypothesize that the use of rapamycin would inhibit the Akt/mTOR signaling pathway, leading to inactive differentiation of OPCs and an overall reduction in myelin protein translation.
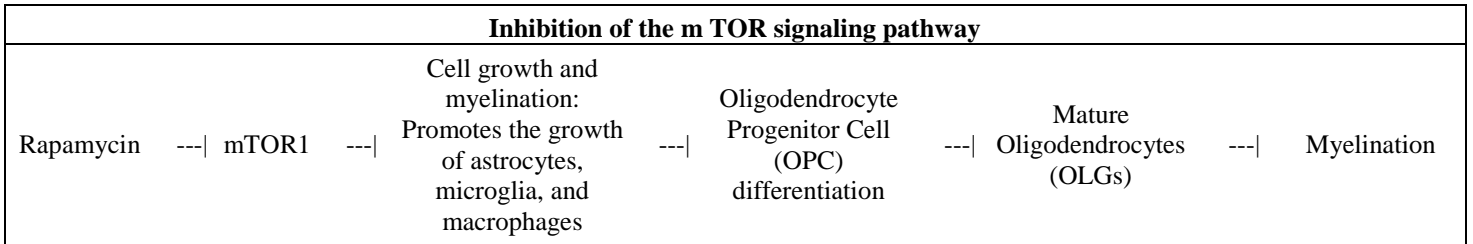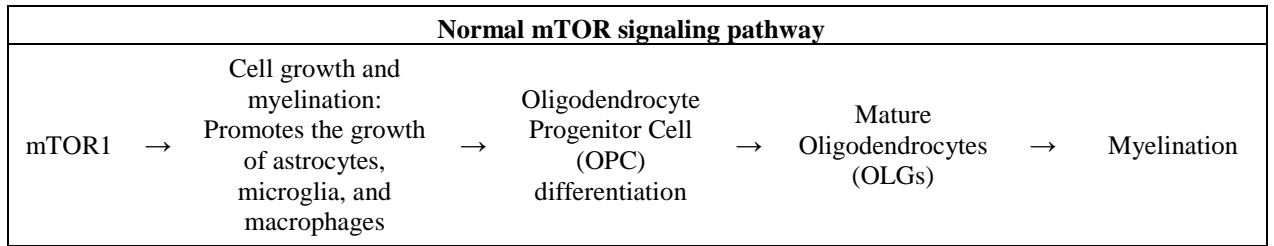
Normal mTOR signaling pathway							
mTOR1	→	Cell growth and myelination: Promotes the growth of astrocytes, microglia, and macrophages	→	Oligodendrocyte Progenitor Cell (OPC) differentiation	→	Mature Oligodendrocytes (OLGs)	→	Myelination

Inhibition of the m TOR signaling pathway									
Rapamycin	---|	mTOR1	---|	Cell growth and myelination: Promotes the growth of astrocytes, microglia, and macrophages	---|	Oligodendrocyte Progenitor Cell (OPC) differentiation	---|	Mature Oligodendrocytes (OLGs)	---| Myelination

Figure 1: Exhibits the mTOR signaling pathway with and without rapamycin inhibition.

DUAL TREATMENT: CUPRIZONE AND RAPAMYCIN

CPZ treatment alone exhibits non-immune mediated demyelination with continual remyelination. Rapid remyelination ensues immediately after treatment is discontinued, with substantial remyelination after 6 weeks of treatment (Matsushima et al., 2001). Ongoing and rapid remyelination following termination is promoted by the progressing OPC surge that is continuously attempting to differentiate into mature OLGs. The OPC surge coincides with an infiltration of microglia, macrophages, and astrocytes, with maximum demyelination at 4-5 weeks (Praet et al., 2014; Matsushima et al., 2001).

In the assessment of models aiming to understand therapeutics, it would be most beneficial to completely separate the two phases of myelination into solely demyelination, followed by remyelination. From our current understanding of rapamycin, this may in fact be possible. Utilization of rapamycin in conjunction CPZ would prevent ongoing remyelination characteristically seen with CPZ, therefore allowing greater demyelination. Inhibition of the differentiating OPC pool would also yield slower remyelination once treatment is removed.

While the CPZ and EAE animal models can mimic the demyelinating effects of MS, the combined CPZ/rapamycin treatment would allow for better understanding of therapeutics due to the ability to separate the demyelination and remyelination phases.

A recent study by Sachs et al., 2014 utilized rapamycin treatment during the 6 week CPZ induced demyelination phase. Investigators of the study used 0.3% CPZ in combination with IP injections 5 days a week, which consisted of rapamycin (10mg/kg) dissolved in a vehicle solution. The results of the study showed that the subjects experienced greater demyelination and an elongated window of remyelination compared to the CPZ and EAE models. This remyelination window provides an immense benefit for future therapeutic assessments (Sachs et al., 2014).

In conclusion, with the use of both compounds, Akt/mTOR signaling is successfully inhibited, therefore suppressing any attempt of OPCs to differentiate. Once CPZ treatment is halted, mitochondria regain normal morphology and function (Praet et al., 2014).

DUAL TREATMENT: MACROPHAGE/MICROGLIAL RESPONSE

MS pathology is characterized by the presence of a leaky blood-brain-barrier (BBB), allowing for the infiltration of inflammatory cells, including self-reactive T-cells. In contrast to this infiltrated immune response, demyelination induced via CPZ treatment occurs in the presence of an intact BBB and without substantial immune response involving B and T cells. This is confirmed by the study by Praet et al., (2014) in which RAG-/- mice (devoid of B and T cells) exhibit similar demyelination to WT mice, confirming lack of B and T cell involvement during CPZ treatment.

Therefore, the only immune cells present in the demyelinated regions are resident microglia and macrophages. The BBB does not permit their transport and the source of their infiltration is unknown (Matsushima & Morell, 2001). The exact contribution may vary during demyelination and remyelination. Their respective roles during de-/remyelination is not fully understood. Previous studies have shown that they are found in the highest concentration within the lesions themselves, and this localization could be a strategy to aid in remyelination, as one of their primary roles is to clean myelin debris.

Interestingly, around 3 weeks of CPZ treatment an elevated infiltration of microglia/macrophages coincides with an increase in OPCs and astrocytes (Praet et al., 2014). Perhaps it would be accurate to propose that rapamycin supplementation could be preventing OPC differentiation via inhibition of infiltrating microglia/macrophages.

Xie et al., (2014) tested this theory by injecting rapamycin into the lateral ventricles of rats, 6 h after a focal stroke was induced. In this study, the goal was to understand the role of mTOR signaling in post-stroke neuroinflammation. Results from the study revealed that IP treatment inhibited polarization toward pro-inflammatory macrophages (M1) and shifted the immune response towards anti-inflammatory macrophages (M2). However, this shift towards M2 macrophages is not a direct effect of rapamycin. The shift towards M2 macrophages was further amplified by a surge of T_{REG} cells, which indicated that IP rapamycin elevates anti-inflammatory T_{REG} cells before proceeding to shift the microglia/macrophages towards M2 anti-inflammatory response. These findings are further confirmed by the macrophage and microglial M1 response via T_{REG} devoid cells.

At 3 weeks of CPZ treatment, remyelination is ongoing as the OPC surge coincides with microglia/macrophage infiltration. However, CPZ treatment supplemented with IP injections of

rapamycin may hinder OPC differentiation, providing greater demyelination compared to CPZ treatment alone.

Furthermore, the role of T_{REG} cells is confirmed by a recent study that enriched units of whole blood from MS patients with $CD25^+$ cells. The cells cultured with expanded with rapamycin exhibited strong suppressor activity with a 91.4% increase in $CD4^+$ cells. These findings revealed that an addition of rapamycin could be potentially applied clinically, as these cultures exhibited increased purity and potency of cells with the phenotype and function of T_{REG} cells (Keever-taylor et al., 2007). However, it should be noted that the sample size included only 3 MS patient's blood samples, which has limited applicability to a broader range of patients and *in-vivo* applications.

DUAL TREATMENT: ASTROCYTES

Astrocytes function within brain homeostasis and immunity. As the most abundant cell in the CNS, their primary role is to support and regulate neural tissue (Mayo et al., 2012). As discussed earlier, CPZ treatment is accompanied by an intact BBB. In the attempt to model MS, the maintenance of the BBB fails to reflect the immune component of the demyelinating disease. While this helps to separate events related to demyelination and remyelination, it also bypasses considerable immune complexities that are clinically and therapeutically relevant (Matsushima & Morell, 2001).

Astrocytes help to maintain the BBB and their activation is associated with demyelinating disorders. Chemokines secreted from astrocytes attract immune cells from blood's peripheral immunity and resident CNS glial cells. The peripheral immune cells include T cells, monocytes, and dendritic cells; resident CNS cells include microglia and OPCs (Mayo et al., 2012).

CPZ treatment, utilizing C57BL/6 mice exhibits gliosis around 3 weeks, with a peak surge around 4-6 weeks. This gliosis is related to the increased number of microglial/macrophages and astrocytes (Gudi et al., 2014). These findings are congruent with work by Komely et al., (1992) in which mice treated for 8 weeks with CPZ exhibited IGF-1 positive astrocytes. A major function of microglia is to stimulate astrocytes to increase the release of chemokines to promote remyelination. After 3 weeks CPZ treatment, OPCs are recruited and differentiate into OLGs. Studies have suggested that this ongoing remyelination may be indirectly facilitated by microglial and astrocytic release of IGF-1.

Within the CPZ model, OPCs are continually attempting to differentiate into OLGs via astrocytes/microglial stimulation. Rapamycin treatment alone exhibits an opposing reaction. Intraperitoneal injections (IP) of rapamycin to post-stroke rats revealed that reactive astrocytes were inhibited and neurons were protected post-stroke (Xie et al., 2014). Previous studies have exhibited similar findings, in which rapamycin completely inhibited mTOR phosphorylation (Lisi et al., 2011). Astrocyte activation is dependent on the mTOR signaling pathway and is sensitive to inhibition via rapamycin (Xie et al., 2014). Furthermore, inhibition of astrocytes via rapamycin could be a possible route of increased demyelination associated with CPZ/rapamycin administration. A reduction in astrocyte recruitment would lead to a reduction in microglia and OPC recruitment (Mayo et al., 2012). Therefore, rapamycin may function to inhibit astrocyte recruitment, which would additionally inhibit OPC differentiation and result in more complete demyelination when combined with CPZ administration

DUAL TREATMENT: MAST CELLS

Mast cells are derived from bone marrow and reside in the CNS where they proliferate in response to various cytokines and growth factors. Interestingly, these cells are directly observed within regions of demyelination. (Skaper et al., 2012). Phosphoinositide 3-kinase (PI3K) signaling regulates mast cell production and homeostasis (Kim et al., 2008). PI3K activates mTORC1 signaling, furthering mast cell development and cell growth associated with the mTOR pathway.

Previous studies have also suggested that mast cells can rapidly cross and diminish the integrity of the BBB further promoting infiltration of immune cells (Mayo et al., 2012). Such findings are confirmed by EAE immune mediated demyelination, in which organisms devoid of mast cells exhibit decreased BBB permeability. Inhibition of mTORC1 yields reduced cell growth and mast cell infiltration. Rapamycin used in conjunction with CPZ inhibits PI3K signaling towards mast cell development via indirect inhibition of mTORC1 (Kim et al., 2008).

DUAL TREATMENT: INSULIN-LIKE GROWTH FACTOR-1

An increase in OPCs and microglia/macrophages at 3 weeks of CPZ treatment coincides with an increase in insulin-like growth factor-1 (IGF-1) (Komoly et al., 1992). In order to release IGF-1, there must be a supportive environment allowing for the remyelination to begin. It comes as no surprise, due to their involvement at other stages, that microglia and astrocytes are responsible for OPC recruitment (Matsushima & Morell, 2001). Previous studies exhibited similar findings, in which mice treated with CPZ for 8 weeks contained IGF-1 positive astrocytes (Komoly et al., 1992).

Activation of mTOR1 is promoted by IGF-1, leading to an increase in mRNA translation and protein synthesis via ribosomal subunit 6S (Johnson et al, 2013; Gudi et al., 2014). As discussed earlier, active myelination is dependent on the Akt/mTOR pathway (Narayanan, 2009), which can be modulated via rapamycin supplementation. OPC differentiation during CPZ treatment can be inhibited by rapamycin, yielding more complete demyelination during CPZ induced demyelination.

BENEFITS OF UTILIZING THE DUAL TREATMENT

In previous trials, rapamycin has been introduced to the organism orally. However, recent studies have utilized IP injections of rapamycin to mimic the pathophysiology of MS. Recent studies by Sachs et al, (2014) and by Narayanan et al, (2009) introduced the dual CPZ/rapamycin treatment. Both groups administered 0.3% cuprizone and IP injections of rapamycin to eight-week old C57BL/6J male mice.

After only 6 weeks of CPZ/rapamycin treatment, there is significantly more demyelination compared to CPZ and EAE models, with the highest proportion of demyelination occurring in caudal regions of the CC, hippocampus, and superior cerebellar peduncles. It would be appropriate to hypothesize that effective inhibition of the Akt/mTOR pathway was achieved. This claim can be confirmed via inactivation of the mTOR pathway following in-vivo treatment utilizing rapamycin. After 4 weeks of treatment MOG reactivity was similar in both the CPZ group and the CPZ/rapamycin group. Increased reactivity of MOG correlates with remyelination, as it is a glycoprotein important in the myelination process (Sachs et al., 2014). However, by 6 weeks of treatment MOG reactivity was barely detectable (4%) in the CPZ/Rapamycin and therefore was significantly more reduced in the CPZ/Rapamycin group, this demonstrates that

administration of rapamycin during CPZ treatment reduces ongoing OPC differentiation into mature OLGs. Inhibition of differentiation is important because it allows complete separation of both de-/remyelination phases, which provides a clearer route to assess whether certain therapeutics are effecting demyelination and/or the remyelination phase. Tagge et al., (2016) demonstrated that CPZ induced demyelination yields complex spatio-temporal patterns of de-/remyelination in the CC. Although the mechanisms behind variable demyelination throughout the CC are unknown, it is possible that certain regions are either less susceptible to demyelination, or are repaired more quickly than others. A model, such as CPZ/rapamycin may offer reliable patterns of pathology that would be beneficial in providing a more complete understanding of the biological processes responsible for demyelination and repair that will ultimately help identify and study new therapeutic approaches.

Remyelination is significantly slowed following removal of the dual treatment, whereas after CPZ administration, remyelination of axons within the corpus callosum is evident one week after termination of treatment. Maximum remyelination was recorded after five weeks of recovery in CPZ-only mice. In contrast, the CPZ/rapamycin treated mice had significantly less remyelination compared to CPZ-only mice after 5 weeks of recovery. To build upon this claim, the CPZ/rapamycin group was comparably slower in the attempt to remyelinate compared to the control group. However, this value increased slowly. Towards the end of 7 weeks off treatment, the CPZ/rapamycin mice still had significantly less myelination compared to the CPZ-treatment group. (Sachs et al., 2014).

The promotion of demyelination and slowed remyelination provided by rapamycin can be attributed to its ability to inhibit any immune or neuroglial cues that would normally lead to remyelination. These include the ability to inhibit microglia and macrophage infiltration

associated with CPZ treatment alone. As discussed earlier, an OPC surge coincides with microglia and macrophage levels; however rapamycin utilizes T_{REG} cells by polarizing microglia and macrophages towards M2 anti-inflammatory response. Research by Keever-Taylor et al., (2007) provided evidence that rapamycin cultured with peripheral blood samples of MS patients selectively inhibits auto-reactive T-cells and spares protective T_{REG} cells. It may be possible that while rapamycin treatment alone is exhibiting an anti-inflammatory response, the slowed remyelination associated with dual in mice could be an indirect effect of rapamycin selectively sparing protective T_{REG}.

Along with selectivity of T_{REG} cells, rapamycin is able to inhibit astrocyte recruitment during CPZ-treatment. Normally, astrocytic migration is followed by infiltration of microglia and OPCs. Inhibition of astrocytes, microglia, and OPCs migration suggest that rapamycin successfully inhibits any attempt of ongoing remyelination during CPZ-treatment, further providing ample demyelination compared to the CPZ-treatment group. Figure 2 highlights the fluctuating cellular interactions during CPZ treatment. Note that the astrocytes and OPC surges at week 4 coincide. Astrocytes are dependent on the Akt/mTOR pathway; following inhibition via rapamycin I expect to see a coinciding reduction of OPCs, resulting in a decline of new mature OLGs.
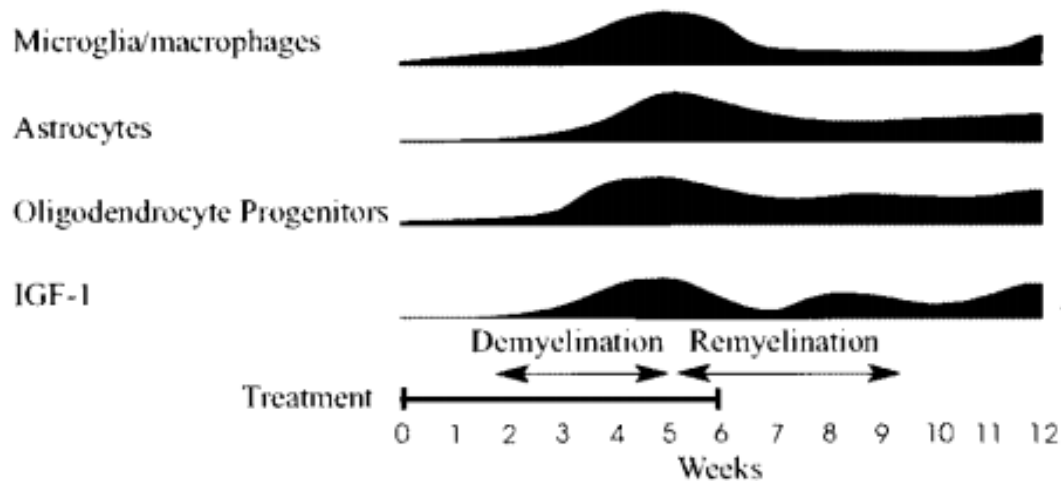
Figure 2: Summary of cellular trends during acute demyelination, followed by removal from CPZ treatment. Diagram was adapted from Mason et al., 2000.

The CPZ model exhibits incomplete demyelination and ongoing remyelination during CPZ-administration. Following CPZ/rapamycin treatment, there is a decrease in expression of MAGs, OPC differentiation into mature OLGs, and a slowed remyelination phase. The changes accompanied by rapamycin treatment allow for the assessment of separate demyelination and remyelination phases. The ability of CPZ/rapamycin to significantly demyelinate specific regions of the CC yields a model to investigate remyelinating therapeutics. Figure 3 outlines a treatment timeline utilizing CPZ compared to the benefit of the proposed CPZ/rapamycin treatment.

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Cuprizone Treatment (0.2%)	Week	Current Understanding of Cuprizone Treatment (0.2%) + Rapamycin
Microglia and Macrophage levels increase	1	Rapamycin inhibits the surge of Microglia, Macrophage, IGF-1, Astrocytes
	2	
Microglia and Macrophage levels increase, myelin genes for MBP, MAG, CAG reduced	3	
Microglia, Macrophage, IGF-1, Astrocytes surge	4	
Complete loss of mature OLGs, Remyelination ~50%	5	
Remyelination ~ 90%	6	MOG activity declined, significantly greater demyelination, little-to-no-remyelination
Microglia, Macrophage, IGF-1, Astrocytes levels decline	7	Remyelination ~20%
	8	
	9	
	10	
	11	
	12	
	13	Remyelination ~70%
	14	
	15	
	16	

Figure 3: Timeline of acute demyelination induced with CPZ versus CPZ in conjunction with rapamycin. Both treatments discontinued diets after 6 weeks. De-/remyelination was associated in the caudal regions of the corpus callosum (CC), hippocampus, and superior cerebellar peduncles, Axons were quantified with sections stained with para-phenylenedia-mine (PPD) (Sachs et al., 2014).

POTENTIAL COMPLICATIONS OF DUAL TREATMENT:

Possible considerations for rapamycin use would be the transient detrimental effects it has on metabolism. These effects are only seen during the first 6 weeks of treatment and include insulin resistance, hyperlipidemia, and glucose intolerance (Fang et al., 2013). Narayanan et al.,

(2009) noted that after 6 weeks of treatment there was a significant decrease in brain size and body weight. By the end of 6 weeks, a pivotal shift in the organism's state took place. By 20 weeks of IP rapamycin treatment, mice exhibited an improved metabolic profile and increased oxygen consumption compared to their baseline states (Fang et al., 2013).

CONCLUDING REMARKS

Utilization of the CPZ model during therapeutic assessment is confounded by continuous attempts of OPCs to differentiate into OLGs during demyelination. The dual CPZ/rapamycin model yields more complete demyelination and a slowed rate of remyelination. The significance of slowed remyelination is measurable, as CPZ treatment group yielded ample remyelination by 6 weeks of recovery. Contrariwise, the CPZ/rapamycin group exhibited significantly less remyelination 6 weeks into treatment removal. The ample demyelination associated with dual treatment offers a novel route to investigate remyelinating therapeutics that may target specific regions of the caudal CC and superior cerebellar peduncles.

Multiple Sclerosis is a disease characterized by demyelination and extensive remyelination. The proposed CPZ/rapamycin model for MS could be more reliable during the assessment of therapeutics, due to the significant demyelination that is largely reversible and the elongated spontaneous remyelination window. Such a model has yet to be utilized in investigations for MS therapeutics and there is substantial reason to believe that this model may provide a reliable model for reversible demyelination and spontaneous remyelination.

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