Continued development of azithromycin as a neuroprotective therapeutic for the treatment of spinal cord injury and other neurological conditions

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Spinal cord injury (SCI) induces a robust inflammatory response largely mediated by resident microglia and infiltrating macrophages across the blood-brain barrier. While these cell populations are capable of promoting repair and regenerative responses, in the days and weeks after SCI they predominately adopt pro-inflammatory profiles known to inhibit recovery and potentiate secondary injury pathways. Continued work is needed to develop clinically viable immunomodulatory therapeutics and promote pro-reparative macrophage responses. Recently we published on the therapeutic benefits of the macrolide antibiotic azithromycin (AZM), which improves locomotor and histological recovery after SCI in 3-month-old female mice (Kopper et al., 2019). Specifically, we initiated AZM beginning 30 minutes, 3, or 24 hours after injury and then daily for 7 days. AZM administration initiated at 30 minutes and 3 hours post-injury improved locomotor function as detected by an open field locomotor scale and significantly improved stepping frequency. The 24-hour time point, however, was ineffective suggesting the importance of early administration. Histologically we observed modest improvements with the 30-minute treatment time point with significantly reduced lesion length and evidence of slight increases in tissue sparing at the lesion epicenter. Previously, we observed that the same AZM dosing strategy after SCI reduces pro-inflammatory microglia and macrophage activation as determined by a diverse panel of inflammatory markers (Gensel et al., 2017). These neuroprotective findings are consistent with recent studies finding AZM to be therapeutically effective in multiple stroke models (Amantea et al., 2016b, 2019) a rat model of retinal ischemia/reperfusion injury (Zheng et al., 2007), and in a rat neonatal hypoxic-ischemic brain injury model (Barks et al., 2019). AZM is the most commonly prescribed antibiotic due in part to its safety profile and large therapeutic index (Durkin et al., 2018). Collectively, these studies highlight the potential for AZM to be developed into a safe, neuroprotective treatment for SCI and other neurological conditions. Here, we highlight additional areas of study that will facilitate the translation of AZM as a neuroprotective agent.

Extending the therapeutic window of AZM treatment would maximize its therapeutic development. Based upon studies in animal models of stroke and SCI, AZM remains effective if the initial dose is delayed up to 3 (oral) or 4.5 (intraperitoneal) hours after SCI and stroke, respectively, with earlier administration time points being most effective (Amantea et al., 2016a; Kopper et al., 2019). This may present a challenge for implementation in SCI. Indeed, the average time of acute methylprednisolone administration in a previous SCI clinical trial was between 8–9 hours after injury (Bracken et al., 1990). An extended therapeutic window may be achieved by investigating alternative routes of administration (e.g., intravenous or intrathecal-we used oral administration in our studies); improving dosing paradigms (initiation time point, concentrations, frequency, and duration) or developing more targeted delivery approaches (i.e., liposomal formations for targeted macrophage delivery). Similarly, continued work in medicinal chemistry holds the potential to improve AZM's therapeutic benefits and/or pharmacokinetics. In our previous work, we were able to introduce a series of changes to the molecular structure of AZM in which its antibiotic activity was reduced but the newly generated derivatives retained their immunomodulatory and neuroprotective effects (Zhang et al., 2019). Continued work in this area could identify a closely related drug with improved therapeutic efficacy. Collectively, given AZM's large therapeutic index and safety profile is it likely that optimization of AZM administration in animal models can be achieved to fully develop AZM as an effective therapeutic in SCI and other neurological conditions.

Insight into AZM's therapeutic mechanisms of action will further facilitate translation. For example, in our recent work we found that AZM did not prevent the overall development of neuropathic pain over the course of 28 days (Kopper et al., 2019), however, previously we found that AZM has analgesic properties when administered 30 minutes prior to pain testing at chronic time points when neuropathic pain (heat hypersensitivity) is already established (Gensel et al., 2019). Microglial activation in the lumbar spinal cord is implicated in chronic pain after SCI yet acute monocyte-derived macrophage infiltration after injury is postulated to contribute to neurodegeneration. The current body of *in vivo* literature has utilized systemic administrations of AZM targeting both microglia, infiltrating macrophages, and likely other cell types yet to be examined. Currently, the relative contribution of each of these cell populations to the therapeutic effects of AZM is unknown, however, it is possible that AZM's effects are localized to one population. Further investigations into the cell-specific effects of AZM may improve therapeutic strategies and the relatively recent introduction of microglia-specific antibodies provides new tools to probe these questions.

As we continue to optimize immunomodulatory therapies for SCI, it is important that we prepare for the logistical challenges of clinical implementation. AZM has a unique benefit among the many promising drug candidates for SCI in that AZM is already Food and Drug Administration approved and heavily utilized by the general population (Durkin et al., 2018). While these properties ease the barrier for human treatment, discontinuities in outcome measures of efficacy in animals models and humans may confound our ability to determine the therapeutic properties of AZM treatment in a clinical setting. In a clinical application there are uncertainties as to what would be the best marker of efficacy in humans. Analyses targeting blood or cerebrospinal fluid would presumably be the first choices given their clinical availability and non-invasive nature, however, the best analyte or cellular outcome to detect efficacy is unknown. Similarly, imaging techniques such as MRI are frequently utilized after SCI, and thus could be a useful tool in quantifying any therapeutic effects. As such, ongoing animal studies involving AZM should begin to test and incorporate blood, cerebrospinal fluid, and/or live animal imaging in order to determine the best approaches to detect AZM's therapeutic efficacy in humans. Fortunately, new SCIs are relatively rare in the United States; however, this may produce a logistical problem in the optimization of biomarkers of efficacy. In this scenario, the SCI research community could benefit from collaborating with the larger stroke field when determining the

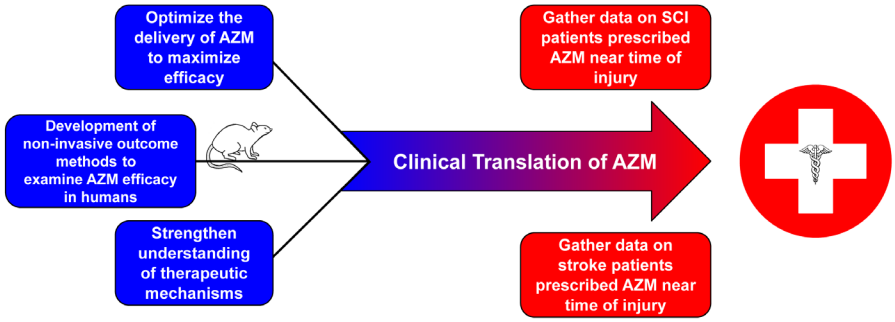

Figure 1 | **Important steps in repurposing of AZM as a neurotherapeutic agent.**
Additional work in animal models is needed to both improve AZM's efficacy and develop outcome measures feasible for use in non-invasive human studies. Application of these outcome measures in the patient population already taking AZM near time of injury could provide key evidence for the transition of AZM into clinical trials and use as a therapeutic for SCI. AZM: Azithromycin; SCI: spinal cord injury.

most useful therapeutic indicators. Once collected, any data indicating therapeutic and anti-inflammatory activities in the patient population would then serve as the final push likely needed to initiate full-scale clinical trials in humans.

Initial clinical studies utilizing AZM should certainly investigate potential improvements in the American Spinal Injury Association Impairment Scale impairment scale, however, this outcome would likely prove to be a difficult threshold to detect efficacy. Given the modest effects of AZM treatment, patient and injury variability during this early stage of investigation would likely make a statistically significant shift in American Spinal Injury Association Impairment Scale grades unlikely. Even in a larger-scale clinical trial, this may still prove to be too demanding of a therapeutic threshold to detect efficacy. In contrast, rodent models of SCI research regularly utilize locomotor recovery as a primary outcome using highly consistent injuries and optimized testing paradigms capable of detecting treatments with smaller effect sizes. The discontinuities between animal and clinical research were recently highlighted at "SCI 2020: Launching a Decade for Disruption in Spinal Cord Injury Research", a meeting hosted by the National Institute of Neurological Disorders and Stroke. Clinicians, individuals with SCI, and researchers at the meeting emphasized the importance of incorporating clinically relevant outcomes such as bladder, bowel, and sexual function, as well as, neuropathic pain and autonomic dysreflexia both into basic research and when determining therapeutic efficacy in the clinic. In our recent works, we investigated the ability of AZM to either prevent or suppress neuropathic pain (Gensel et al., 2019; Kopper et al., 2019), however, AZM's impacts on these other important clinical outcomes are currently unknown. Therefore, continued research into AZM's impact on other key outcomes in animal models could highlight specific measures to consider when designing clinical studies.

As an Food and Drug Administration approved drug with an excellent safety history, AZM holds great promise as a therapeutic to treat SCI and other neuroinflammatory conditions. Although the current research in animal models of neurological conditions is promising, the clinical variability in the human population and AZM's relatively modest effect size will likely become a challenge in detecting efficacy. To address this, as summarized in **Figure 1**, we need to better understand AZM's underlying mechanisms, improve its efficacy by optimizing dosing paradigms, and begin developing approaches to detect therapeutic effects non-invasively in humans. Once these challenges are overcome AZM will have greatly improved chances of moving towards successful clinical implementation as a neuroprotective treatment.

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