Selective Sema3A inhibitor SM-216289 promotes axon regeneration and functional recovery in spinal cord injury

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Spinal cord injury (SCI) is a life-altering condition that is especially difficult to heal due to the genetic makeup of the central nervous system (CNS) and inhibitory molecules present in SCI lesion sites. A variety of proteins in SCI lesion sites transduce inhibitory signals, preventing neurite outgrowth. One of these proteins is semaphorin 3A (Sema3A), which collapses growth cone expansion by dissembling actin networks. Xanthofulvin (SM-216289), a drug cultured from Penicillium, has been identified as a selective Sema3A inhibitor that blocks Sema3A from binding to its receptors. Therefore, it is suspected to inhibit growth cone collapse in a pharmaceutical setting. This review investigates the efficacy of SM-216289 in promoting axon regeneration and functional recovery in vitro and in vivo for SCI, specifically, by analyzing a study conducted by Kaneko and colleagues in 2006. There is convincing evidence that SM-216289 is efficacious in inhibiting growth cone collapse and enhancing functional recovery, as enhanced upregulation of growth-associated proteins, migration of Schwann cells, and reestablishment of hindlimb control were observed in SM-216289 treated rats. However, further research must be conducted regarding the drug's integration with other therapeutic strategies.

I. INTRODUCTION

Spinal cord injury (SCI) is damage to the spinal cord and interrupts the mechanism of communication between the brain and body. This results in partial or complete loss of sensory and motor function below the site of injury [1]. Further, SCI can cause damage to spinal cord tracts such as the corticospinal tract (CST) and serotonergic raphespinal tract (SRT). The CST plays an important role in motor function and when lesioned, incites ipsilateral paralysis among other conditions [2]. The SRT is a crucial aspect of serotonin signaling that allows the neurotransmitter to control locomotion, so injury to this tract results in limited limb control [3].

SCI is a prevalent and serious form of injury, with the USA alone accumulating 18,000 new cases per year on top of the approximately 295,000 pre-existing ones [4]. Additionally, lifetime SCI expenses for each patient in the USA range from $1,200,000 to $5,000,000 [4]. As SCI cases remain at this growth rate and cost, it's imperative that efficacious therapies be instituted–especially as SCI significantly decreases patients' quality of life [5].

However, the limited regenerative capacity of the adult mammalian central nervous system (CNS) has prevented the emergence of a therapeutic intervention for centuries. Inhibitory signaling and the lack of regeneration-associated genes (RAGs) in CNS neurons are the main mechanisms by which axon regeneration is suppressed (glial and fibrotic scar tissue surrounding the lesion site also acts as a physical barrier for regeneration in the CNS) [6]. Proteins such as tenascin-C, chondroitin sulfate proteoglycans (CSPGs), Nogo-A, myelin-associated glycoprotein (MAG), oligodendrocyte myelin glycoprotein (OMgp), and semaphorin 3A (Sema3A) have associations with such inhibitory signaling [7].

Neutralizing inhibitory molecules has proven to be a promising avenue for SCI treatments. For instance, nogo proteins found at SCI lesion sites have been neutralized *in vivo* using the function-blocking antibody IN-1, which directly inhibits Nogo-A. This has produced results including neurite outgrowth of up to 11mm caudal to the lesion site as well as regained dexterity and fine motor movement control in primates [1,8,9].

Similar to these therapies, the drug xanthofulvin (SM-216289) has been suggested to be a highly selective Sema3A inhibitor capable of inducing axon regeneration [10,11]. Sema3A is an especially important molecule to investigate in SCI treatment research because of its close interactions with and potential upregulation of other inhibitory proteins such as CSPGs [12,13]. Further, it is expressed at elevated levels in the CNS, whereas CSPGs are expressed in various tissues throughout the body; thus, Sema3A inhibition has the potential to have a widespread therapeutic effect.

Sema3A is a chemorepulsive protein that is part of the class-III semaphorin family. Semaphorins are the largest family of axonal guidance and directional growth molecules, subdivided into eight classes [14]. Semaphorins contribute to the development of both the nervous and cardiovascular systems, and class-III semaphorins have been specifically noted to contribute to immunity, tumor progression, angiogenesis, and lymphangiogenesis [14,15]. Though some semaphorins are membrane-bound, class-IIIs are mainly secreted by fibroblasts [15]. Sema3A has been shown to collapse growth cone expansion in SCI due to its chemorepulsive properties, hence its original name, "collapsin" [16]. This suggests that inhibiting Sema3A from transducing its inhibitory signals may be a promising approach for axon regeneration in SCI.

This review aims to investigate the role of SM-216289 as an inhibitor for Sema3A to come to a conclusion on its efficacy and prospect as a treatment for spinal cord injury. To help reach this conclusion, a 2006 exploratory study by Kaneko and colleagues will be summarized [11].

II. SEMA3A SIGNALING AND IMPACT ON REGENERATION

i. Sema3A

Sema3A is a protein that guides the direction of axonal growth by chemorepulsion. Sema3A signaling disassembles actin networks, resulting in microtubule retraction and collapse [17]. This property of Sema3A makes its signaling a crucial component of neural growth and angiogenesis [18]. The specific repulsiveness of Sema3A during embryonic development is regulated by its cleavage patterns and binding sites [14]. Sema3A is upregulated during neural development and diminishes with neural maturation; however, its expression levels can be heightened as a result of traumatic injuries like SCI.

ii. Sema3A Signaling

Sema3A must bind to a transmembrane neuropilin-plexin complex in order to initiate signal transduction. Like all semaphorins, Sema3A contains an N-terminal sema domain that regulates receptor interactions. Unlike most semaphorins, class III semaphorins cannot adequately bind to plexins alone, hence the necessity for neuropilins. There are nine types of neuropilins, but Sema3A exclusively binds to NP-1 [15]; the specificity of class IIIs is suggested to be determined by the presence of a Lys497 sidechain on sema proteins [14]. Disulfide bonds between Sema3A and NP-1 stabilize the structure of the complex, though all components interact at several binding sites. Sema3A and NP-1 bind specifically to class A plexins, most commonly plexinA4. This receptor complex consisting of one neuropilin and one plexin allows for Sema3A to conduct intracellular signaling.

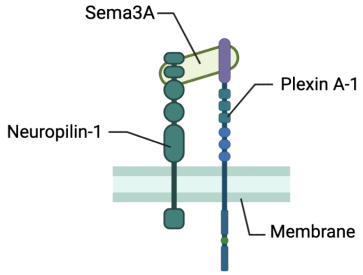

Figure 1: Sema3A/NP-1/P-A1 membrane complex. Created using BioRender.

iii. Sema3A in SCI Lesion Sites

Sema3A signaling in lesion sites collapses growth cones at the tip of the expanding axons of neurons, preventing regeneration and thus functional recovery in SCI. The protein's signaling disassembles actin networks, resulting in microtubule retraction and collapse [17]. Sema3A has been shown to exist in lesion sites for up to 4 weeks post-injury, peaking in upregulation between 1-2 weeks [11]. This suggests that Sema3A must be targeted expediently following injury. SEMA3A induction has been shown to take place at the lesion site of SCI and its respective mRNA is mainly found at the epicenter of the glial scar; thus, Sema3A binds to neurons at this site [11,19].

iv. Sema3A Impact on Remyelination

Additionally, Sema3A has been demonstrated to not only affect neurons but to also have repulsive effects on Schwann cell migration [11]. Oligodendrocyte breakdown is subsequential to axotomy, which limits neurons' abilities to conduct signals; myelination, the crucial role that oligodendrocytes take on, allows action potentials to reach the synapse [20]. Further, oligodendrocytes do not have the capacity to regenerate [21]. Their breakdown results in the release of myelin-associated inhibitory proteins such as Nogo-A, MAG, and OMgp [21]. Because of the proximity of the CNS and PNS in SCI, Schwann cell migration to lesion sites is a plausible solution to achieve remyelination. In one experiment by Kaneko and colleagues, Schwann cells were cultured in a Sema3A-Fc fusion stripe assay, which measured the growth decisions of Schwann cells in response to a Sema3A-populated stripe [11]. The study found that Schwann cells tended to avoid Sema3A-populated areas, suggesting their sensitivity to the presence of the protein. Thus, in addition to affecting axon regeneration by inducing growth cone collapse, it seems that Sema3A limits remyelination, a crucial aspect of axon regeneration and functional recovery, in SCI by repulsing Schwann cells.

III. SM-216289 CHARACTERIZATION AND EFFICACY IN VITRO AND IN VIVO

i. SM-216289 Pharmacological Profile

SM-21629 is a selective Sema3A inhibitor found in the fermented broth of *Penicillium* sp. SPF-3059 [10]. The compound has been shown to inhibit Sema3A by binding to it, whereas other Sema3A inhibitors such as lavendustin A and olomousine operate post-receptor [10,11,22].

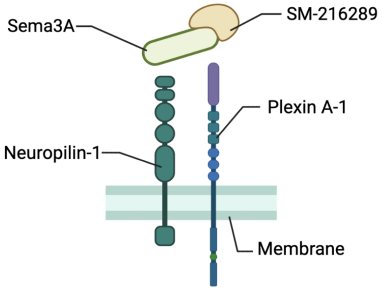

Figure 2: SM-216289 binding to Sema3A inhibits the creation of a Sema3A/NP-1/P-A1 membrane complex. Created using BioRender.

In a study by Kikuchi and colleagues, a collagen co-culture with Sema3A-expressing COS7 cells and a DRG explant found that Sema3A mRNA and protein expression was not impacted by SM-216289 administration [10]. This suggests that the inhibition conducted by SM-216289 alters Sema3A activity. Additionally, an experiment by Kaneko [11] tested the specificity of SM-216289. HEK293T cells were transfected with plasmids set to express Nogo-A, MAG, OMgp, Sema3B, and Sema3F. SM-216289 was cultured in a DRG explant with Nogo-A, MAG, and OMgp to investigate whether it inhibited other proteins associated with regeneration. Likewise, the molecule was cultured with Sema3B and Sema3F to investigate whether it inhibited other class-IIIs, specifically those present in the sympathetic ganglia. SM-216289 was unable to prevent growth cone collapse initiated by Nogo-A, MAG, and OMgp, and showed no effect on Sema3B and Sema3F, suggesting that it is a highly specific inhibitor for Sema3A.

Kaneko and colleagues have also examined the impact of SM-216289 on intracellular signaling by measuring cell growth in growth assays. Non-neuronal KB cells were utilized to assess whether SM-216289 would impact intracellular signaling in a general context. The growth of these cells is dependent on epidermal growth factor, a protein that can be inhibited by gefitinib. KB cells were incubated with either SM-216289 or gefitinib and after 3 days, gefitinib inhibited cell growth as expected. SM-216289 also exhibited significant cell growth inhibition, but only when concentrations were higher than those required for the inhibition of Sema3A *in vitro*. Kaneko and colleagues were able to determine that SM-216289 caused an insignificant change in existing signaling pathways through this analysis of growth, further solidifying its role as a highly-specific Sema3A inhibitor.

ii. Evidence From Kaneko et al., 2006

In addition to the previously described experiments, Kaneko and colleagues have used a wide variety of *in vitro* and *in vivo* SCI models to examine whether Sema3A neutralization by SM-216289 promotes axon regeneration following SCI. The *in vivo* models featured complete transection injuries; while complete SCIs made up approximately 32.5% of SCI cases since 2015, this model offers the benefits of replicability and reliability [4].

SM-216289 application in neuronal cultures:

Two other prominent compounds for inhibiting Sema3A-induced growth cone collapse have been identified: lavendustin A and olomoucine, tyrosine kinase and Cdk inhibitors, respectively. As previously mentioned, these inhibitors act intracellularly as opposed to directly on Sema3A. SM-216289, lavendustin A, and olomoucine were identified as the three most promising Sema3A inhibitors from a screening of over 140,000 compounds. To compare these inhibitors, growth cone collapse assays using DRG explants were performed, and SM-216289 exhibited much stronger inhibition. Further, lavendustin A and olomoucine were demonstrated to be toxic at higher concentrations, implying that SM-216289 was the safest and strongest known Sema3A inhibitor at the time of investigation.

SM-21629 application in SCI:

Kaneko and colleagues conducted several *in vivo* experiments to assess the efficacy of SM-216289 in SCI models. In one experiment investigating the drug's relationship with extracellular matrix proteins, adult rats were administered SM-216289 through an osmotic mini-pump across the span of 4 weeks. Immunohistochemical analyses performed 14 weeks after injury found that both neurofilament and growth-associated protein 43 (GAP43) had been significantly upregulated in SM-216289-treated rats. The results suggest that the presence of SM-216289 positively impacts the upregulation of proteins associated with axon growth and repair. However, another model demonstrated that rats treated with SM-216289 had fibronectin-positive scar tissue 2 weeks post-transection. Though this is typical of SCI scar formation, fibronectin promotes the growth of fibroblasts: the proteins that secrete Sema3A [23]. After 14 weeks, the same rats were positive for glial fibrillary acidic protein (GFAP), and wider expansion of fibronectin from the lesion epicenter was observed. This suggests that SM-216289 has no impact on the action of reactive astrocytes and fibroblasts, and thus the expansion of SCI scar tissue. Upon further investigation, peptidergic nociceptive C fibers, indicated to cause "abnormal [and debilitating] sensory functions," were present in significantly higher concentrations in SM-216289-treated rats compared to controls. The study suggests that C fiber content in lesion sites is generally minimal, and the upregulation of neurofilament and GAP43 in rats treated with SM-216289 acts as a protective factor.

In another model, the study tested the regeneration of axons in various spinal tracts including the CST and SRT. When spinal cords of rats were transected and treated with SM-216289, no regeneration was observed in the CST; however, GAP43 and NP-1 expressing serotonergic axons in the SRT crossed the lesion site in the SM-216289 condition, though overall regeneration was limited. This suggests that SM-216289 slightly enhances the regeneration of SRT axons; however, its inability to enhance CST regeneration limits the extent to which the drug may promote functional recovery, as the CST is essential in enabling voluntary movement.

SM-216289 and functional recovery:

Although CST regeneration was not observed in Kaneko's experiment following SM-216289 treatment, the

SRT regeneration observed was noted as a crucial factor in functional recovery. Through the administration of serotonin neurotoxin DHT, the study found that serotonergic regeneration in the SRT is responsible to some extent for functional recovery, which SM-216289 enhances.

As previously mentioned, remyelination is crucial to establishing functional recovery. It was found that rats treated with SM-216289 had significantly higher levels of Schwann cells at spinal cord lesion sites. This suggests that SM-216289 alleviates the repulsive effects of Sema3A on Schwann cells *in vivo*. This characteristic of SM-216289 is especially significant because peripheral myelin does not contain inhibitory myelin-associated proteins such as Nogo-A, MAG, and OMgp, and suggests protection against growth inhibition if faced with re-injury [7,24]. By enabling remyelination, SM-216289 allows for the recovery of functional action potential conduction in regenerated axons.

In addition to investigating certain aspects contributing to functional recovery, the study measured the functional recovery of rats using the Basso-Beattie-Bresnahan (BBB) locomotor rating scale. SM-216289-treated rats were shown to have significantly greater hindlimb control compared to controls who exhibited extremely limited control. The researchers confirmed that axon regeneration contributed to the difference in scores by retransecting rats 10 weeks after the initial transection. They found that the rats' scores dropped to 0 and remained low, demonstrating that previously seen recovery was not due to a compensatory response.

IV. DISCUSSION

There is convincing evidence to suggest that SM-216289 is an efficacious Sema3A inhibitor capable of enhancing axon regeneration and reinstating locomotor function in SCI. *In vitro* experiments by Kikuchi and colleagues in 2003 identified that SM-216289 selectively and continuously inhibits Sema3A, providing a strong basis for the study's investigation in 2006. Kaneko and colleagues demonstrated that SM-216289 administration to SCI lesion sites promotes functional recovery not only by inhibiting Sema3A signaling but also by encouraging GAP43 and neurofilament expression and Schwann cell migration to the lesion site. Further, some regeneration of the SRT was observed, though limited. While the study provides strong evidence for SM-216289 as an efficacious treatment for SCI, there are certain limitations of the treatment that must be addressed. While some regeneration was observed in the SRT, none was observed in the CST, an essential spinal tract that enables voluntary movement. Though functional recovery was observed in Kaneko and colleagues' study, it is imperative for a SCI treatment to facilitate CST axon regeneration for full functional recovery to be achieved. Further, Kaneko and colleagues found that lesion sites tested positive for GFAP and fibronectin, suggesting that SM-216289 may not act on these proteins. SM-216289 may be more effective if combined with treatments that mediate CST axon regeneration such as AAV-assisted co-expression of insulin-like growth factor 1 and osteopontin [25], the inhibition of GFAP and fibronectin, or treadmill therapy [26], and this is an area for future research.

The study notes the limitation that regenerated axons were not connecting to their appropriate targets. In 2014, Kaneko investigated a possible solution to this limitation by testing the efficacy of continuous treatment of a newer Sema3A inhibitor, vinaxanthone (SM-345431), in hindlimb motor function recovery [27]. SM-345431 is a natural product related to SM-216289, synthesized in a similar dimerization reaction and suggested to have similar regenerative effects on SCI neurite outgrowth [28]. In this study, SM-345431 was administered to lesioned rats along with treadmill therapy, shown to increase CST function [26], in hopes of observing the "rewiring" of regenerated axons. While this treatment delivery system resulted in increased axon regeneration and limited functional recovery, the main conclusion drawn by this study was centered on the importance of combining treatments for SCI. By combining pharmacotherapy with treadmill training, the study was able to observe enhanced levels of functional recovery compared to the use of SM-216289 alone. A more recent study, published by Ivakhnitskaia and colleagues in 2021, examined SM-345431 inhibition of Sema3A during neural growth in DRG models [29]. It was found that while enhancing embryonic neurite outgrowth, SM-345431 was ultimately unsuccessful in inhibiting Sema3A in adult PNS neurons. There was also evidence suggesting that SM-345431 does not always target Sema3A specifically, concluding that more research is needed to determine the efficacy of the treatment.

Another study took a different approach to Sema3A inhibition by blocking an intracellular protein, rho-associated protein kinase 2 (ROCK2).[30] ROCK2 is present in the CNS and unlike Sema3A, its expression increases with age [31]. The protein integrates inhibitory growth signals, contributing to the prevention of neurite outgrowth following injury [32]. Zhang and colleagues demonstrated that ROCK2 blockage in an optic crush attenuated the inhibitory effects of Sema3A.

There has been limited research into the therapeutic benefits of SM-216289 in SCI or any other condition since Kaneko's 2006 study; however, other methods of Sema3A inhibition have been investigated. It is important that researchers investigate various avenues for treatment because of the complexity and diversity of SCI; a wider scope of understanding offers valuable insight into

applicability. For example, Kaneko's 2014 study suggests that SM-345431 may be more applicable than SM-216289 in a clinical context because it demonstrates better stability in less invasive administration techniques [27]. In addition, successful intracellular inhibition of Sema3A signaling using ROCK2 suggests that Sema3A can be inhibited after binding to its receptors, not explicitly before binding like the mechanisms of SM-216289 and SM-345431, opening up possibilities for future investigation. Nevertheless, there is strong evidence suggesting SM-216289 to be efficacious, and further research should be conducted to investigate the drug in combination with other SCI treatments.

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