Tamoxifen and Src kinase inhibitors as neuroprotective/neuroregenerative drugs after spinal cord injury

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Introduction
Spinal cord injury (SCI) is a detrimental condition that affects mostly young adult individuals (National SCI Statistical Center, 2014). Patients with this condition are subject to drastic changes in their lifestyle, as well as economic stress. After the trauma to the cord, several events take place at the lesion epicenter and surrounding areas (rostral and caudal). These changes start from the first millisecond to months after the lesion, and this eventually lead to the clinical symptoms that are observed in the patient. Among these symptoms are the loss of body functions such as voluntary muscle movement rendering the patient paraplegic or tetraplegic (depending on the location of the injury), lack of somatosensory information, decreased breathing capacity, limited bowel and bladder control, and in some situations development of chronic pain.

SCI is initiated by the physical impact to the cord that triggers molecular and cellular changes immediately after injury resulting in an acute phase where necrosis, axotomy, damage to blood vessels, ischemia, excitotoxicity, increase in the production of free radicals and edema occurs (Hulsebosch, 2002). Inflammation follows necrosis, facilitating axonal degeneration and demyelination at the lesion epicenter (Donnelly and Popovich, 2008). In the chronic phase, further inflammation and demyelination continues, as well as programmed cellular death (apoptosis), reactive gliosis and the formation of a gliotic scar (Hulsebosch, 2002; Fitch and Silver, 2008). Neuroprotective compounds are necessary to reduce the number of dead cells (neurons, astrocytes and/or oligodendrocytes) at the lesion epicenter and surrounding areas, and regenerative drugs are needed to promote axonal outgrowth (by either increase in the sprouting and/or regeneration process) across the damaged region. Axonal outgrowth could be achieved by either promoting or stimulating neurite outgrowth or by reducing or blocking the expression of repulsive/repellent factors at the injury site. Therefore, multi-active drugs are required to target most of the cellular events initiated by the physical insult to the spinal cord and as a result may result in some functional locomotor recovery.
Neuroprotective effect of tamoxifen

The use of estradiol as a neuroprotective hormone is very well known in central nervous system conditions like cerebrovascular stroke, brain trauma and spinal cord injury (Dhandapani and Brann, 2002; Yune et al., 2004; Sribnick et al., 2005; Garcia-Segura and Baltazar, 2009; Arevalo et al., 2010; Etgen et al., 2011; Sirriphorn et al., 2012). This hormone promotes cell survival either by an increase in the expression of anti-apoptotic genes (Segarra and Lee, 2004; Scott et al., 2012) or by a decrease in pro-apoptotic genes (Chaovipoch et al., 2006; Sribnick et al., 2006), resulting in some functional locomotor recovery (Sribnick et al., 2010). Another neuroprotective mechanism proposed for estradiol after injury is by its steroidal structure. Several investigators propose that the phenol hydroxyl ring provides antioxidant and anti-inflammatory activities (Sugioka et al., 1987; Behl et al., 1997; Winterle et al., 2001). However, the mitogenic effect of estradiol in tissues like breast and uterus, deep vein thrombosis or pulmonary embolism restricts the use of this hormone as a therapeutic drug for spinal cord injury patients (Cummings et al., 2002; Sare et al., 2008; Laliberté et al., 2011). Therefore, the consideration of tamoxifen as a neuroprotective and regenerative drug is under investigation in our laboratory and others (Don Carlos et al., 2009; Tian et al., 2009; Ismailoglu et al., 2010; Franco et al., 2013; Guptarak et al., 2014; Mosquera et al., 2014).

Tamoxifen is a selective estrogen receptor modulator (SERM) and an FDA approved drug that interacts with estrogen receptors, producing estrogenic or anti-estrogenic effects. The final outcome depends on the target tissue and the expression of co-activators or co-repressors that interact with the activated estrogen receptor (McDonnel and Wardell, 2010). For this reason, tamoxifen is used mostly for the treatment of cancer, due to its antagonistic activity in tissues like breast and uterus. On the other hand, tamoxifen acts as a neuroprotective drug in pathologies like amyotrophic lateral sclerosis (Traynor et al., 2006), ischemic brain injury (Dhandapani and Brann, 2002; Kimelberg et al., 2003; Mehta et al., 2003; Zhang et al., 2007) and spinal cord injury (Tian et al., 2009; Ismailoglu et al., 2010; Guptarak et al., 2014; Mosquera et al., 2014).

Tamoxifen reduces the edema formation in adult rats after SCI and results in some locomotor recovery (Tian et al., 2009; Ismailoglu et al., 2010; Guptarak et al., 2014; Mosquera et al., 2014). This compound crosses the blood brain barrier (Biegon et al., 1996) and could produce some behavioral improvement through a reduction in several molecular/cellular mechanisms related to the formation of reactive oxygen species, inflammatory molecules or the formation of reactive astrocytes (Zhang et al., 2007; Barreto et al., 2009; Arevalo et al., 2011; Sun et al., 2013). In our laboratory, we observed an improvement in functional locomotor behavior when ovariectomized female rats were treated with tamoxifen. Significant changes were observed in the Basso, Beattie and Bresnahan (BBB) open field test (Mosquera et al., 2014), as well as in the number of animals that crossed the round beam test at 7, 14, 21 and 28 days post injury (Figure 1). The narrow beam crossing test was used to monitor the contribution of equilibrium and balanced posture (mediated through the vestibulospinal and proprioception related tracts) on walking. Rats crossed a 3 feet long round bar (2.5 cm diameter) suspended about 15 inches from the ground (Merkler et al., 2001). The percent of rats in each group able to cross the bar after SCI, with or without the use of their hindlimbs, was determined weekly. Estradiol and tamoxifen treated rats showed a significant higher capacity for crossing the round beam at 7, 14, 21 and 28 days post injury than non-treated control group. Fifty percent of the rats in the estradiol (n = 12) and tamoxifen (n = 10) groups were able to cross the round beam without paw positioning but maintaining some balance by the first week. In contrast, less than 0.01 % of the control rats (n = 13) were able to cross the beam. This pattern was kept until the 4th week where 75% of the estradiol-treated rats were able to cross the beam with or without paw positioning and only 0.2% of the control group crossed. The locomotor recovery was associated with an increase in the amount of white mater spared tissue and a decrease in the superoxide activity (Mosquera et al., 2014). Estradiol or tamoxifen may upregulate the expression of glutathione and/or superoxide dismutase proteins, or these multi-active compounds could work as free radical scavengers to reduce reactive oxygen species after trauma to the spinal cord (Prokai and Simpkins, 2007). Post-treatment experiments demonstrated that tamoxifen administration after SCI produced a significant locomotor recovery in the BBB open field test and beam crossing test, and this effect is related to an increase in the amount of spared tissue, increase in neurofilament immunoreactivity caudal to the lesion epicenter and reduction in the reactive gliosis (unpublished results: Colon et al., 2014). Additional mechanisms that may explain the locomotor improvement by tamoxifen administration after

![Figure 1](https://example.com/image1.png)
SCI could be a reduction in the edema, a diminution in the apoptotic process, a decline in the production of inflammatory cytokines (TNFα & IL-1β), and a decrease of myelin loss and inhibitory proteins (Tian et al., 2009; Ismailoglu et al., 2010; Liu et al., 2010; Guptarak et al., 2014). Similar results with tamoxifen were observed after brain injury and cerebral ischemia (Zhang et al., 2007; Liu et al., 2010; Franco-Rodriguez et al., 2013; Sun et al., 2013). Therefore, tamoxifen (an FDA approved drug) should be considered as a neuroprotective agent to treat spinal cord injury conditions (Figure 2A).

Neuroprotective/regenerative effect of PP2

Investigators are attempting to enhance axonal outgrowth after spinal cord injury either by infusing factors that promote/stimulate neurite outgrowth (Lu and Tuszynski, 2008) or by blocking the factors that have a non-permissive/repulsive effect on regeneration or sprouting (Bouquet and Nothias, 2007). Oligodendrocytes and reactive astrocyte produce these repellent factors. Among the repulsive factors are NOGO, MAG, OMgp, chondroitin sulfate and the Eph receptors (Chen et al., 2000; Wilson et al., 2002; Grados-Munro and Fournier 2003; McGee and Strittmatter 2003; Fitch and Silver, 2008). The complex intracellular signaling by which these proteins hold their effects is through activation of kinases that promote growth cone collapse. The aforementioned NOGO-A is activated by Src (Yokoyama et al., 2006) and the signaling cascade activated by EphA receptors after ligand binding also requires Src activation to induce the repulsive activity (Knoll and Drescher, 2004).

The Src-tyrosine kinases (SFKs), is the largest family of non-receptor tyrosine kinases. The SFKs are widely expressed in many cell types and in different subcellular compartments (Thomas and Brugge, 1997). SFKs induce cellular responses associated with proliferation, growth control, survival, differentiation and cytoskeletal arrangements (Thomas and Brugge, 1997; Kalia et al., 2004; Zhao et al., 2009). SFKs can be switched from an inactive to an active state through control of its phosphorylation state, or through protein interactions. SFKs are the meeting point of several signaling pathways.
associated with neuropathologies like stroke, Alzheimer’s disease, epilepsy and central nervous system trauma (Len-ny et al., 2004; Jadhav et al., 2007; Liang et al., 2009; Liu et al., 2014). This family of kinases can phosphorylate or mediate the activation of central nervous system proteins such as epidermal growth factor (EGF), extracellular regulated kinase (ERK), Eph, NOGO, myelin associated glycoprotein (MAG) (Thomas and Brugge, 1997; Georgakopoulos et al., 2006; Yokoyama et al., 2006; Slack et al., 2008; Wu et al., 2011; Tanaka et al., 2013). After intracerebral hemorrhage in the striatum, blockade of SFKs with PP1-inhibitor, decreased the number of TUNEL-stained cells and reduced behavioral abnormalities (Ardizzzone et al., 2007). In addition, the activation of SFK mediates changes in the blood brain barrier permeability and promotes edema by phosphorylation of metalloproteinase, tight junction proteins and other BBB proteins. The access of SFK inhibitors, like PP1 and PP2, to the endothelial cells from rat brain after its systemic administration inhibits post-ischemic Src activation and vascular leakage, which resulted in the reduction of brain edema (Liang et al., 2009). Moreover, several studies in the adult spinal cord focus in the pain mediated by Src after nerve injury. The induced neuropathic pain by transection of spinal nerve appears to be related to N-methyl-D-aspartate receptor (NMDA) receptor currents potentiated by SFKs (Katsura et al., 2006). The nerve injury induces activation of SFKs in microglia of the dorsal root ganglion but also in the spinal cord. This activation contributes to the development of hypersensitivity to mechanical stimulation that has been associated to an increased ERK phosphorylation in microglia but not with astrocytes or neurons. Another signaling molecule associated with inflammatory hyperalgesia is EphrinB2. EphrinB2 induces tyrosine phosphorylation of the NMDA receptor-2B subunit via SFKs (Slack et al., 2008).

In the spinal cord, SFKs are also involved in the repulsion of the axonal growth cone during development (Thomas and Brugge, 1997; Kalia et al., 2004). SFK have also been associated with primary and secondary damage after spinal cord injury. SFKs, as mentioned before, has been related to the activation of NMDA receptor and inflammatory pain but also to the early inflammatory response mediated by uncontrolled microglia stimulation. Histological evidence from spinal cord tissue treated with 1-(1,1-di-methylethyl)-3-(4-methylphenyl)-1H-pyrazolo[3,4-d] pyrimidin-4-amine (PP1) after 10 minutes of compression show a decrease in the edema, macrophage infiltration and inflammation (Akiyama et al., 2004). SFKs have also been associated with secondary damage after injury (Akiyama et al., 2004). During secondary damage, the activation of SFK mediated by vascular endothelial growth factor (VEGF), leads to stimulation of endothelial cells survival, angiogenesis and edema (Akiyama et al., 2003). In terms of neuroprotection, the inhibition of SFKs reduced and restricted the macrophages infiltration to the lesion area as early as 3 days after injury (Akiyama et al., 2004). In models of cerebral ischemia and surgically induced brain injury, as occur in spinal cord injury, blockade of SFK prevented VEGF-mediated signaling and vascular leakage, reduction in brain edema and decreased breakdown of the blood brain barrier (Jadhav et al., 2007; Liang et al., 2009). Deposition of fibrinogen in the central nervous system after damage to the blood brain barrier, either after a spinal cord transection or contusion, inhibits neurite outgrowth through SFK activation (Schachtrup et al., 2007).

Until now, no study has elucidated the action of SFKs in long-term recovery after spinal cord injury. In our laboratory, we have demonstrated with histological, immunohistochemical and behavioral studies that long-term blockade of SFKs with PP2 in lesioned rats not only produced long-term functional locomotor recovery, but also augmented spared tissue and increased serotonin fibers caudal to the lesion epicenter (Rosas et al., 2014). Animals treated with PP2 after SCI presented higher scores in the BBB open field test and also demonstrated a better performance in the beam crossing test (Rosas et al., 2014). In contrast to other studies related to neuropathic pain, the effect of PP2 in locomotor recovery was not associated with a reactive gliosis response or the amount of microglial cells present at the injury site. We suggest that phosphorylation of ephexin1 by Src kinase after spinal cord injury may be involved in the collapse of axonal growth cones, as has been demonstrated during CNS development after EphA receptor (Knöll and Drescher, 2004), generating a repulsive microenvironment at the injury site. When PP2 was administered to the injured rats the level of SFKs activation was reduced and the activation of ephexin1 decreased, resulting in a better environment for axonal outgrowth. Because SFKs are associated with different events present after spinal cord injury, more studies are necessary to elucidate other key elements upstream or downstream in its signaling cascade that leads to inhibition of locomotor recovery.

**Conclusions and future directions**

The information discussed previously supports the consideration of tamoxifen and PP2 in SCI conditions. These drugs demonstrated their neuroprotective activity because of their effect in functional locomotor recovery and increased spared tissue after SCI. Decrease in ROS, inflammation and apoptosis are possible mechanisms regulated by TAM (Figure 2A). However, additional studies are required to determine which genes are activated in these cellular processes. Moreover, if tamoxifen also provides some regenerative activity and the proteins involved in any axonal outgrowth are also unclear. Blockade of Src activation with PP2 demonstrated, in the other hand, some regenerative activity due to an increase in serotonin fibers caudal to the lesion epicenter (Figure 2B). Although some molecular mechanisms have been related to tamoxifen and PP2 activity after SCI, further experiments are warranted to establish the role of these drugs in the activation of anti-apoptotic genes, downregulation of pro-apoptotic proteins, changes in the expression of proteins that may generate a repulsive environment for axonal regeneration and expression of cytoskeletal proteins related to neurite outgrowth. Moreover, in order to determine the future use of
tamoxifen or SFK inhibitors (PP1 or PP2) for the treatment of human spinal cord injury, more studies should be aimed to determine the optimal doses and therapeutic window for their use. Finally, the spinal cord injury field should consider the neuroprotective and neuroregenerative effects associated with the concomitant use of both drugs (tamoxifen and PP1/PP2) as a therapy for SCI.

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