UNIVERSITY OF CALIFORNIA SAN DIEGO

Effects of M_1R Antagonism in Treating Chemotherapy Induced Peripheral Neuropathy

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by

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The Thesis of Markos Zewdie Kassahun is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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ABSTRACT OF THE THESIS

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Chemotherapy induced peripheral neuropathy (CIPN) is a debilitating condition that affects up to 70% of patients undergoing chemotherapy treatment. CIPN causes a mix of small and large fiber damage as well as motor dysfunction that occurs less frequently but varies patient to patient. CIPN is categorized as a “dying-back” axonal degeneration in which

neurodegeneration proceeds in a distal-to-proximal manner. There is currently no FDA-approved prophylactic or treatment for CIPN. In vitro research has shown that rat sensory neurons exposed to chemotherapeutics exhibited reduced neurite outgrowth, but neurons that were concurrently exposed to the muscarinic acetylcholine receptor 1 (M_1R) antagonist pirenzepine (PZ) were protected from chemotoxicity. To investigate the practical application of this effect, indices of CIPN were measured in Swiss Webster mice treated with chemotherapeutics paclitaxel, oxaliplatin, and bortezomib. Subcutaneous PZ treatment protected against and reversed indices of neuropathy and neuropathic pain in each drug class. The translational therapeutic potential of PZ was explored by testing varied routes of administration and dose frequencies. Topical PZ had no effect on transient tactile allodynia but was able to reverse MNCV slowing in mice with paclitaxel induced CIPN. Dose frequency was examined as a possible variable in topical PZ efficacy. Topical PZ reversed tactile allodynia at each dose frequency and reversed mild MNCV slowing. My data suggests that M_1R may be a viable target in preventing and reversing CIPN.

INTRODUCTION

Peripheral neuropathy is a condition caused by damage to the peripheral nervous system (PNS), which can include sensory nerves, motor nerves, or autonomic nerves. Sensory nerve damage leads to problems in perception of pain, temperature and touch, while motor nerve damage leads to impaired limb movement and autonomic nerve damage disrupts involuntary organ function. Peripheral neuropathy can be triggered by a variety of conditions including diabetes, HIV, and drugs used to treat cancer (NIH: National Institute of Neurological Disorders and Strokes, 2020). Currently there is no cure for any type of peripheral neuropathy.

Peripheral Nerve and the Dorsal Root Ganglion

The dorsal root ganglia (DRG) are a component of the PNS located adjacent to the vertebral column and contain the cell bodies of pseudo-bipolar neurons whose axon bifurcates, with one projection innervating target organs and the other entering the spinal cord (Berta et al., 2017). DRG neurons that have large cell bodies are known to give rise to axons that are classified as highly myelinated and fast-conducting Aβ-fibers (Berta et al., 2017). Those that have small cell bodies have axons that are typically thinly myelinated Aδ-fibers or unmyelinated C-fibers (Berta et al., 2017). Within the DRG, neuronal cell bodies are separated from one another by a wrap of satellite glial cells (Krames 2014). DRG neurons express assorted sodium, potassium, and calcium channels that function in transduction, translation, and modulation of sensory information (Krames 2014), including the TRPV and TRPA receptor families, which are particularly highly expressed in nociceptors, (Berta et al., 2017). DRG neurons are transducers of peripheral nociceptive pain signals from external stimuli and damage to the DRG, sensory axons or adjacent tissues can produce chronic neuropathic pain (Berta et al., 2017). Moreover, as DRG

neurons are not protected by the blood nerve barrier, they are vulnerable to large and small toxic molecules present in blood (Krames 2014). Consequently, the DRG and associated sensory nerves are a target for both acute and chronic pain therapeutics (see below).

Chemotherapy Induced Peripheral Neuropathy

Chemotherapy induced peripheral neuropathy (CIPN) is a debilitating side effect of chemotherapy treatment. The toxic properties of chemotherapeutics are able to prevent the unregulated proliferation of cancer cells, but at the same time can lead to peripheral nerve damage thereby causing CIPN. CIPN is a dose-limiting side effect, burdening patients with the decision to either continue with treatment that is neurotoxic or reduce the therapy that is crucial toward killing cancer cells. This type of dilemma is common since CIPN affects up to 70% of patients that undergo chemotherapy (Hopkins et al., 2016).

CIPN currently does not have an effective treatment approach other than modification of chemotherapy dose regimen. CIPN can also persist for months after cessation of chemotherapy treatment, a phenomenon referred to as coasting (Staff et al., 2017). Coasting effects have been reported in chemotherapeutics that fall under the class of platinum drugs or vinca alkaloids, whereas it has not been reported in the other drug classes such as taxanes and proteasome inhibitors (Staff et al., 2017). Despite these differences, there is growing evidence that a variety of peripheral neuropathies, including CIPN, may share common pathogenic mechanisms downstream of diverse initiating events (Höke, 2012). Therapeutics under development for other forms of peripheral neuropathy may therefore be applicable to CIPN.

CLINICAL PATHOPHYSIOLOGY OF CIPN

In this section, CIPN symptoms are discussed and give a glimpse of the day-to-day life of patients.

Sensory Dysfunction

The most common form of damage seen in CIPN is damage to sensory neurons and their perikarya in the DRG. The pathology of CIPN is described as a "dying-back" primary axonal degeneration (Eldridge et al., 2020) in which neurodegeneration proceeds in a distal-to-proximal manner (Fukuda et al., 2017). This manifests as pain, sensory loss and weakness in the hands and feet in what is referred to as a "glove-and-stocking" distribution (Fukuda et al., 2017; Brewer et al., 2016).

Sensory symptoms of CIPN can include paresthesia (a burning prickling sensation), dysesthesia (a painful or itchy sensation), allodynia (painful sensation to a normally innocuous stimulus), hyperalgesia (an exaggerated painful sensation to a normally painful stimulus), or hypoalgesia (a lack of sensation to a painful stimulus) (Deuis et al., 2017; Han & Smith, 2013; Boland et al., 2010). These symptoms can persist even after discontinuation of treatment, a phenomenon referred to as coasting. (Han & Smith, 2013; Quasthoff & Hartung, 2002). In CIPN caused by platinum-based therapeutics, coasting effects can be seen up to several months post therapy (Staff et al., 2017).

CIPN frequently causes a mix of both large and small fiber nerve damage, (NIH: National Institute of Neurological Disorders and Strokes, 2020) with some variation depending on the specific chemotherapeutic agent. It has been suggested that damage to touch sensing low threshold myelinated Aβ fibers causes a slight decrease in mechanical threshold, so that they report previously innocuous touch stimuli as noxious (Djouhri & Lawson, 2004). Conversely,

there is evidence that tactile allodynia following peripheral nerve injury is due to impulses carried to the CNS along residual Aβ fibers (Djouhri & Lawson, 2004).

Small myelinated and unmyelinated fibers produce different pain sensations. Aδ-fibers are small myelinated fibers important for transmitting short intense or first pain information quickly due to their myelination (Djouhri & Lawson, 2004). This fiber type allows quick responses to sharp painful stimuli like stepping on a nail. Damage to these fibers can lead to impaired sharp pain detection (Flatters et al., 2017). C- fibers are unmyelinated fibers that are important for long, dull, or burning pain transmission (Djouhri & Lawson, 2004). Damage to C-fibers can cause loss of heat sensation, lack of temperature awareness and unnoticed burning of the skin. (Han & Smith, 2013).

As CIPN is a distal neuropathy, the earliest manifestations may be detected as loss of intraepidermal nerve fibers (IENFs). IENFs are bare nerve endings derived from dermal Aδ and C fibers that shed their ensheathing Schwann cells when they cross the dermal/epidermal junction (Boyette-Davis et al., 2011). Studies have shown that areas of the epidermis that experience markedly reduced IENF density are correlated with greater dysfunction in thermal sensitivity along with mechanical sensitivity (Boyette-Davis et al., 2011; Shun et al., 2004; Walk et al., 2007). Because IENF density can be measured in skin biopsies, it can serve as an early biomarker for CIPN-induced damage to Aδ and C fiber types.

Motor Dysfunction

Motor symptoms occur less frequently in CIPN in comparison to sensory symptoms (Zajączkowska et al., 2019) although the severity of deficits varies from patient to patient (Staff et al., 2017). Motor dysfunction is characterized by muscle weakness that extends to distal portions of the body (NIH: National Institute of Neurological Disorders and Strokes, 2020).

Other symptoms include impaired movement and balance, with CIPN patients being 3 times more likely to experience falls (Zajączkowska et al., 2019). The presence of both sensory and motor deficits causes CIPN to be categorized as an axonal sensorimotor neuropathy (Staff et al., 2017).

The sensory and motor disorders reported in rodent models of CIPN reflect the experience of patients undergoing chemotherapy. At present there is no FDA-approved therapy to prevent, reverse or attenuate symptoms of CIPN and severity of particularly sensory symptoms ultimately leads to dose reduction or halting of cancer treatment. My research investigates the efficacy of a new therapeutic approach to preventing or reversing indices of neuropathy in rodent models of CIPN. Three chemotherapeutic models of CIPN will be examined, each of a different drug class.

Chemotherapeutics

Paclitaxel

Paclitaxel (PX) is a taxane antitumor drug widely used to treat breast and ovarian cancer (Scripture et al., 2006). Its mechanism of action is to promote stabilization of microtubules in order to prevent unregulated cell division, with abnormal bundles of microtubules disrupting cell proliferation and preventing tumorigenesis. However, PX is also believed to have adverse effects on neuronal development and function because of its effect on microtubule assembly, since microtubules play a role in neurite outgrowth and growth cone mechanics via microtubule elongation (Scripture et al., 2006; Daniels, 1975; Bray & Hollenbeck, 1988; Kobayashi & Mundel, 1998). PX in high doses and with continuous exposure causes CIPN, and patients with pre-existing conditions are at even greater risk (Scripture et al., 2006). PX induced CIPN leads to

development of a predominant sensory peripheral neuropathy with a less frequent and mild distal motor neuropathy (Scripture et al., 2006).

Sensory Disorders: When administered to rats, PX at a variety of dose regimens produced heat hyperalgesia, mechano-allodynia, mechano-hyperalgesia, and cold allodynia (Polomano et al., 2001). PX accumulates in the DRG as it can penetrate the blood DRG barrier via fenestrations in local capillaries, leading to sensory dysfunction (Zheng et al., 2011)

Motor Disorders: Lack of access of PX to the CNS and spinal cord due to the blood-brain and blood-spinal cord barriers means that motor neuron cell bodies located in the ventral horn are relatively spared. Motor function, coordination and large myelinated fiber motor nerve conduction velocity (MNCV) are reported as being not significantly affected by PX treatment (Xiao et al., 2011).

Oxaliplatin

Oxaliplatin (OXA) is a platinum-based compound that, when used in combination with folinic acid and 5-Fluorouracil, is a first line and adjuvant treatment for colorectal cancer (Starobova & Vetter, 2017). OXA's mechanism of action relies on its cytotoxic effects via DNA damage (Alcindor & Beauger, 2011). As a platinum-based compound, it promotes the formation of platinum-based DNA adducts which activate pro-apoptotic pathways leading to tumor degradation (Zajączkowska et al., 2019). By inhibiting transcription factors that would normally have a strong affinity for platinum, OXA is able to prevent transcription of cancer cells from occurring (Alcindor & Beauger, 2011). Despite OXA having such toxic effects on cancer cells it is limited in use due to the onset of CIPN (Alcindor & Beauger, 2011). OXA induced CIPN is a predominant sensory peripheral neuropathy, with motor axons typically spared (Xiao et al., 2012).

Sensory Disorders: Symptoms of OXA induced CIPN include mechano-allodynia, mechano-hyperalgesia, and cold-allodynia (Xiao et al., 2012). Unlike PX induced peripheral neuropathy, heat hyperalgesia is not common (Xiao et al., 2012). Spontaneous discharge of sensory afferents is reported in OXA treated rats and has been suggested to contribute to the development of allodynic and hyperalgesic pain states (Xiao et al., 2012). This is typically seen in acute peripheral neuropathy, whereas in chronic peripheral neuropathy OXA accumulates in the DRG and causes mitochondrial dysfunction (Canta et al., 2015).

Motor Disorders: There is a decrease in large myelinated sensory nerve conduction velocity in rat models of OXA, whereas no decrease in large myelinated MNCV was detected (Xiao et al., 2012).

Bortezomib

Bortezomib (BTZ) is a proteasome inhibitor used to treat multiple myeloma (Zajączkowska et al., 2019). BTZ, like other chemotherapeutics, causes a dose-dependent neurotoxicity resulting in a distal symmetrical sensory neuropathy (Zajączkowska et al., 2019). Coasting effects can be present years after cessation of treatment (Zajączkowska et al., 2019). Like other chemotherapeutics, higher doses of BTZ lead to greater prevalence of CIPN in patients (Zheng et al., 2012). BTZ mechanism of action targets proteasomes in multiple myeloma cells, which rely heavily on the ubiquitin-proteasome system to perform degradation of misfolded proteins. This results in degraded particles being remodeled into functional proteins (Ri, 2016). When the 26s proteasome is inhibited by BTZ, misfolded proteins are not degraded and accumulate in the ER lumen and cytosol leading to stress-related apoptosis (Ri, 2016). BTZ has become a widely used treatment for multiple myeloma, but one adverse side effect that can result is sensory CIPN (Ri, 2016).

Specific characteristics of BTZ that may contribute to nociceptive hypersensitivity seen in CIPN include increased production of ceramide, sphingosine-1 phosphate (S1P), and dihydrosphingosine-1-phosphate (DH-S1P) (Zajączkowska et al., 2019). Sphingolipid metabolism is increased in astrocytes due to BTZ's action of upregulating production of tumor necrosis factor alpha and interleukin 1-beta (Zajączkowska et al., 2019). These proinflammatory cytokines are known to contribute to neuropathic pain when they accumulate in the dorsal horn. It has been proposed that cytokine upregulation leads to S1P binding to S1P receptors on astrocytes, which promotes increased release of presynaptic glutamate in the dorsal horn, thereby contributing to neuropathic pain (Zajączkowska et al., 2019).

Sensory Disorders: Sensory dysfunction occurs following repeated BTZ treatment and is characterized by a painful length-dependent small-fiber predominant axonal sensory neuropathy. (Staff et al., 2017; Chaudhry et al., 2008). In rats, this manifests as mechano-allodynia, mechano-hyperalgesia, and cold allodynia (Zheng et al., 2012). Heat hypersensitivity is not reported, but heat hypoalgesia has been seen to develop following high doses of BTZ in mice (Zheng et al., 2012; Bruna et al., 2010; Meregalli et al., 2010).

Motor Disorders: Mice treated with BTZ developed deficits in large myelinated fiber sensory, but not MNCV (Bruna et al., 2010).

MOLECULAR MECHANISMS OF CIPN

There are a variety of molecular mechanisms that are proposed to contribute to CIPN including interactions with DNA, ion channels, mitochondria, glutamate neurotransmission, and kinases (Eldridge et al., 2020).

DNA Interaction: One of the many key mechanisms that lead to the development of CIPN is the interaction between chemotherapeutics and DNA. Chemotoxicity has shown to impact both

nuclear and mitochondrial DNA (Canta et al., 2015). Platinum compounds such as cisplatin and OXA are known to interact with DNA, leading to the formation of platinum-DNA adducts that inhibit transcription and ultimately lead to cell death, cell cycle arrest, or apoptosis (Canta et al., 2015). Animal models of OXA and cisplatin treatment have shown that platinum accumulation in the DRG led to morphological abnormalities in DRG neurons such as a decrease in size of neuronal soma, nucleus, and nucleolus (Canta et al., 2015).

Mitochondrial Dysfunction: Physiological effects of anticancer drugs vary from class to class, but mitochondrial dysfunction is reported in multiple classes of chemotherapeutics, including OXA, BTZ, and PX. Each of these chemotherapeutics causes enlargement, swelling, and vacuolation of mitochondria (Waseem et al., 2017) leading to the suggestion that mitotoxicity is a point of convergence in the pathogenic cascades of diverse CIPN-inducing agents. One of the main contributing factors to mitochondrial degeneration is the opening of the mitochondrial Permeability Transition Pore (mPTP) (Canta et al., 2015). This intricate complex has a variety of responsibilities as a high conductance channel in the inner membrane of the mitochondria (Canta et al., 2015). mPTP is a sensitive pore, meaning that specific conditions must be maintained because of its multiple roles as it contains a voltage-dependent anion channel and mitochondrial phosphate carrier which, if not functioning properly, may lead to the pore opening (Canta et al., 2015; Kroemer et al., 2007; Baines 2009). Opening of the mPTP leads to a series of adverse events including ATP depletion, increase of reactive oxygen species (ROS) and Ca^{2+} release, along with vacuolization and mitochondrial swelling that ultimately leads to cell death (Canta et al., 2015; Bernardi et al., 2006).

Within the peripheral nervous system, 90% of mitochondria are located within the axons (Canta et al., 2015). Consequently, opening of the mPTP can have dramatic effects on peripheral

nerves, including impeding ATP production and ATP-dependent processes such as axonal transport. It has been reported that axons of both sensory and motor neurons exhibit chemotherapeutic-induced mitochondrial dysfunction, which has the potential to cause neuronal dysfunction and distal degeneration (Waseem et al., 2017; Vincent et al., 2002).

Ion Channels: CIPN is reported to cause disruption of axonal electrical activity via dysregulation of neuronal membrane ion channels. Three channel types (sodium, potassium, and calcium channels) are considered to play a role in promoting pain in CIPN.

Voltage-gated sodium channels (Na_V) have been shown to be associated with CIPN in both in vitro and in vivo studies (Boyette-Davis et al., 2018). Na_V 1.7 was found to be upregulated in rat DRG tissue that received PX or OXA treatment (Boyette-Davis et al., 2018; Li et al., 2017). Na_V channels are important for initiating and promoting action potentials in neurons, and their upregulation by chemotherapy can lead to small fiber hypersensitivity and ectopic activity (Zajączkowska et al., 2019). A previous study has demonstrated this by injecting OXA into the rat hind paw which led to an intense short duration of mechanical and cold allodynia. Patch-clamp recordings showed OXA treatment acted on certain isoforms of Na^+ channels (Adelsberger et al., 2000). Subsequent in vivo studies confirmed Na_V 1.7's involvement in OXA induced peripheral neuropathy. Researchers administered a tocainide congener NeP1 to OXA treated rats. NeP1 was previously found to have a dose dependent on $Na_V1.7$ by patch clamp recording. Oral administration of NeP1 led to reversal of mechanical hyperalgesia in OXA treated rats, confirming Na_V 1.7's involvement (Ghelardini et al., 2010).

Along with Na_V 1.7, Nav 1.9 has also been shown to be involved in OXA induced peripheral neuropathy by being responsible for cold-triggered nociception. This was determined when $Na_V.9^{-/-}$ mice underwent a cold ramp test where temperature decreased from 25 to 0°C.

Compared to wild type mice, $Na_V.9^{-/-}$ mice exhibited a significant reduction of jumps as temperature decreased, suggesting $Na_V.9$'s importance for cold nociception (Lolignier et al., 2015).

Like Na_V channels, voltage-gated potassium channels (K_V) may also play a role in promoting CIPN symptoms. Normally, K_V channels are important for hyperpolarization, but recent studies have shown that after OXA administration, K_V channels in DRG neurons are downregulated leading to neuronal membrane hyperexcitability (Zajączkowska et al., 2019; Thibault et al., 2012). This results in ectopic nociceptive activity in neurons, which corresponds with development of CIPN in rodent models (Boyette-Davis et al., 2018; Zhang & Dougherty, 2014).

Voltage-gated calcium channels (Ca_V) are the third type of channel involved in promoting CIPN symptoms. Calcium homeostasis, which is regulated by various transport and sequestration mechanisms to maintain the free intracellular calcium levels at nanomolar levels, prevents ectopic activity in peripheral neurons (Starobova & Vetter, 2017; Siau and Bennett, 2006). Other mechanisms to regulate calcium levels include extracellular influxes via calcium channels and release from internal calcium stores in the ER and mitochondria by membrane pumps (Starobova & Vetter, 2017; Siau & Bennett, 2006). Changes in intracellular calcium concentration can influence membrane excitability, which may contribute to the development of PX and OXA induced CIPN (Starobova & Vetter, 2017; Carozzi et al., 2015). An example of this is seen in the OXA metabolite oxalate, which is a calcium chelator (Starobova & Vetter, 2017). Local injection of oxalate into the footpad of mice caused spontaneous nocifensive behavior as well as mechanical allodynia (Starobova & Vetter, 2017; Deuis et al., 2013).

Glutamate Neurotransmission: Excessive glutamate receptor activation in the spinal cord is a hallmark of neuropathic pain (Yamamoto et al., 2017; Liu et al., 2008; Woolf & Salter, 2000; Grace et al., 2014). Studies have shown that in PX-treated rats, downregulation of spinal glutamate transporters was a feature seen after PX treatment (Cata et al., 2006). Wide dynamic range (WDR) neurons of PX treated rats exhibited exaggerated after discharges in response to a single cutaneous electrical stimulus compared to vehicle treated rats (Cata et al., 2006). WDR neurons of PX treated rats also exhibited a greater propensity to wind up from repeated application of an electrical stimulus (Cata et al., 2006). Immunohistochemistry showed that in PX treated rats, the staining intensity of glutamate transporters was significantly reduced (Cata et al., 2006). These results support the suggestion that downregulation of spinal glutamate transporters following PX administration contributed to excess activity in spinal neurons and subsequent hyperalgesia (Cata et al., 2006).

Kinase Activity in the DRG: Another mechanism associated with peripheral nerve sensitization is the expression of kinases. MAPK has been repeatedly shown to be a contributor to the pathogenesis of CIPN (Boyette-Davis et al., 2018). Studies have shown that in rodent models of PX induced CIPN, MAPK caused hyperalgesia by activating TLR4 receptors on the DRG (Boyette-Davis et al., 2018; Li et al., 2015). Normally, MAPK is involved in signal transduction that regulates processes such as cell proliferation, cell differentiation, and cell death (Morrison, 2012). MAPK isoforms such as p38 and ERK have been shown to play a role in nociceptor excitability (Asiedu et al., 2016). Pain that is caused by CIPN is triggered by many factors that act on sensory neurons: chemokines, growth factors, and increases in MAPK and mTOR signaling (Asiedu et al., 2016). Along with MAPK, other studies have shown PX treatment to activate microglia and astrocytes, leading to macrophage infiltration of the spinal cord (Boyette-

Davis et al., 2011; Peters et al., 2007). This leads to an increase in proinflammatory cytokines such as TNF-alpha and IL-6, which cause hypersensitivity of dorsal horn neurons (Boyette-Davis et al., 2011; O'Brien et al., 1995; Zaks-Zilberman et al., 2001). The role of kinases in CIPN damage ultimately causes ectopic activity in the DRG which in turn may contribute to spontaneous pain and hyperalgesia.

CURRENT STATE OF THE ART TREATMENTS

There is no FDA-approved treatment for CIPN, so the only way to mitigate symptoms is through reduction of treatment regimen, or complete cessation of treatment. Despite there not yet being an effective treatment for CIPN, studies in rodent models have investigated efficacy of a variety of drugs that were developed for other conditions to identify potential therapeutics including antidepressants, opioids, and gabapentinoids (Hopkins et al., 2016).

Antidepressants: The commonly used antidepressant duloxetine was shown to significantly inhibit mechanical allodynia in PX-treated mice and BTZ treated rats (Hopkins et al., 2016; Smith et al., 2013). In clinical studies, patients receiving OXA responded better to duloxetine than patients receiving PX treatment. (Hopkins et al., 2016; Smith et al., 2013). Another antidepressant shown to improve quality of life in CIPN patients is amitriptyline (Hopkins et al., 2016; Kautio et al., 2008). When administered topically in combination with baclofen and ketamine, it alleviated sensory neuropathy in CIPN patients (Hopkins et al., 2016; Barton et al., 2011). Experiments conducted in rodents have examined amitriptyline administration in PX and OXA models of CIPN. Repeated intraperitoneal injections of amitriptyline produced antinociceptive effects in PX treated rats 24 hours after the first injection, and antinociceptive effects continued after subsequent injection (Hopkins et al., 2016; Xiao et al., 2008). In a rat

OXA model, intraperitoneal injections of amitriptyline did not prevent cold and mechanical hypersensitivity development, but daily oral administration of amitriptyline did (Hopkins et al., 2016; Zhao et al., 2014; Sada et al., 2012).

Opioids: There is not a history of opioid use to treat CIPN (Hopkins et al., 2016). However, different opioids have been used as positive controls in preclinical studies, where they have inhibited symptoms ranging from cold allodynia to mechanical hypersensitivity evoked by OXA (Hopkins et al., 2016; Ling et al., 2008; Ling et al., 2007; Kanbara et al., 2014; Han et al., 2014; Balayssac et al., 2009). Morphine inhibits PX induced hypersensitivity at high doses, as well as OXA induced mechanical hypersensitivity (Hopkins et al., 2016; Flatters & Bennett, 2004; Pascual et al., 2010; Xu et al., 2011; Ling et al., 2008; Ling et al., 2007). Oxycodone has also been reported to reverse PX and OXA induced mechanical allodynia, whereas fentanyl administration has given mixed results (Hopkins et al., 2016; Kanbara et al., 2014; Mori et al., 2014). The disconnect between efficacy in animal models of CIPN and lack of clinical use is due to concern with addictive potential in patients (Kaley & Deangelis, 2009). There is no published evidence that opioids help in CIPN, but research has shown opioids to be used in conjunction with other neurotropic drugs to treat neuropathic pain in patients, but the effects were addictive (Majithia et al., 2016).

Gabapentinoids: Gabapentin was originally developed as an anticonvulsant but is now used widely, alongside its second generation derivative pregabalin, to treat post-herpetic neuralgia, diabetic neuropathy, spinal cord injury pain, and neuropathic cancer pain (Magnowska et al., 2018). Preclinical studies have shown that gabapentin can reduce mechanical hypersensitivity in PX-treated rats and prevent and reverse cold allodynia in mice and rats induced by OXA (Hopkins et al., 2016; Xiao et al., 2007; Zhao et al., 2014; Ling et al., 2007). Despite positive

results seen in preclinical studies in rodent models of CIPN, there has been a lack of efficacy in gabapentin attenuated neuropathic pain caused by CIPN. In a previous study, a randomized control phase 3 trial was conducted on patients with CIPN from all classes of chemotherapeutics. Patients were administered gabapentin at 2700mg/day for 6 weeks, followed by a 2-week washout period then a 6-week crossover. Gabapentin failed to produce any effect on neuropathic pain by CIPN (Kaley & Deangelis, 2009; Rao et al., 2007). This demonstrates why CIPN drug development faces obstacles, because success in preclinical models has yet to translate in the clinical setting. Side effects are present with gabapentin administration, but research has suggested that variations that exist in gabapentin's effect in humans could be due to the route of administration selected (Quintero, 2017; Comoglu, 2013; Aulton, 2002). Clinical trials have shown that common side effects include somnolence, dizziness, ataxia, and fatigue (Quintero, 2017; Ramsay et al., 1995).

ANTIMUSCARINICS AS NOVEL THERAPEUTICS FOR PERIPHERAL NEUROPATHY

Cholinergic Receptors

Cholinergic receptors are receptors that respond to acetylcholine and are primarily classified by whether they are also activated by either nicotine or muscarine (Tiwari et al., 2013). Neuronal cell bodies in the DRG express both cholinergic receptor types (Tata et al., 2003; Biagioni et al., 1999, 2000), while sensory neurons also express choline acetyltransferase (ChAT) a key enzyme in acetylcholine synthesis (Tata et al., 2003; Biagonni et al., 1999). As primary sensory neurons in culture secrete acetylcholine and are sensitive to cholinergic receptor antagonists, they appear to operate a cholinergic autocrine feedback system (Calcutt et al., 2017).

Muscarinic Receptors

The muscarinic receptor family consists of five metabotropic receptors: M1, M2, M3, M4 and M5 (Scarr, 2012). M1, M3, and M5 receptors couple to Gαq/11 subunits, while M2 and M5 couple to Gαi/o subunits that when activated, trigger secondary messenger cascades in the neurons that express them and activate multiple signal transduction pathways (Scarr, 2012). The carboxyl terminal end of the third intracellular loop shows homology within M1, M3 and M5 receptors and between M2 and M4 receptors (Tiwari et al., 2013). This homology is implicated in downstream effects the receptors can cause when stimulated. M1 and M3 have been shown to take part in the mobilization of intracellular calcium resulting in calcium mediated events (Tiwari et al., 2013). Calcium ions are one of the numerous signal transduction molecules that convey growth cone guidance information (Lowery & Vactor, 2009; Gomez et al., 2006). Muscarinic acetylcholine receptors in the PNS are found on autonomic effector cells innervated by post ganglionic parasympathetic nerves (Tiwari et al., 2013) and sensory neurons (Calcutt et al., 2017).

Muscarinic regulation of neurite outgrowth

Screening studies using DRGs of adult rats identified antimuscarinics as agents that enhanced neurite outgrowth (Calcutt et al., 2017). This is consistent with prior studies reporting spontaneous release of acetylcholine from the growth cones of non-mammalian neurons and acetylcholine-mediated signaling via both muscarinic and nicotinic receptors in which nicotinic signaling was positive for growth whereas muscarinic signaling was negative (Erskine & McCaig, 1995; Young & Poo, 1983). Acetylcholine signaling via muscarinic receptors is also known to regulate growth cone motility in embryonic sensory neurons and cause mobilization of

intracellular calcium (Tata et al., 2003; Yang et al., 2004). Calcium is among many signal transduction molecules that conveys guidance information to growth cones (Lowery & Vactor, 2009; Gomez et al., 2006). Subsequent studies showed that mammalian adult sensory neurons express a peripheral form of choline acetyltransferase (pChAT), and when maintained in vitro-secrete acetylcholine. As these mammalian sensory neurons also express muscarinic receptors (Calcutt et al., 2017; Bernardini et al., 1999, 2004; Bellier & Kimura et al., 2007; Tata et al., 2003) they thus appear to undergo cholinergic constraint of neurite outgrowth via an autocrine negative feedback system.

Of the muscarinic receptors expressed by adult sensory neurons, pharmacological studies using selective and specific agonists and antagonists identified the muscarinic acetylcholine receptor 1 (M_1R) as being responsible for suppressing neurite growth. This was confirmed in molecular studies where M_1R was either overexpressed or deleted (Calcutt et al., 2017). Adult sensory neurons overexpressing M_1R showed reduced neurite outgrowth whereas sensory neurons from M_1R knockout mice showed enhanced neurite growth in vitro. A potential mechanism downstream of M_1R was identified by studies showing that neurons derived from diabetic rodents exhibited reduced levels of mitochondrial proteins and decreased respiratory capacity, and that administration of M_1R antagonists rescued respiratory capacity and promoted neurite outgrowth (Calcutt et al., 2017). This also raised the possibility that M_1R antagonists could be viable therapeutics for peripheral neuropathies via their capacity to enhance mitochondrial activity, given that multiple types of peripheral neuropathy have been linked to mitochondrial dysfunction (Höke, 2012).

Preclinical Studies of Antimuscarinics

In studies to explore the therapeutic potential of M_1R antagonism it was demonstrated that the selective M_1R antagonist pirenzepine (PZ) was able to prevent and reverse multiple structural and functional indices of peripheral neuropathy and neuropathic pain in diabetic mice and rats. PZ was also able to prevent and reverse loss of small sensory fibers in the cornea of a mouse models of HIV protein-induced peripheral neuropathy (Calcutt et al., 2017). Exploratory studies using DRG neurons of normal rats that were isolated and exposed to PX or OXA indicated that these chemotherapeutics also reduced neurite outgrowth and that this disorder was prevented by concurrent exposure to PZ. The potential for muscarinic antagonism to protect in vitro models of CIPN prompts my research described in this thesis that will investigate whether efficacy extends to indices of CIPN in rodent models.

Pirenzepine

PZ is a selective antimuscarinic agent that is used to treat peptic ulcers by controlling gastric secretions **(**Ishimori & Yamagata, 1982). Double blind clinical trial data has shown that PZ administration and duodenal ulcer healing exhibited a positive relationship. As doses of PZ increased, the healing rate of duodenal ulcers increased as well (Texter Jr & Reilly, 1982). PZ administered at daily oral doses of 100 - 150mg produced significant ulcer healing in patients (Texter Jr & Reilly, 1982). Common side effects included dry mouth and blurred vision which are seen in more clinically relevant doses. Other side effects include constipation, diarrhea, headache, and mental confusion (Carmine & Brogden, 1985). PZ is tolerated by most patients, but in rare cases PZ may lead to antimuscarinic effects on the gastrointestinal, genitourinary system, or the heart (Carmine & Brogden, 1985). Previous animal studies have shown PZ to be a

competitive inhibitor of muscarinic acetylcholine receptors, but unlike other anticholinergics it selectively blocks muscarinic receptors (Carmine & Brogden, 1985).

METHODS

INDUCTION OF CIPN BY CHEMOTHERAPEUTICS

The chemotherapeutics listed below, when administered in sufficient doses, can cause the dose-limiting side effect of CIPN.

Paclitaxel Treatment Regimens

Previous work in mice have used varied administration approaches to evaluate how it may influence the severity of the CIPN that develops. One study used a 4mg/kg cumulative dose in male ddY mice and administered this dose by - single intraperitoneal injection, staggered intraperitoneal injections over four days and by single dose intravenous injection to compare if route of administration had an effect on symptom severity. Route of administration had no effect on the severity of mechanical allodynia or thermal nociceptive threshold (Matsumoto et al., 2006). For my study, we selected to use the repeated intraperitoneal administration regimen rather than a single dose in order to mimic a course of chemotherapy in the clinical setting (Höke & Ray, 2014). Saline, Cremophor EL and ethanol were used to dissolve PX (Höke & Ray, 2014). For my experiments, PX was dissolved in a 1:1:2 solution of ethanol, Cremophor, and saline. Injections were administered intraperitoneally every other day until the target cumulative dose was reached.

Paclitaxel PZ Reversal Study: 50 adult female Swiss Webster mice (Envigo) were grouped as follows: N = 10 control, N = 10 PX + vehicle (0.9% sterile saline), N = 10 PX + 0.1mg/kg PZ, N

= 10 PX + 1mg/kg PZ, N = 10 PX + 10mg/kg PZ. Mice receiving PZ were pre-treated subcutaneously with their respective doses for three days prior to the first PX injection. PZ solutions were made by first mixing a 1mg/ml stock solution of 10mg/kg PZ using 0.9% sterile saline, and the subsequent concentrations were made by diluting them with 0.9% sterile saline. PZ treatment was halted during PX injections, so that PZ did not influence development of PX neurotoxicity. PX was administered intraperitoneally at 1mg/kg every other day for five days to a cumulative dose of 5mg/kg. This dose regimen was selected based on preliminary work that demonstrated PX administered in a high dose format of 20mg/kg produced CIPN in female Swiss Webster mice, and was prevented by PZ administration (Calcutt et al., 2017). I wanted to see if CIPN could be produced in a low dose format of 5mg/kg, and whether PZ could produce the same neuroprotective effects seen in high doses of PX. Previous work has mentioned that PX administered at 4mg/kg was a clinically relevant dose, so we also intended to make our dose clinically relevant (Matsumoto et al., 2006). Following PX injections, PZ was administered daily (five days) every week through the remainder of the study.

Paclitaxel Topical PZ Reversal: 60 adult female Swiss Webster mice (Envigo) were grouped as follows: N = 10 control, N = 10 PX + vehicle (0.9% sterile saline), N = 10 PX + 1% PZ, N = 10 PX + 3% PZ, N = 10 PX + 5% PZ, N = 10 PX + 10% PZ. Topical PZ concentrations were produced by a mixture of 0.5mL hydrogel and varying amounts of PZ. 1% PZ was made using 5mg PZ, 3% used 15 mg PZ, 5% used 25mg PZ, and 10 % used 50mg PZ. Mice received a 1mg/kg intraperitoneal injection of PX every other day for five days for a cumulative dose of 5mg/kg. Following PX administration, mice receiving PZ were administered topical PZ treatment once daily (five days) on the left hind leg through the remainder of the study.

Paclitaxel Topical PZ Reversal Dose Frequency: 50 adult male Swiss Webster mice (Envigo) were grouped as follows: N = 10 control, N = 10 PX + vehicle (0.9% sterile saline), N = 10 PX + 10% PZ 1/Wk, N = 10 PX + 10% PZ 3/Wk, N = 10 PX + 10% PZ 5/Wk. Topical PZ solutions were made following the same protocol in the previous PX topical experiment. Mice were treated with PX at 4mg/kg every other day for five days for a cumulative dose of 20mg/kg. An increase of PX concentration was selected due to the previous PX topical PZ reversal study exhibiting transient toxicity that was resolved within four weeks following administration. Previous work has demonstrated that PX administered at a cumulative dose of 20mg/kg caused CIPN in female Swiss Webster mice, and was prevented by subcutaneous administration of PZ (Calcutt et al., 2017). I wanted to investigate how topical PZ would work against CIPN in a high dose format. Following PX treatment, mice received topical PZ on the left hind leg for the remainder of the study.

Oxaliplatin Treatment Regimens

In previous experiments, OXA was administered intraperitoneally in male C57Bl/6 mice at 3mg/kg daily for five days, followed by five days of rest for two cycles, ultimately for a cumulative dose of 30mg/kg. This dosing protocol led to the development of acute neuropathy with cold hyperalgesia and mechanical allodynia. This dose regimen approximates human therapeutic doses relative to body weight (Ta et al., 2009). For my experiments OXA was dissolved in a solution of 5% glucose using 0.9% sterile saline. Following previous protocols, injections were administered intraperitoneally every other day until the desired cumulative dose was reached.

Oxaliplatin PZ Reversal: 40 adult female Swiss Webster mice were grouped as follows: N = 10 control, N = 10 OXA + vehicle (0.9% sterile saline), N = 10 OXA + 1mg/kg PZ, N = 10 OXA +

10mg/kg PZ. Mice receiving PZ were pre-treated with their respective concentrations for three days prior to the first OXA injection. Following PZ pre-treatment, OXA was administered intraperitoneally at 5mg/kg every other day for four days, for a cumulative dose of 20mg/kg. PZ treatment was halted during OXA injections, so that PZ did not influence development of OXA neurotoxicity. I selected this dose regimen based on previous work that has demonstrated high doses of OXA leading to CIPN (Ta et al., 2009). I also wanted to match the high dose concentration used in preliminary studies of PX induced neuropathy where PZ treatment prevented CIPN development (Calcutt et al., 2017). I wanted to see if the same neuroprotective effect would be present across different drug classes. After OXA injections, experimental groups continued to receive PZ treatment daily (five days) for the remainder of the study.

Bortezomib Treatment Regimens

In a previous study using C57BL/6 mice, BTZ was dissolved in DMSO and diluted in 0.9% sterile saline. BTZ was administered at 0.4mg/kg by intraperitoneal injection three times a week for four weeks for a cumulative dose of 4.8mg/kg. This treatment regimen was comparable to treatment of patients undergoing chemotherapy (Boehmerle et al., 2014). For my experiment, BTZ was dissolved in 0.9% sterile saline and delivered by intraperitoneal injection every other day until the cumulative dose was reached.

Bortezomib PZ Reversal Study: 30 adult female Swiss Webster were arranged as follows: N = 10 control, N = 10 BTZ + vehicle (0.9% sterile saline), N = 10 BTZ + 10mg/kg PZ. PZ pre-treatment was administered for three days prior to the first BTZ injection. PZ treatment was halted during BTZ injections, so PZ did not influence development of BTZ neurotoxicity. Following pre-treatment, BTZ was administered at 1mg/kg every other day for five days by

intraperitoneal injection. This dose regimen was selected based on previous experiments that have demonstrated CIPN developing from a similar cumulative dose regimen that was clinically relevant (Boehmerle et al., 2014). The BTZ concentration I selected caused four mice to die so treatment was halted. The resulting cumulative dose was 2mg/kg. This demonstrated how genetic background may influence the inconsistencies in drug potency seen (Höke & Ray, 2014). Following BTZ injections, PZ was administered daily (five days) for the remainder of the study.

BEHAVIORAL TESTS

Behavioral tests can be viewed as illustrating a relationship between input and output systems. In animal studies, the input is the stimuli that the researcher imposes on the animal and the output is the animal's reaction to that stimulus. (Le Bars et al., 2001). There are various forms of pain that can be implied through behavioral tests (Deuis et al., 2017):

- Allodynia, defined as a painful response to an otherwise innocuous stimulus.
- Hyperalgesia, defined by exaggerated pain to an already noxious stimulus.
- Hypoalgesia, defined as decreased sensitivity to a noxious stimulus.

Despite widespread use, behavioral tests also have weaknesses. The focus on pain-like behaviors is purely inferential, as perception of pain cannot be directly measured in animals (Deius et al., 2017). However, studies developing rodent models of CIPN that replicate patient symptoms have focused on measuring evoked pain-like behaviors to mimic recording pain levels of patients (Flatters, 2017). In rodent models of CIPN, pain is inferred through the rodent's reaction to a nociceptive stimulus such as paw withdrawal (Deius et al., 2017).

Tests such as the von-Frey filament test rely on the physiological state of the tissue being stimulated which could influence the responses evoked by nociceptors (Le Bars et al., 2001).

This overlay of physiological factors makes it difficult to determine an accurate nociceptive threshold when the state of the tissue could be the reason for a premature reaction.

A second weakness is that behavioral tests aren't really measuring nociceptive stimuli. For stimuli to be truly nociceptive, the stimuli should produce lesions or damage to the tissue (Le Bars et al., 2001). Researchers are really inferring pain from evoked pain behaviors to increasing stimulus intensity rather than at a nociceptive threshold. Despite these tests having these drawbacks, they provide a viable and widely accepted means of investigating nociception to mechanical, thermal, chemical, or electrical stimuli.

The recent emergence of non-stimulus evoked behavior tests has become more popular as a means to measure nociception. This is in part due to animal models using stimulus evoked measurements of nociception failing to translate into the clinic (Deuis et al., 2017; Mogil, 2009). A great example of a successful non-stimulus test is the conditioned place preference test (CPP), which uses preference as a surrogate for ongoing pain (Jin et al., 2020). To overcome the limitations set by the current state of behavioral assays, the goal is to eventually move to non-reflexive testing as opposed to reflexive testing, measuring spontaneous as opposed to evoked responses and using broader parameters of 'quality of life' measurements (Mogil, 2009). Non-stimulus evoked behavior tests such as the CPP have been able to rule out results that have suggested significant effects from behavioral assays. In one example, CPP ruled out an association of a TRPA1 channel antagonist affecting mechanical hyperalgesia due to the effect not translating in the CPP test (Jin et al., 2020; Wei et al., 2013). This suggests the need to start including operant measures in behavioral assays for chronic pain. Despite this potential, drawbacks prevent the normalization of these techniques. Operant tests tend to require more labor for training animals, they are more time consuming and more costly (Mogil, 2009). Due to

these limitations, researchers remain more likely to use stimulus evoked behavior as the primary measurement for pain state.

All methods discussed below have been published in detail (Jolivalt et al., 2016).

Manual von-Frey Filament Test: Allodynia to light touch is a commonly reported feature of CIPN in patients and animal models and reflects sensory dysfunction in large myelinated sensory fibers (Han & Smith, 2013; Höke & Ray, 2014; Djhouri & Lawson, 2004). Von-Frey filaments are used in both clinical and pre-clinical studies to measure allodynia to light touch and the 50% withdrawal threshold, which represents the stimulus intensity required to produce a response in an animal 50% of the time. It is a commonly used approach in quantifying allodynia in rodents (Chaplan et al., 1994). Dixon, who developed the up down technique associated with this test, demonstrated that optimal calculation of 50% withdrawal threshold using this technique requires at least 6 responses in the animal (Chaplan et al., 1994).

In preparing for this assay, rodents are placed on a wire grid surface mounted on an elevated metal stand. The spaces between the mesh allows applied filaments to access the plantar surface of the paw. When working with mice, mice are placed inside 250mL glass beakers to limit movement. If rats are used, they are placed inside plastic boxes that also restrain movement. Once mice or rats are placed up on the stand, they are left to acclimate to the conditions for 15 minutes prior to testing. Following acclimation, plastic von-Frey filaments of varying diameter and thus defined applied pressures on buckling are used to poke the plantar surface of the hind paw of rodents. Rodents are poked with filaments of increasing pressure when there is an absence of reaction, and of decreasing pressure when a reaction is evoked. Rodents are poked with an individual filament five times (1/sec) to elicit a response before an increase or decrease in filament is made. Once a change in response occurs, responses to the next four tests are

recorded to allow calculation of the 50% withdrawal threshold for the hind paw. The sequence of von-Frey filaments used is: 3.22, 3.61, 3.84, 4.08, 4.31, 4.56, 4.74, 4.93, and 5.18. The range of filaments used for mice is 3.22 - 4.74, whereas for rats it is 3.84 - 5.18. When measuring allodynia from collected data, individual measurement outliers within a set of data that are defined as two or more standard deviations outside the mean are excluded. This is to obviate occasional unrepresentative behavioral responses such as when animals freeze and become temporarily unresponsive.

Heat Sensitivity Test: Another key feature of clinical CIPN is the presence of heat hypoalgesia or hyperalgesia (Han & Smith, 2013; Dougherty et al., 2004; Cata et al., 2006; Attal et al., 2009; Nahman-Averbuch et al., 2011; Zajączkowska et al., 2019; Bernhardson et al., 2007). The presence of either of the two conditions has been linked to the increase or decrease of TRPV1 expression levels in C-fiber nociceptors (Han & Smith, 2013; Pabbidi et al., 2008). The Hargreaves thermal apparatus is used to test paw heat sensitivity.

To begin, the apparatus is turned on and left to heat up until the surface on which rodents will stand is constant at 30°C. Once the desired temperature is reached, a heating element fixed to a mobile arm is maneuvered until it is directly below a temperature probe located on the under surface of the apparatus. Heating is activated and temperature recorded every 5 seconds for a total of 20 seconds. Once temperatures are recorded at 0, 5, 10, 15 and 20 seconds, a time-temperature curve is produced. During a given session in which the thermal sensitivity test is conducted, three time-temperature curves are produced, during the beginning, middle, and end of the experiment. From 30°C, surface temperature at the probe should increase at a rate of 1°C per second. This is important to document, as the rate at which the temperature probe heats the animal's skin dictates what type of sensory afferents are activated. C-fiber nociceptors typically

activate at a heating rate of 0.9°C per second, whereas Aδ-fibers are typically activated when skin is heated at a rate of 6.5°C per second (Yeomans & Proudfit, 1996).

Mice or rats are placed on the 30°C surface of the Hargreaves apparatus inside 250mL beakers (for mice) or plastic boxes (for rats) to constrain movement. Prior to experimentation, rodents acclimate to the conditions for 15 minutes. Once animals are ready, the arm of the apparatus is moved so that the heating element is located underneath the plantar surface of one hind paw. Heating, which will increase surface temperature by 1°C per second, is initiated until the rodent lifts its paw because the heat stimulus is perceived as noxious. When the rodent lifts its paw, the heating mechanism automatically turns off. If the animal urinates on the surface, the surface must be cleaned before recording as heat transfer from the surface to the paw is disrupted. Measurements are taken three times for each paw of each rodent and the median of each triplicate is used to represent the withdrawal latency in seconds for that paw. The latency may then be used to calculate the withdrawal heat using the time-temperature curves described above.

Rotarod Test: Performance on a rotarod tests combined sensory and motor function and thus indicates whether other forms of dysfunction may contribute to behavioral tests that focus on sensory function. For example, impaired motor function can impede paw withdrawal latencies independent of sensory dysfunction.

To perform this test, the commercially produced rotarod device (Stoelting Inc.) is adjusted to a range of 4RPM to 40RPM. The device consists of 1.25-inch diameter rods for mice, and larger rods for rats. Once activated, the rotating rod will gradually increase in speed over 120 seconds and then maintain a rate of 40RPM for another 180 seconds. Mice or rats are placed on the rod facing toward the researcher. The rod rotates backwards so that the rodents move toward

the researcher in order to maintain balance. Rodents perform two trial runs to get them acclimated to the moving rod. Three consecutive trials are then conducted, and each trial concludes by either timing out or the rodent falling from the rod. Measurements are reported as maximal RPM achieved, time to fall, and distance traveled prior to fall.

ELECTROPHYSIOLOGY

Nerve Conduction Velocity: Nerve conduction studies provide a way to measure the function and integrity of large myelinated sensory and motor nerves but are not suitable for testing thinly myelinated Aδ-fibers and unmyelinated C-fibers (Tavee et al., 2019). Slowing of nerve conduction velocity (NCV) is an important indicator of large fiber peripheral neuropathy in humans and animals.

Animals are initially placed in an induction chamber where they are anesthetized via exposure to isoflurane. Once the animals are anesthetized, they are placed on a heated water pad, which allows the rodents to maintain their core body temperature while anesthetized by exposure to a mixture of oxygen and 4% isoflurane. Core body temperature is monitored by the placement of a rectal thermometer and maintained at 37°C. If body temperature falls below 37°C, a heat lamp is used to increase body temperature. The rectal thermometer and the heat lamp are connected to a temperature controller. This temperature controller will turn on the heating lamp when body temperature read by the rectal thermometer falls. Once the desired temperature is reached, the heating lamp will turn off. It is important that prior to use of the heating lamp, a covering is placed on top of the animal to prevent burns to the skin and ears.

Two fine needle recording electrodes (Grass Instruments) are placed into the interosseous muscles between the second and third, and third and fourth toes to record a stimulus-evoked

electromyogram (EMG). A grounding electrode is then placed into the skin at the back of the neck and a stimulating electrode placed at the sciatic notch or Achilles tendon. These electrodes are connected to a PowerLab stimulator which delivers a 200mV, 50μsec duration square wave stimulus every 2 seconds. Resulting M waves of the electromyogram (EMG) are used to calculate MNCV whereas H waves are used to calculate sensory nerve conduction velocity.

The stimulating electrode is placed at the Achilles tendon to record the first of the M wave pairs (M1). The stimulating electrode is then removed and placed at the sciatic notch to record the second M wave of the pair (M2). The time lag between the stimulus and each M wave is recorded in ms and (M2 – M1) calculated to give the difference in latency between the two peaks. The window for acceptable peak-peak latencies was 0.3-0.8 ms. This process is done three times followed by measuring the distance between the sites where the stimulation electrodes were placed. Once determined, the median of the M wave latencies is divided by the length between the stimulation sites to determine the nerve conduction velocity in m/s. This process is repeated when calculating sensory nerve conduction velocity except H waves are measured. Mice typically lack H waves because of their higher susceptibility to anesthesia-induced spinal block. The H wave involves a spinal reflex arc, which is easily blocked in mice causing no H wave to be detected. See below for images of assays:

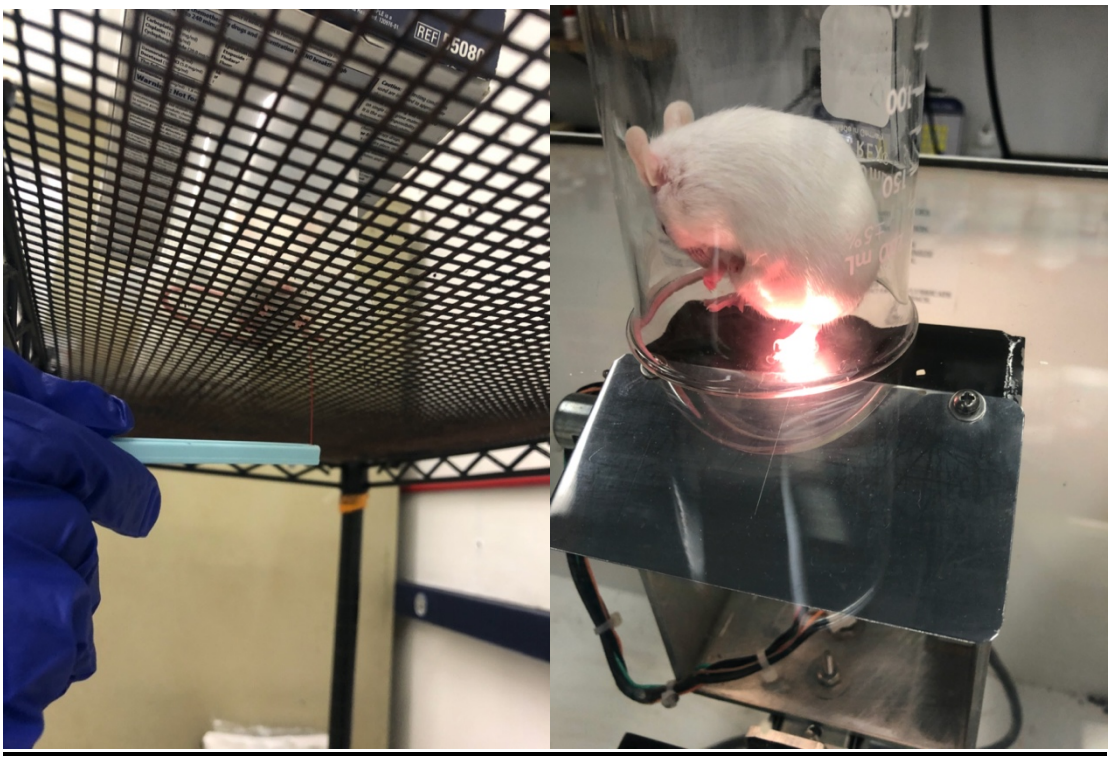


Figure 1.1: Images of Manual von-Frey and Hargreaves Heat Sensibility Test

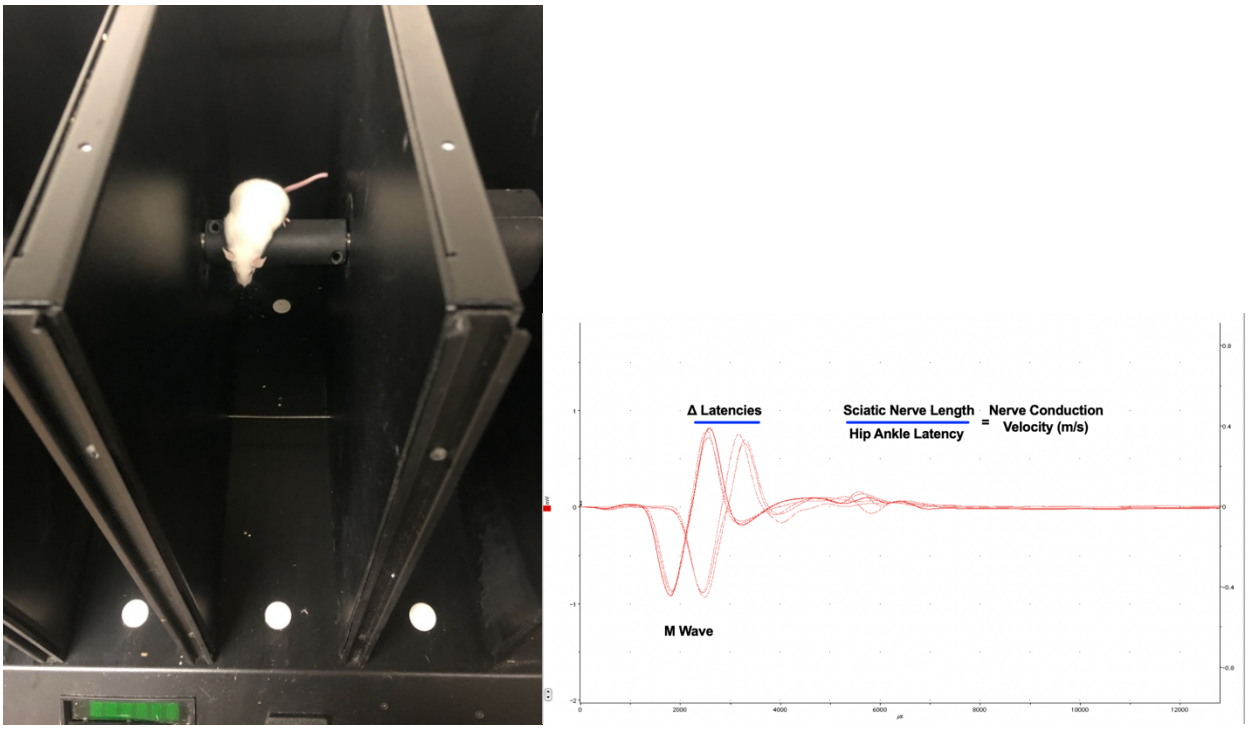


Figure 1.2: Images of Rotarod test and MNCV recording

RESULTS

STUDY #1: PACLITAXEL NEUROPATHY AND EFFICACY OF SYSTEMIC PIRENZEPINE TREATMENT

Purpose: To determine a) efficacy of subcutaneously injected PZ at varying concentrations in preventing or reversing indices of neuropathy and neuropathic pain in female Swiss Webster mice b) if a dose dependent effect is present.

Design: Adult female Swiss Webster mice received PZ at their assigned doses (0.1, 1.0 and 10.0 mg/kg/day) by subcutaneous injection starting three days prior to PX administration. PX was administered at a dose of 1mg/kg given on alternate days for a cumulative dose of 5mg/kg (see methods for details). Experimental groups were withdrawn from PZ treatment during the period of PX treatment, in order to prevent any possible effect of PZ on PX neurotoxicity. Once the PX regimen was complete, mice returned to PZ treatment throughout the remainder of the study.

Health Status: No mice died unexpectedly during the study. At study end, mice underwent a rotarod balance test to assess sensorimotor function. There was no significant difference in rotarod performance (time prior to falling) between any group (**Fig. 2.1**), indicating absence of sensorimotor dysfunction and supporting viability of using stimulus-evoked behavioral assays to assess sensory function.

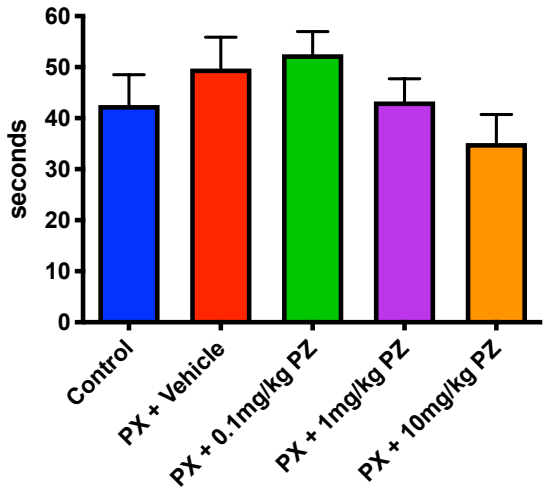
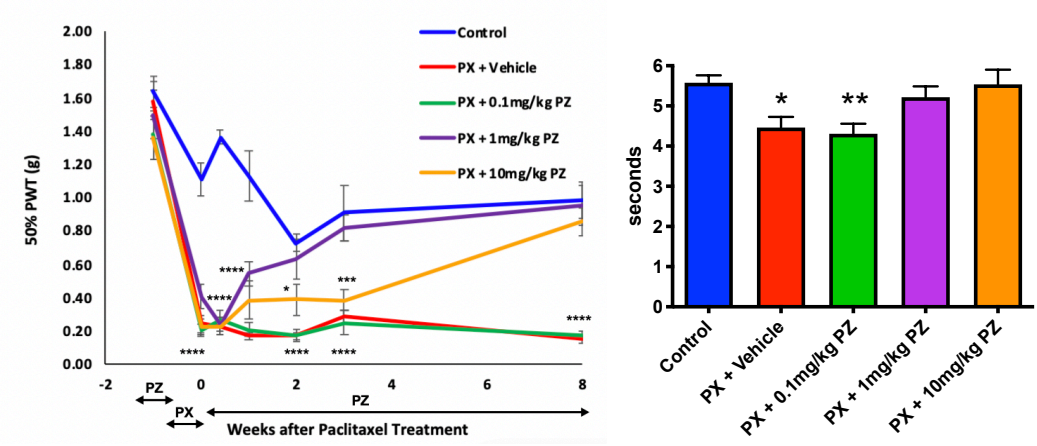


Figure 2.1: Rotarod performance in all study groups: Rotarod performance was measured at the end of the study. Data are group mean±SEM of N=10/group. Statistical comparison vs control by one-way ANOVA with Dunnett's post-hoc test.

Sensory Function: Paw withdrawal from light pressure applied to the plantar surface via von-Frey filaments was the primary measure of painful neuropathy and was measured weekly throughout the study (**Fig. 2.2A**). All PX-treated groups developed significant allodynia (response to lower applied pressure) by the end of the PX treatment regimen ($P<0.0001$ vs control: **Fig. 2.2A**). PX mice treated with vehicle remained significantly allodynic for the subsequent eight weeks, as did PX mice treated with 0.1 mg/kg/day PZ. In contrast, mice treated with 1.0 or 10.0 mg/kg/day PZ returned to values that were not significantly different from control mice, with the 1.0 mg/kg/day treated mice recovering faster than the 10.0 mg/kg/day treated group.

Paw heat withdrawal latency was measured at the end of the study (**Fig. 2.2B**). Vehicle-treated PX mice exhibited significant ($P<0.05$ vs controls: **Fig. 2.2B**) heat hyperalgesia, as did PX mice that received 0.1mg/kg/day PZ ($P<0.01$ vs controls: **Fig. 2.2B**). In contrast, PX mice that received 1.0 or 10.0 mg/kg/day PZ were not significantly different from controls.

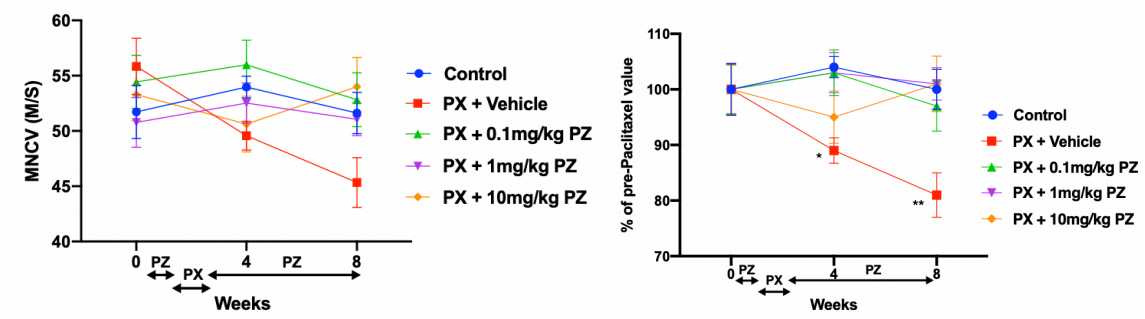
A) B)



Figures 2.2: Paw tactile (A) and heat (B) evoked responses in all study groups: Data are group mean±SEM of N=10/group. Statistical comparisons by two-way (A) and one-way (B) ANOVA with Dunnett's post-hoc test. **** = P<0.0001, *** = P<0.001, ** = P<0.01, * = P<0.05 vs control.

Motor Function: PZ treatment was able to protect against significant large fiber MNCV slowing induced by PX. Figures **2.3A** and **2.3B** demonstrate how variability at baseline can hide therapeutic effects. When expressed as absolute MNCV (m/s), considerable between-group variation was present before onset of PZ or PX treatment, despite randomization of animals to groups (**Fig. 2.3A**). Vehicle-treated PX mice showed a marked trend to reduced MNCV in the eight weeks following the PX regimen but this was not significantly different from control mice at any time. Expressing data as the percent change of each mouse from its pre-PX value resolved the variability (**Fig. 2.3B**). By week four, the vehicle treated PX group exhibited a significant (P<0.05 vs controls: **Fig. 2.3B**) decrease in MNCV that was exaggerated by week eight (P<0.01 vs controls: **Fig. 2.3B**). Mice treated with any dose of PZ did not develop a significant decrease in MNCV compared to controls at any time.

A) B)



Figures 2.3: Motor nerve conduction velocity in all study groups: Values were calculated as MNCV (A) and percent change from week 0 values (B). Data are group mean±SEM of N=10/group. Statistical comparisons by two-way ANOVA with Dunnett's post-hoc test. ** = P<0.01, * = P<0.05 vs control.

Discussion Points (see General Discussion for expansion)

- The PX regimen caused a severe and protracted tactile allodynia that was not prevented by pre-treatment with PZ. PZ produced a dose dependent reversal of allodynia with the 1mg/kg dose resolving allodynia faster than the 10mg/kg dose. Efficacy of PZ in reversing allodynia confirms our previous report (Calcutt et al., 2017). The present experiment showed that a lower dose of PZ (1mg/kg/day) is sufficient to reverse tactile allodynia.
- PX caused MNCV slowing and PZ across all doses was able to protect against slowing from occurring. The 0.1mg/kg dose was able to protect against MNCV slowing whereas it was unable to reverse tactile allodynia. As both assays measure aspects of large fiber function, PZ may have a larger impact on electrophysiological function than behavioral function. Alternately, PZ may more readily protect MNCV than large sensory fibers (tactile responses)
- PX treatment caused heat hyperalgesia at the end of the study. PZ treatment at 1mg/kg and 10mg/kg were able to protect against PX induced heat hyperalgesia whereas the 0.1mg/kg

dose was unsuccessful. This experiment along with the results of the manual von-Frey test suggest that 0.1mg/kg PZ is unable to protect against various forms of sensory dysfunction.

Again, I would like to acknowledge Dr. Nigel Calcutt and Katie Frizzi for allowing me to use unpublished data in this section "Paclitaxel Neuropathy and Efficacy of Systemic Pirenzepine Treatment".

STUDY #2: PACLITAXEL NEUROPATHY AND EFFICACY OF TOPICAL PIRENZEPINE TREATMENT

Purpose: To determine a) efficacy of topically delivered PZ against indices of neuropathy and neuropathic pain in female Swiss Webster mice b) determine if a dose dependent effect is present.

Design: Adult female Swiss Webster mice received PX at a dose of 1mg/kg given on alternate days for a cumulative dose of 5mg/kg (see methods for details). Once the PX regimen was complete, mice received vehicle (hydrogel) or PZ at daily doses of 1%, 3%, 5% and 10% in hydrogel applied for twenty minutes per day to the left hind paw.

Health Status: One mouse in the control group died unexpectedly. Body weight was measured throughout the study. Neither PX nor PZ treatment had any effect on body weight (**Fig. 3.1**) indicating no effect on general metabolic health.

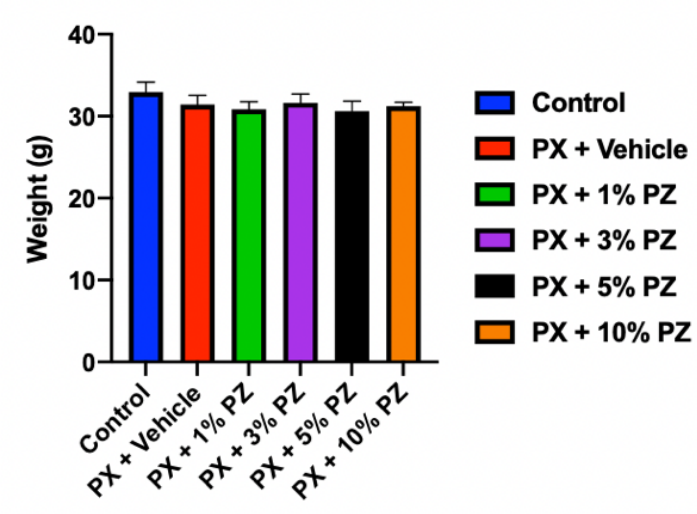
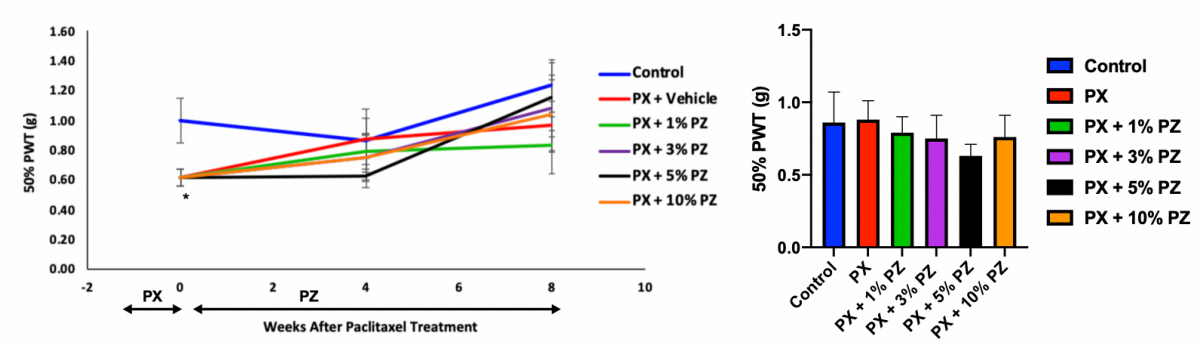


Figure 3.1: Body weight in all study groups: Body weight was measured weekly. Data are group mean±SEM of N=9-10/group. Statistical comparison vs control by one-way ANOVA with Dunnett's post-hoc test.

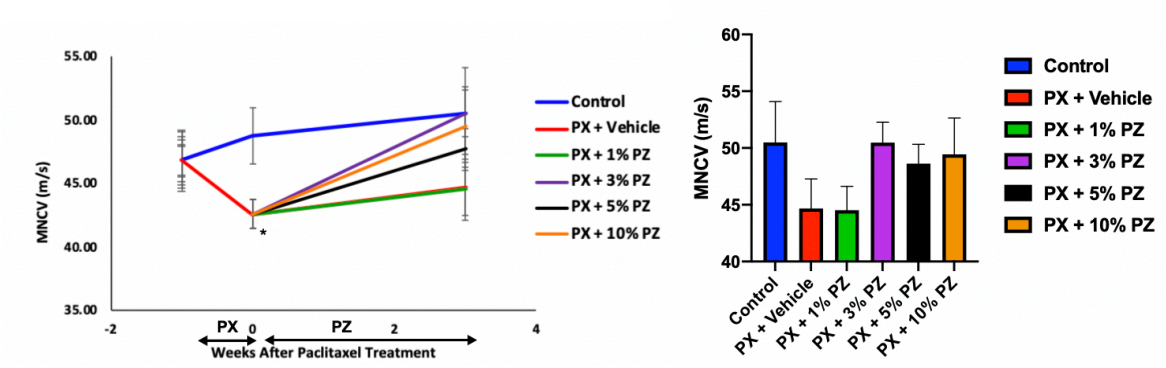
Sensory Function: Paw withdrawal from light pressure applied to the plantar surface via von-Frey filaments was the primary measure of painful neuropathy (**Fig. 3.2A, B**). PX-treated mice developed significant allodynia by the end of the PX treatment regimen ($P<0.05$ vs controls: **Fig. 3.2A**). Due to variability seen amongst PX treated mice at the week zero timepoint, PX-treated mice were expressed as a cohort since tactile allodynia was expected to occur immediately after PX treatment. PX mice treated with vehicle spontaneously reverted to control values by week four, whereas PZ-treated mice either returned to control values or remained mildly allodynic **(Fig. 3.2B)**.



Figures 3.2: Paw tactile evoked responses in all study groups: A) Tactile illustrated across the entire study B) Tactile values at week 4. Data are group mean±SEM of N=9-10/group. Statistical comparisons by two-tailed unpaired T-test at week 0 (A) and one-way ANOVA with Dunnett's post-hoc test at week 4 (B). * = P<0.05 vs control.

Motor Function: PX treatment induced significant MNCV slowing (P<0.05 vs controls: **Fig. 3.3A**) compared to control values at the week zero time point. Due to variability seen amongst PX treated mice at the week -1 and zero time point, PX-treated mice were expressed as a cohort due to variability at baseline and following PX treatment since MNCV slowing was expected to occur. MNCV of PX-treated mice that received daily vehicle treatment remained stable over time whereas mice treated with topical PZ, showed a dose-dependent trend to returning to control values, although there was no statistically significant difference between any group at study end (**Fig. 3.3B**).

A) B)



Figures 3.3: Motor nerve conduction velocity in all study groups: A) MNCV illustrated across entire study B) MNCV values at final time point. Data are group mean±SEM of N=9-10/group. Statistical comparisons by two-tailed unpaired T-test at week 0 (A) and one-way ANOVA with Dunnett's post-hoc test at week 3 (B)., * = P<0.05 vs control.

Discussion Points

- The degree of allodynia achieved following completion of the PX regimen was less marked than study #1 and mice reverted to normal more quickly than anticipated (four weeks > eight weeks in study #1). This was despite using the same sex, strain of mouse, age and the same PX dosing regimen. We have no explanation for this but decided to use a higher dose PX regime in future studies to try and achieve more marked allodynia.
- Some groups of PZ-treated mice appeared to have delayed recovery of allodynia compared to vehicle-treated PX mice. This was unanticipated, given that systemic PZ accelerated recovery from PX-induced allodynia (Study #1). It was noted that tactile assays were performed within hours of topical treatment and we speculated that topical delivery might

induce an acute allodynia. The design of future studies was modified so that tactile tests were performed no less than 24 hours after the last topical treatment.

- PX induced initial MNCV slowing of a similar magnitude to that seen in Study #1. However, unlike study #1 values did not continue to decline over time but stabilized. The relatively small difference between controls and vehicle-treated PX mice along with within-group variability in MNCVs meant that there was no statistically significant difference between any group at study end. Despite lack of significance at study end, data suggests that topical PZ produced a dose dependent trend to reverse MNCV slowing.

STUDY #3: PACLITAXEL NEUROPATHY AND EFFECTS OF DOSE FREQUENCY IN TOPICAL PIRENZEPINE TREATMENTS

Purpose: To determine a) whether increased PX dose generated a more robust allodynia in mice b) efficacy of topically delivered 10% PZ against indices of neuropathy and neuropathic pain in male mice c) the optimal dose frequency for topical delivery.

Design: PX was administered to adult male Swiss Webster mice at a dose of 4mg/kg given on alternate days for a cumulative dose of 20mg/kg (see methods for details). Once the PX regimen was complete, mice received vehicle (hydrogel) or 10% PZ in hydrogel applied for twenty minutes on the left hind paw for one, three, or five days per week.

Health Status: No mice died unexpectedly during the study. Body weight was measured throughout the study, and PX treatment was shown to have no effect on body weight (**Fig. 4.1**) indicating there was no effect on general metabolic health.

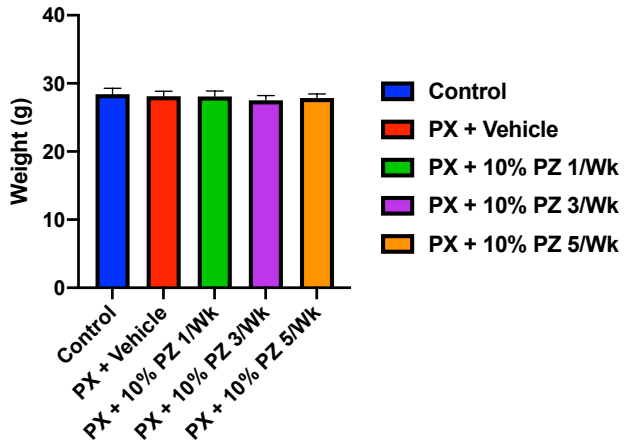
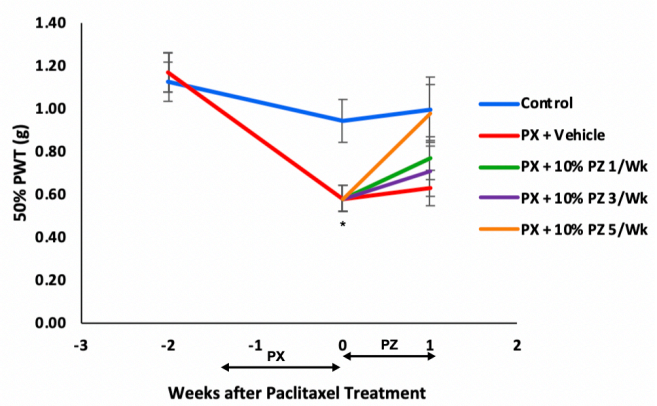


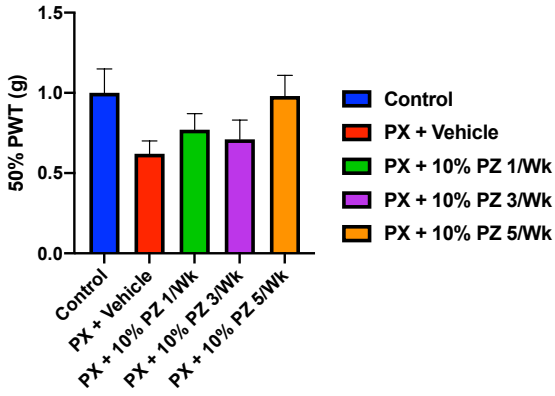
Figure 4.1: Body weight in all study groups: Body weight was measured weekly. Data are group mean±SEM of N=10/group. Statistical comparison vs control by one-way ANOVA with Dunnett's post-hoc test.

Sensory Function: Paw withdrawal from light pressure applied to the plantar surface via von-Frey filaments was the primary measure of painful neuropathy (**Fig. 4.2A, B)**. PX-treated mice developed significant allodynia ($P < 0.05$ vs controls: **Fig. 4.2A**) following PX treatment (week zero). Due to variability seen amongst PX treated mice at the week zero timepoint, PX-treated mice were expressed as a cohort since tactile allodynia was expected to occur immediately after PX treatment. PX-vehicle treated mice did not remain statistically different from control mice at the final time point, but topical PZ treated mice did exhibit higher paw withdrawal threshold (PWT) following PX treatment suggesting a frequency dependent trend that was a result of PZ administration (**Fig. 4.2B).**

A)



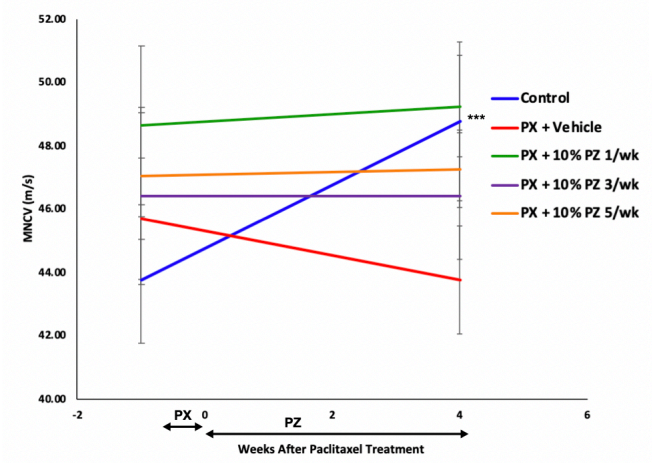
B)



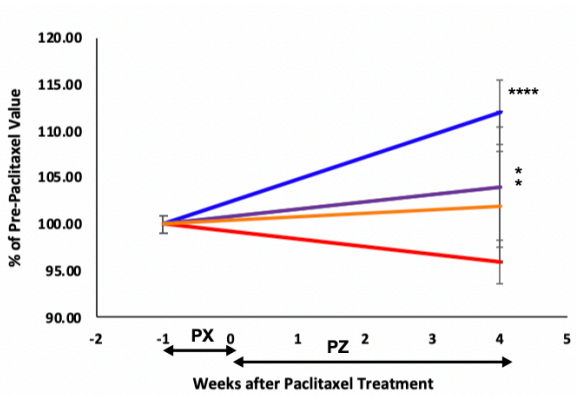
Figures 4.2: Paw tactile evoked responses in all study groups: A) Tactile illustrated across entire study B) Tactile values represented at final time point. Data are group mean±SEM of N=10/group. Statistical comparisons by two-tailed unpaired T-test at week 0 (A) and one-way ANOVA with Dunnett's post-hoc test at week 1 (B). * = P<0.05 vs control.

Motor Function: MNCV measurements were expressed as both absolute velocity (**Fig. 4.3A**) and percent change from baseline (**Fig. 4.3B**) to account for variability in baseline measurements. Control mice exhibited a significant increase in MNCV during the study (p<0.001, two tail paired t test: **Fig. 4.3A**) whereas PX administration caused mild MNCV slowing in the hind limb of vehicle treated mice (**Fig. 4.3A**). When represented as percent change from baseline, control mice were significantly (P<0.0001 vs PX: **Fig. 4.3B**) different from PX-vehicle treated mice at the final time point. Topical PZ treated mice at dose frequencies 1/wk and 3/wk were also significantly (P<0.05 vs PX: **Fig. 4.3B**) different from PX-vehicle treated mice. The 5/wk topical PZ group was not statistically different than PX-vehicle treated mice but exhibited a higher pre-PX value suggesting a topical PZ effect.

A)



B)



Figures 4.3: Motor nerve conduction velocity in all study groups: A) MNCV shown as absolute velocity across entire study B) MNCV values represented as percent change from baseline across entire study. Data are group mean±SEM of N=10/group. Statistical comparison by two-tailed paired T-test (A) and one-way ANOVA with Dunnett's post-hoc test at week 4 (B) **** = P<0.0001, *** = P<0.001, * = P<0.05 vs PX.

Discussion Points

- 20mg/kg PX caused a significant tactile allodynia in male mice (P<0.05 vs controls: **Fig. 4.2A**). PZ intervention led to a trend towards frequency dependent attenuation of tactile allodynia.
- Male control mice, unlike females, are still on a growth curve hence the increase in MNCV. Male mice, like females, exhibited MNCV slowing as a response to PX.
- Topical PZ intervention protected against MNCV slowing but did not exhibit a frequency dependent effect. When represented as percent change from baseline, at the final time point topical PZ dose frequency groups 1/wk and 3/wk were significantly (P<0.05 vs PX: **Fig. 4.3B**) different from PX-vehicle treated mice. The 5/wk treatment group was not statistically different from the PX-vehicle treated group, but trends suggest topical PZ still protected

against MNCV slowing (**Fig. 4.3A, B)**. Data overall suggests topical PZ intervention had an effect on MNCV.

- This study shows that PZ therapy can extend to males and that topical delivery of PZ is a viable therapeutic regimen, with 10% being optimal for impacting both neuropathy and neuropathic pain.

STUDY #4: OXALIPLATIN NEUROPATHY AND EFFICACY OF SYSTEMIC PIRENZEPINE TREATMENT

Purpose: To determine a) if the efficacy of PZ extends to different classes of cancer drugs that cause CIPN and b) if a dose dependent effect is present.

Design: Adult female Swiss Webster mice received PZ at their assigned doses (1.0 and 10.0 mg/kg/day) by subcutaneous injection starting three days prior to OXA administration. OXA was administered at a dose of 5mg/kg given on alternate days for a cumulative dose of 20mg/kg (see methods for details). Experimental groups were withdrawn from PZ treatment during the period of OXA treatment, in order to prevent any possible effect of PZ on OXA neurotoxicity. Once the OXA regimen was complete, mice returned to PZ treatment throughout the remainder of the study.

Health Status: One mouse in the control group died unexpectedly. OXA treatment had no impact on body weight, meaning there was no effect on general metabolic health (**Fig. 5.1**).

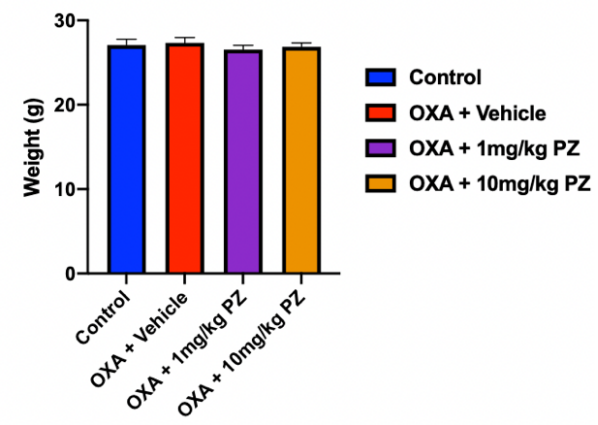


Figure 5.1: Body weight in all study groups: Body weight was measured weekly. Data are group mean±SEM of N=9-10/group. Statistical comparison vs control by one-way ANOVA with Dunnett's post-hoc test.

Sensory Function: Paw withdrawal from light pressure applied to the plantar surface via von-Frey filaments was the primary measure of painful neuropathy. OXA-treated mice developed a significant severe tactile allodynia (P<0.001 vs controls: **Fig. 5.2**) at week zero. Tactile allodynia increased in severity at week 1 while still being statistically significant (P<0.01 vs controls: **Fig. 5.2**) from control mice. Mice pre-treated with PZ (1 or 10mg/kg) also exhibited a drastic decrease in PWT, suggesting PZ pre-treatment had no neuroprotective effect. Systemic administration of PZ through the remainder of the study had no significant effect since all OXA treated mice showed attenuated allodynia over time and approached values of control mice by nine weeks post OXA treatment.

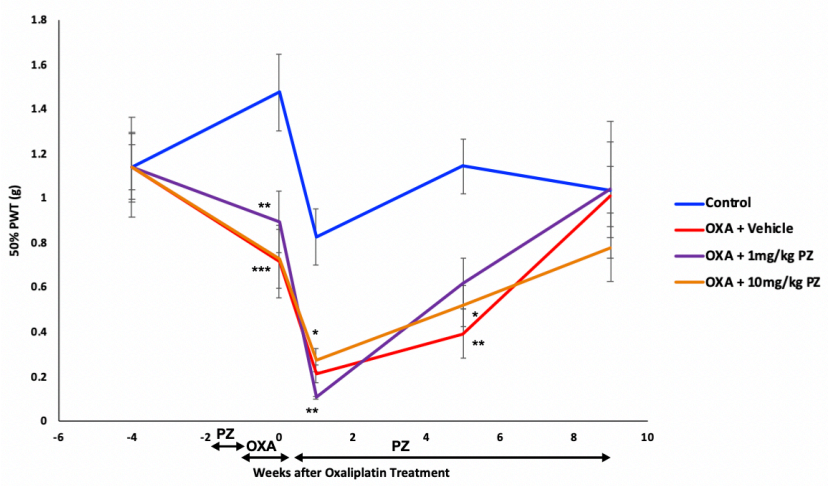


Figure 5.2: Paw tactile evoked responses in all study groups: Data are group mean±SEM of N=9-10/group. Statistical comparison by two-way ANOVA with Dunnett's post-hoc test. *** = P<0.001, ** = P<0.01, * = P<0.05 vs control.

Motor Function: MNCV was measured at three timepoints within the study. MNCV was significantly (P<0.05 vs controls: **Fig. 5.3A**) lower in vehicle treated OXA mice than control mice at the week one time point. When represented as percent change from baseline vehicle treated OXA mice were significantly (P<0.0001 vs controls: **Fig. 5.3B**) different than control mice. PZ treated mice remained stable throughout the remainder of the study whereas vehicle treated OXA mice returned to level similar to that of control mice.

A) B)

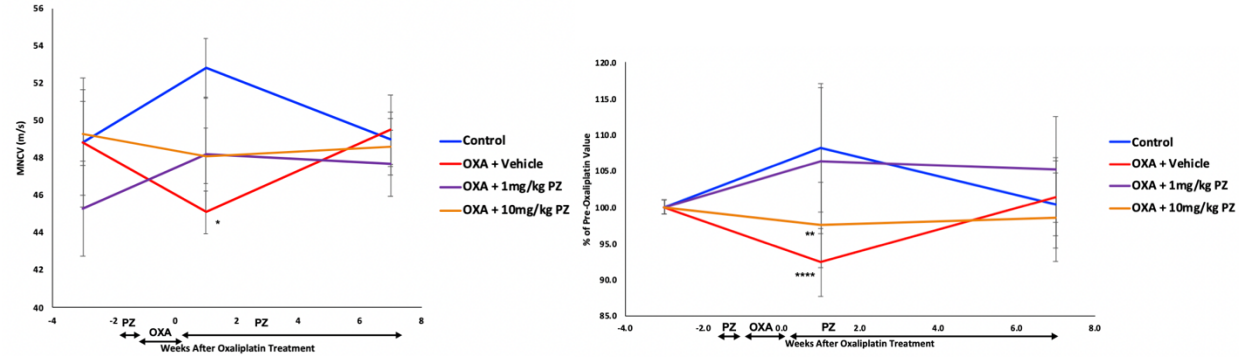


Figure 5.3: Motor nerve conduction velocity in all study groups: A) MNCV shown as absolute velocity across entire study B) MNCV values represented as percent change from baseline across entire study. Data are group mean±SEM of N=9-10/group. Statistical comparison by two-way ANOVA with Dunnett's post-hoc test. * = P<0.05 vs control.

Discussion Points

- Administration of OXA caused CIPN in mice, as indicated by an acute onset tactile allodynia that resolved by week nine post treatment and also an acute onset MNCV slowing.
- Neither the magnitude of initial allodynia nor the resolution of allodynia was markedly affected by PZ (1 or 10mg/kg). This contrasts with prior data showing efficacy of systemic (study #1) and topical (study #3) PZ against PX-induced allodynia.
- The acute onset MNCV slowing detected in OXA-treated mice was attenuated by PZ at both doses. Efficacy against MNCV slowing is consistent with similar efficacy in CIPN induced by PX (Studies 1-3).

Additional Study #4

Purpose: To determine any acute effect of 10mg/kg PZ on paw tactile response thresholds in control and CIPN mice.

Design: At the week one time point of the above study, control and OXA-treated mice were tested for paw tactile response thresholds before (baseline) and after single dose injection of 10mg/kg PZ.

Sensory Function: 10mg/kg PZ had caused an increase in PWT at the 120 minute time point in control mice (**Fig. 5.4**). Mice with OXA-induced CIPN displayed allodynia at thirty minutes post delivery that resolved by sixty minutes post-delivery.

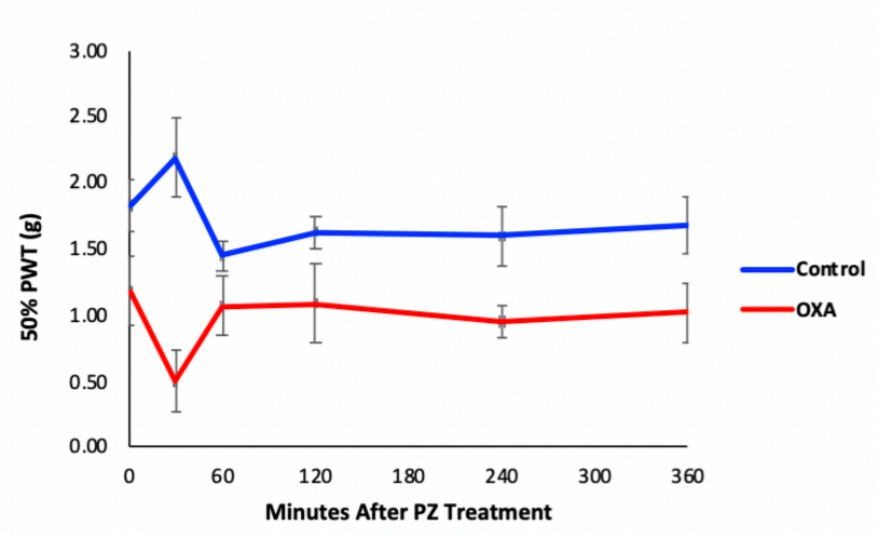


Figure 5.4: Paw tactile evoked responses following 10mg/kg PZ: Data are group mean±SEM of N=9-10/group. Statistical within group comparison by two-way ANOVA with Tukey's post-hoc test.

Discussion Points

- Control and CIPN mice react differently to acute 10mg/kg PZ.
- Acute allodynia in CIPN supports caution in not measuring paw tactile thresholds immediately following treatment and modifying study design to make measurements 24 hours after last treatment.
- This experiment must be repeated using topical PZ to determine if acute allodynia extends to different routes of administration.

STUDY #5: BORTEZOMIB NEUROPATHY AND EFFICACY OF SYSTEMIC PIRENZEPINE TREATMENT

Purpose: To determine if the efficacy of PZ extends to cancer drugs of different classes.

Design: BTZ was administered to adult female Swiss Webster mice at a dose of 1mg/kg given on alternate days for an intended cumulative dose of 5mg/kg (see methods for details and below for modification). Mice received vehicle or 10 mg/kg PZ by subcutaneous injection for five days per week. PZ treatment began three days prior to the induction of CIPN but experimental groups were withdrawn from PZ treatment during the period of BTZ treatment, in order to prevent any possible effect of PZ on BTZ neurotoxicity. Once the BTZ regimen was complete, mice returned to PZ treatment throughout the remainder of the study.

Health Status: Four of twenty mice died during the BTZ treatment regimen following the second intraperitoneal injection. Treatment was suspended so that surviving mice received a total cumulative dose of 2mg/kg. Body weight was measured throughout the study. BTZ treatment had no effect on body weight suggesting that there was no effect to metabolic health of the surviving mice (**Fig. 6.1**).

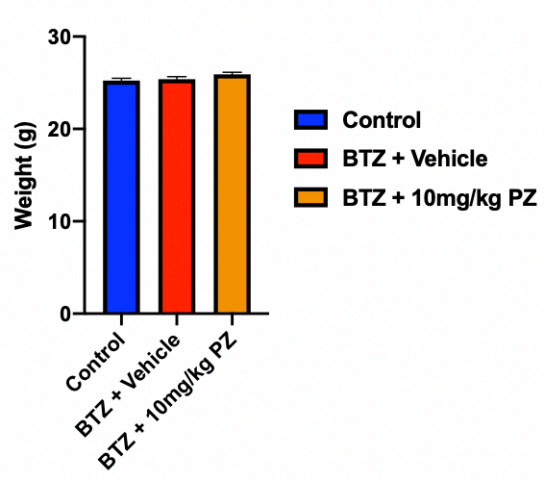
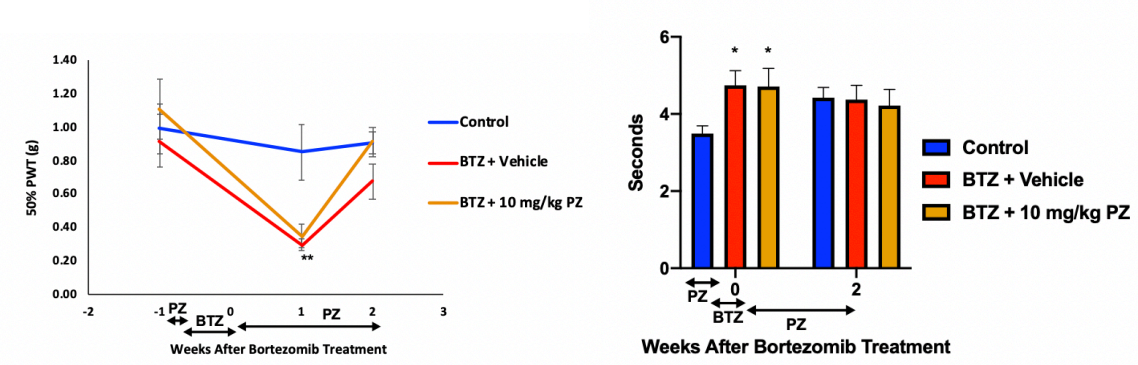


Figure 6.1: Body weight in all study groups: Data are group mean±SEM of N=7-10/group. Statistical comparison vs control by one-way ANOVA with Dunnett's post-hoc test.

Sensory Function: Paw withdrawal from light pressure applied to the plantar surface via von-Frey filaments was the primary measure of painful neuropathy. BTZ-treated mice that received vehicle developed significant ($P<0.01$ vs control: **Fig. 6.2A)** allodynia when measured one week after the end of the BTZ treatment regimen and similar allodynia was seen in BTZ mice that were pre-treated with PZ. Allodynia reverted to normal in both BTZ treated groups, but PZ treated animals saw accelerated recovery suggesting a PZ effect was present.

BTZ administration caused an acute heat hypoalgesia exhibited at the week zero time point. Heat hypoalgesia was present in the BTZ treated mice at the end of the treatment regimen and this was not prevented by pre-treatment with PZ causing a significant ($P<0.05$ vs controls: **Fig. 6.2B**) difference in heat withdrawal latency at the week zero time point. Hypoalgesia resolved within two weeks of BTZ treatment in both BTZ treated groups, so no effect of PZ treatment could be detected.

A) B)



Figures 6.2: Paw tactile (A) and heat (B) evoked responses in all study groups: Data are group mean±SEM of N=7-10/group. Statistical comparison by two-way ANOVA with Dunnett's post-hoc test. ** = P<0.01, * = P<0.05 vs control.

Motor Function: MNCV was measured at three time points: baseline, immediately after BTZ treatment, and three weeks after the completion of the BTZ treatment regimen. No significant differences were seen between any group at any time.

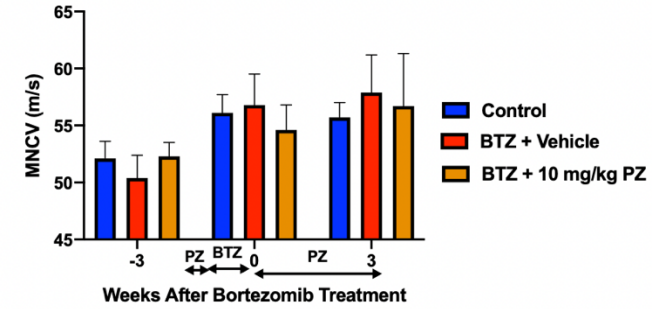


Figure 6.3: Motor nerve conduction velocity in all study groups: Data are group mean±SEM of N=7-10/group. Statistical comparison vs control by two-way ANOVA with Dunnett's post-hoc test.

Discussion Points

- Two 1mg/kg doses of BTZ were lethal to four of twenty mice. The proposed dose regimen of 5mg/kg was based on previous experiments that had demonstrated CIPN developing in C57Bl/6 mice using a similar (4.8mg/kg) cumulative dose regimen (Boehmerle et al., 2014) which had only caused the death of one mouse.
- BTZ caused acute tactile allodynia within two days that partially resolved within two to three weeks. This is a novel finding for BTZ-induced CIPN and aligns with models of PX and OXA CIPN described in earlier sections of this thesis. Prior work has demonstrated that single dose injection of 0.2, 0.5, and 1mg/kg BTZ in C57Bl/6 mice produced dose dependent effects lasting up to eleven days post treatment (Höke & Ray, 2014; Trevisan et al., 2013).
- Systemic PZ treatment appears to accelerate recovery of allodynia and this is consistent with my prior findings in PX-CIPN (Study 1), but not of OXA-CIPN (study 4), although in the latter measurements, allodynia had already spontaneously resolved in vehicle treated mice making difficult to discern if a genuine PZ effect was present.
- The acute paw heat hypoalgesia caused by BTZ is consistent with previous literature for this model (Bruna et al., 2010) and distinguishes the model from PX-CIPN where mice showed heat hyperalgesia. The absence of effect of PZ on heat hypoalgesia in the BTZ-CIPN mice suggests differential effects on different aspects of sensory dysfunction – efficacy against painful symptoms (allodynia) but not sensory loss (hypoalgesia)
- The lack of effect of BTZ on MNCV is consistent with previous literature showing that BTZ treatment in mice caused slowing of large fiber sensory nerve conduction velocity

(SNCV) but not MNCV (Bruna et al., 2010). BTZ-CIPN differs from both PX-CIPN (studies 1-3) and OXA-CIPN (study 4) which show acute MNCV slowing. This may reflect relative accessibility of different chemotherapeutics and their doses to motor neuron cell bodies in the spinal horn, where cells are protected from blood borne toxins by the blood brain barrier (see discussion for further comment).

DISCUSSION OF FINDINGS

TREATING PACLITAXEL INDUCED PERIPHERAL NEUROPATHY BY M_1R ANTAGONIST PIRENZEPINE

Modeling of CIPN in rodent models presents a number of issues, and these issues are present across multiple chemotherapeutics. For example, in PX CIPN, the mode of administration to mice varies as some researchers use intraperitoneal injections and others intravenous (Höke & Ray, 2014). Intravenous injections give the most clinically relevant model, but researchers tend to use intraperitoneal injections because it is less difficult to perform (Höke & Ray, 2014). A recent study has also shown that the age and genetic background of mice can influence the degree of CIPN development. In a previous study, PX at 4mg/kg was administered by intraperitoneal injection over four days in ten different inbred mouse strains. Statistically significant variations in mechanical allodynia were noted across all strains (Höke & Ray, 2014). This may explain why the cumulative dose range to cause CIPN in mice is so varied. Previous literature has shown that a successful cumulative dose to cause PX-induced CIPN in mice falls within the range of 4mg/kg - 38mg/kg (Eldridge et al., 2020; Food & Drug Administration, 2005; Freireich et al., 1966; Liston & Davis, 2017). With such a large dosing range and variables of age, genetic background and mode of administration there is really no uniform accepted model

of PX. In my PX studies, cumulative doses of both 5mg/kg and 20mg/kg were able to produce significant tactile allodynia and large fiber MNCV slowing.

In Study #1, administration of 5mg/kg of PX led to tactile allodynia, heat hyperalgesia, and MNCV slowing (Figure 2.2, 2.3). The presence of tactile allodynia suggests dysfunction of Aβ fibers as, despite being low-threshold mechanoreceptors responsible for light touch sensations, these fibers are also implicated in neuropathic pain sensations arising from light touch (Djouhri & Lawson, 2004).

PX administration caused significant tactile allodynia across all PX treated mice and pre-treatment for three days with PZ was not neuroprotective against onset of tactile allodynia. However, continued daily subcutaneous administration of PZ (1 and 10mg/kg) after the end of the PX regimen was able to reverse tactile allodynia whereas 0.1mg/kg PZ did not. This experiment expands on previous work which showed reversal of tactile allodynia in PX treated mice that were administered 10mg/kg PZ (Calcutt et al., 2017), by establishing the dose range for efficacy (>0.1mg/kg).

PX treatment also produced heat hyperalgesia that was present eight weeks following the end of PX treatment. Mice treated with 1mg/kg or 10mg/kg PZ were protected against increased heat sensitivity, whereas 0.1mg/kg was without effect. This is consistent with the dose range for impact of PZ on tactile allodynia. In this assay, the heat increased at a rate of 1.0°C per second, which selectively activates C-fiber nociceptors (Yeomans & Proudfit, 1996). Thus, my experiments have shown that efficacy of PZ extends across sensory Aβ and C fibers. Future experiments may investigate to see if this pattern of sensory dysfunction amelioration extends to Aδ-fibers, which can be investigated using pressure tests to measure mechanical hyperalgesia.

Along with multiple forms of sensory dysfunction, MNCV slowing was exhibited following PX treatment. MNCV slowing was analyzed using both absolute values and percent change from baseline. Unlike tactile allodynia, PZ protected against MNCV slowing at all three doses, including 0.1 mg/kg/day. This suggests motor dysfunction may be more sensitive to PZ than sensory dysfunction. This may be surprising, as motor neuron cell bodies are located behind the blood brain barrier (BBB) in the ventral horn of the spinal cord whereas sensory neuron cell bodies are located in the dorsal root ganglia, which lie outside the BBB and have fenestrated capillaries that allow easy access of blood-borne drugs. It is plausible that electrophysiology is a more sensitive assay for detecting efficacy of PZ than behavioral tests such as the tactile and thermal tests.

Based on positive results in the PZ reversal study, the topical PZ reversal study examined whether a different route of administration could be effective in ameliorating CIPN. Having used multiple doses in the previous study, we used multiple formulations of topical PZ to examine if a dose dependent effect was present. A previous study used topical PZ in diabetic mice at a variety of concentrations to successfully ameliorate diabetic peripheral neuropathy (Jolivalt et al., 2020). In that study, 10% topical PZ was the only concentration that displayed neuroprotective effects on MNCV, whereas tactile allodynia was prevented by doses at or below 1% (Jolivalt et al., 2020). It is notable that this is the inverse of my findings with systemic PZ, where MNCV was more sensitive to PZ than tactile allodynia.

PX treatment caused a transient tactile allodynia across all treated groups that resolved within four weeks of PX administration. This resolution occurred more quickly than in my previous study (study #1), in which allodynia persisted for at least eight weeks. I have no explanation for this as I used the same PX treatment regimen, sex, age, and strain of mice. The

lack of statistical difference between PX-vehicle treated mice and control mice prevents any demonstration that topical PZ resolved tactile allodynia. It is possible that more frequent testing of mice between weeks 1-4 may have identified a time point where allodynia was present, and efficacy of PZ could be assessed. However, at weeks four and eight, mice treated topically with PZ showed either no effect or a trend to delayed recovery from tactile allodynia in comparison to the PX-vehicle treated group. This contradicts study#1 in which PZ treatment accelerated recovery. One explanation for this could be that tactile testing was conducted within hours after topical treatment, so that any acute pro-allodynic effect could disrupt identification of chronic anti-allodynic effects. This possibility was addressed in a separate study using mice with OXA-induced CIPN (see below). Future studies in PX CIPN with topical PZ were modified to prevent disruption by acute allodynic effects of PZ.

PX administration caused significant MNCV slowing that was present immediately after cessation of the PX regimen. This is consistent with study#1. However, unlike study#1 MNCV slowing did not increase in severity as the study continued. A dose-dependent attenuation of MNCV slowing was seen with 1% PZ being not effective. Efficacy of PZ by topical delivery is consistent with my earlier finding using systemic delivery. This suggests a clinically viable route of delivery for PZ to treat neuropathy in PX-CIPN, as applying a topical solution will be more acceptable to patients than systemic injection. This was also a reversal study, with PZ not administered until after the end of the PX regimen. This means that in a clinical setting, only patients who develop CIPN will need treatment with PZ, rather than having to treat all patients who start PX chemotherapy. This is important as meta-analysis shows that PX-induced peripheral neuropathy has affected 44% to 98% of patients (Duggett et al., 2017; Seretny et al., 2014).

In the final PX study, we investigated whether dose frequency played a role in the efficacy of topical PZ therapy. The effect of dose frequency on peripheral neuropathy has been examined in a previous study of female Swiss Webster diabetic mice using 2% topical PZ (Jolivalt et al., 2020). In that study, following topical PZ treatment that was administered five days a week, diabetic mice had paw thermal responses similar to those of control mice, whereas diabetic mice treated with vehicle or 2% PZ for one or three days a week remained significantly different from control mice (Jolivalt et al., 2020).

Of the four topical PZ concentrations used in study #2, we selected the 10% concentration as most likely to show efficacy against PX neurotoxicity. Previous experiments using mice with diabetic peripheral neuropathy have shown stronger concentrations of topical PZ to be more effective. Thus 2% topical PZ was able to prevent tactile allodynia and loss of IENF, but it was only when 10% topical PZ was used that MNCV slowing was also prevented in both ipsilateral and contralateral limbs (Jolivalt et al., 2020). This guided our choice of using the strongest concentration of topical PZ for our dose frequency study.

Further study design modifications were to increase the cumulative dose of PZ from 5 to 20 mg/kg, to measure allodynia at earlier time points after the PX regimen ceased and to measure tactile responses only 24 hours or more after the last topical treatment with PZ. The increase in dose was done in response to the unexpectedly mild allodynia and rapid reversal of allodynia seen in study #2 and the suspicion that topical PZ may cause an acute transient allodynia that disrupts measurement of pain relief (discussed above). Operational concerns also meant that I was only able to include measurements made after one and two weeks of PZ treatment.

PX caused a significant tactile allodynia in male mice at the week zero timepoint, and PZ intervention led to a trend suggesting attenuation of tactile allodynia. A frequency dependent

effect was present, as each topical PZ treated group saw attenuation of allodynia following one week of topical PZ treatment. The 5/wk group caused the greatest recovery of allodynia whereas the 1/wk and 3/wk groups saw less marked recovery, but greater recovery than PX-vehicle treated groups. At the final time point, all PX treated groups were no longer statistically different compared to control mice, but the upward trend seen across each topical PZ treated group suggests that topical PZ impacted neuropathic pain regardless of dose frequency since each PZ treated group exhibited higher PWTs than PX-vehicle treated mice (Figure 4.2 A, B).

PX treatment caused mild MNCV slowing when represented as both absolute m/s and percent change from baseline (Figure 4.3A, B, C). Topical PZ treatment following PX administration protected against MNCV slowing at all dose frequencies (Figure 3.3A). Efficacy of PZ corroborates my previous PZ treatment data of studies #1 and #2. Moreover, as once weekly topical treatment with PZ was equally effective as three or five days per week, this data suggests that motor nerves do not require continuous exposure to PZ for efficacy, as topical PZ washes out of the blood within 24 hours of delivery (Jolivalt et al., 2020).

In summary, the M_1R antagonist PZ was shown to be successful in reversing indices of PX induced neuropathy, with generally greater efficacy against MNCV slowing. PZ was also shown to have versatility in its administration route since it was able to protect against and reverse MNCV slowing by either subcutaneous injection or ipsilateral topical administration. Finally, PZ was effective against tactile allodynia and MNCV slowing when given intermittently, suggesting a modification of neuropathy phenotype rather than simple acute receptor blocking pharmacology. These findings support advancement of PZ, potentially as a weekly topical administration, as a therapy for PX-induced CIPN.

TREATING OXALIPLATIN INDUCED PERIPHERAL NEUROPATHY BY M_1R ANTAGONIST PIRENZEPINE

Development of OXA induced peripheral neuropathy is influenced by genetic polymorphisms that determine what CIPN features develop. Various mouse strains exposed to OXA show significant differences in behavioral features and severity of CIPN (Salat, 2020). Polymorphisms in the SCN9A gene, which is associated with the Na_V 1.7 ion channel have been to protect against OXA induced CIPN shown in human patients (Salat, 2020; Sereno et al., 2017). As with PX CIPN, genetic variability and dosing schedule make it difficult to establish a uniform model of OXA. However, unlike PX there is one commonly accepted mode of administration - intraperitoneal injection (Höke & Ray, 2014). Ranges of cumulative OXA levels that typically cause CIPN in mice range from 3 - 30 mg/kg (Eldridge, 2020; Food & Drug Administration, 2005; Friereich et al., 1966; Liston & Davis, 2017). I selected a cumulative dose of 20mg/kg, as this falls within range of what is commonly used and accepted.

OXA produced significant tactile allodynia that was present one day after OXA administration and increased in severity by day eight but began to resolve within one month and had returned to control values by two months. PZ, which was given for three days prior to the OXA regimen, did not have a neuroprotective effect. Moreover, restarting PZ treatment after the end of the OXA regimen did not accelerate recovery of allodynia, although the slow testing frequency (only at one and two months) may mean that early effects were missed.

Nevertheless, this is consistent with study #2, where transient tactile allodynia in PX-vehicle treated mice was followed by a rapid recovery that prevented resolution of any efficacy of PZ. Unfortunately, assays were conducted within less than 24 hours of the previous PZ dose and thus I cannot exclude any acute allodynic effect of PZ. This later possibility was therefore

addressed in an adjuvant study in which control and OXA mice received a single dose of 10mg/kg PZ and were tested for tactile responses over subsequent hours. PZ caused a trend of acute hypoalgesia in control mice that resolved within an hour, whereas in mice with OXA-CIPN it produced a transient allodynia that also resolved within an hour. It is not yet clear what about OXA-CIPN modifies this acute response to PZ. However, there is some precedence for M_1R antagonists having differential effects in nerve from normal vs neuropathic rodents. For example, the specific M_1R antagonist MT7 increased AMPK phosphorylation (activation) in trigeminal ganglia and also promoted corneal sensory nerve growth when applied topically to the eye of mice with OXA-induced CIPN but did not have similar effects in control mice (Saleh et al., 2020). As AMPK activation is associated with increased mitochondrial activity and decreased mitochondrial activity has been linked to CIPN, it is possible that the acute allodynia is due to PZ having an immediate effect on mitochondrial respiration in neurons of OXA-treated mice that produces a transient release of ROS, which is known to promote allodynia.

The role of AMPK being linked to CIPN is corroborated by literature showing western blots of OXA treated mice exhibiting decreased protein levels of P-AMPK. Intervention by the specific M_1R antagonist MT7 protected CIPN mice from decrease of P-AMPK (Saleh et al., 2020). Indirectly, this data demonstrates how PZ may contribute to acute allodynia.

AMPK augments PGC-1 alpha activity, which regulates mitochondrial function, but in order to augment this activity, AMPK must first be phosphorylated by the upstream activator CamKKB (Saleh et al., 2020). Once phosphorylated by CamKKB, AMPK forms a complex that modulates mitochondrial biogenesis, function, and regeneration (Saleh et al., 2020; Feige & Auwerx, 2007; Hardie, 2008; Marcelo et al., 2016). CamKKB, AMPK, and PGC-1 alpha each rely on each other through a downstream pathway, and in cases of mitochondrial dysfunction this

pathway may be affected. In the case of P-AMPK, the presence of decreased levels of P-AMPK as a result of OXA-CIPN suggests that OXA toxicity may cause downregulation of CamKKB during periods of high energy demand such as when nerves are degenerating. CamKKB activation has key requirements that include rise of intracellular calcium along with availability of calmodulin (Saleh et al., 2020; Marcelo et al., 2016). Mechanisms that contribute to development of OXA-CIPN include dysregulation of calcium homeostasis along with altered function of respiratory chain causing an increase of ROS (Starobova & Vetter, 2017). Thus, it is possible that the presence of acute allodynia following PZ treatment is due to PZ already exacerbating present mitochondrial dysfunction through its attempt to ameliorate respiration capacity, which may inadvertently lead to release of ROS and contributing to the neuropathic pain state. Future experiments can determine if mechanisms of OXA-CIPN such as increase of ROS and calcium homeostasis dysregulation are exacerbated acutely by PZ administration.

Another possibility is that because we did not measure tactile responses in vehicle treated control and OXA-CIPN mice, the different behavioral responses to PZ could simply reflect different responses to handling or the injection. This can be addressed by repeating the studies to include control and OXA-CIPN mice that received an injection of vehicle.

OXA treatment caused significant MNCV slowing in OXA-vehicle treated mice that was detected within one week of finishing the OXA regimen and resolved by week seven. PZ treatment appeared to have a partial effect at the week one time point. For future experiments, MNCV needs to be tested at greater regularity. Having the second time point and the final time point be separated by six weeks may have led to effects being missed.

This study established a model of OXA-induced CIPN in mice that showed acute tactile allodynia and mild MNCV slowing that resolved in seven weeks. PZ treatment appeared to

partially ameliorate MNCV slowing. This suggests that M_1R antagonism may be a viable therapy for multiple forms of CIPN.

TREATING BORTEZOMIB INDUCED PERIPHERAL NEUROPATHY BY M_1R ANTAGONIST PIRENZEPINE

The chemotherapeutic BTZ is a proteasome inhibitor that produces a novel form of CIPN that is not fully understood (Höke & Ray, 2014). Studies have attempted to model clinical administration of BTZ in rodents, but neurophysiologic results seen in animals were not replicable in patients (Höke & Ray, 2014; Cavaletti et al., 2007). BTZ, like other chemotherapeutics has produced variable manifestations of neuropathy that make developing a uniform model difficult. The dose of BTZ used is dependent on the route of administration as well as the dosing schedule. In one study, female Sprague-Dawley rats were administered intraperitoneal injections at 0.2 mg/kg for five consecutive days, and this resulted in no deaths, whereas another study administering the same dose intravenously in female Wistar rats three times a week for eight weeks led to eight deaths (Höke & Ray, 2014; Zheng et al., 2012; Meregalli et al., 2012). In mice, there is also no standard model of BTZ CIPN due to the lack of consistency across studies (Höke & Ray, 2014). One study administered various single doses (0.2, 0.5, 1mg/kg) of BTZ by intraperitoneal injection in C57Bl/6 mice and saw a dose dependent neuropathy develop lasting up to eleven days following injection, whereas another study using Swiss OF1 female mice administering a 1mg/kg dose subcutaneously twice a week for six weeks saw a mild to moderate sensory neuropathy (Höke & Ray, 2014; Trevisan et al., 2013; Bruna et al., 2010).

Given the lack of guidance from the literature, I originally attempted to administer a 1mg/kg dose of BTZ every other day for five days by intraperitoneal injection. However, prior

to completing the dosing schedule, this led to the death of four of twenty mice at the cumulative dose of 2mg/kg. I therefore halted the dosing schedule and investigated neuropathy in the remaining mice. BTZ caused acute tactile allodynia detected one week after dosing ceased that partially resolved at two weeks. Vehicle treated BTZ mice saw recovery of tactile allodynia. However, PZ treated group experienced a trend to accelerated recovery of tactile allodynia that appears similar to my earlier findings in PX-CIPN.

BTZ had no impact on large fiber MNCV. My results corroborate what has been previously seen in a similar model (Bruna et al., 2010).

BTZ has been shown to induce a mixed large and small fiber axonal sensory neuropathy, which is why we also measured paw thermal response latency (Zajączkowska et al., 2019). Heat hypoalgesia has been reported following high doses of BTZ in mice (Zheng et al., 2012; Bruna et al., 2010; Meregalli et al., 2010). BTZ treatment caused significant acute heat hypoalgesia that resolved within two weeks and was not prevented by PZ. Since hypoalgesia of BTZ-vehicle treated mice and PZ treated mice resolved at the same time, further studies will need to examine shorter timepoints to determine if PZ has an effect.

GENERAL DISCUSSION AND FUTURE STUDIES

PART 1: OVERVIEW OF CIPN

CIPN affects up to 70% of patients that undergo chemotherapy, so many individuals are in need of a therapy (Hopkins et al., 2016). CIPN has no currently FDA-approved method of treatment. Modeling neuropathic pain is a difficult task since animal studies can only measure stimulus evoked and non-stimulus evoked behaviors (Deuis et al., 2017). Since pain cannot be directly measured in animal models, behavioral tests causing evoked behavior are the closest estimate. Along with this, more problems stem when attempting to standardize studies because

research to date has used varied administration routes, dosing schedules, and concentrations of chemotherapeutic agents (Höke & Ray, 2014). In my research, I investigated how antagonizing M_1R can prevent or reverse CIPN in different chemotherapeutic drug classes. The M_1R antagonist PZ, attenuated, prevented or reversed specific indices of CIPN. This supports further development of PZ as a treatment that can lead to improvement of quality of life in cancer patients receiving chemotherapy

PART 2: CIPN AND MITOXICITY

As discussed above (see Introduction), many molecular mechanisms are involved in the development of CIPN. Each of the drug classes I investigated induce mitotoxicity, causing enlargement, swelling, and vacuolation of mitochondria (Waseem et al., 2017). One of the key contributors to mitochondrial degeneration is opening of the mPTP which leads to a series of adverse events such as ATP depletion, increase of ROS and Ca^{2+}, which contribute to cell death (Canta et al., 2015). This is pertinent since 90% of mitochondria in the peripheral nervous system are located within axons, and events such as those listed can contribute to neuronal dysfunction and distal degeneration of both sensory and motor axons (Waseem et al., 2017; Vincent et al., 2002). Damage to the mitochondria can also disrupt expression and function of mitochondrial proteins, which in turn affects respiratory chain function and ATP production (Staff et al., 2017).

PART 3: MITOCHONDRIA, CIPN AND M_1R

PGC1-alpha has been shown to likely be a downstream target of AMPK, since AMPK directly phosphorylates PGC1-alpha leading to increases in PGC1-alpha protein action on the PGC1-alpha promoter (Jäger et al., 2007). Literature has shown that AMPK driven increases of mitochondrial gene expression requires PGC1-alpha, thus showing how the two are dependent

on each other. Previous work has shown that neurons derived from diabetic rodents exhibited reduced levels of mitochondrial proteins and decreased respiratory capacity that are restored by administration of M_1R antagonists (Calcutt et al., 2017). A recent study from my laboratory and collaborators showed reduced P-AMPK in trigeminal ganglia of mice with OXA-CIPN, indicative of reduced signaling through the AMPK/PGC1a pathway that regulates mitochondrial function (Saleh et al., 2020). PGC1-alpha plays an important role in oxidative metabolism of brown fat and muscle by increasing mitochondrial biogenesis (Jäger et al., 2007). Further, P-AMPK was greatly enhanced by topical delivery of a M_1R antagonist to the cornea, a part of the eye that receives sensory innervation from the trigeminal ganglion (Saleh et al., 2020). This supports the idea that PZ is acting by restoring mitochondrial function in models of CIPN. In future research I would like to perform assays of mitochondrial respiratory function using my laboratories oroboros respirometer and protein assays for mitochondrial proteins by western blotting to see if PZ administration can rescue respiratory capacity and mitochondrial protein levels in the peripheral nerve of mice with CIPN induced by PX, OXA and BTZ.

PART 4: IENF LOSS, CIPN AND M_1R INDUCED PROTECTION/RECOVERY

The ability of M_1R antagonism to promote increased mitochondrial respiration in peripheral nerves has been associated with a capacity to promote nerve growth and regeneration (Calcutt et al., 2017; Saleh et al., 2020). In vitro studies show that multiple M_1R antagonists, including PZ, promote neurite outgrowth from sensory neurons derived from the DRG of normal, diabetic and CIPN mice (Calcutt et al., 2017; Saleh et al., 2020) which are a model of axonal regeneration following transection. In vivo studies also showed that loss of IENF in diabetic mice could be prevented and reversed by systemic or topical PZ treatment (Calcutt et al., 2017; Jolivalt et al., 2020). IENFs represent the peripheral terminal regions of small sensory Aδ

and C fiber types that shed their ensheathing Schwann cells when they cross the dermal/epidermal junction (Boyette-Davis et al., 2011).

One of the earliest manifestations of CIPN is loss of IENF density, which has been correlated with greater dysfunction in thermal sensitivity along with mechanical hyperalgesia/allodynia (Boyette-Davis et al., 2011; Shun et al., 2004; Walk et al., 2007). My studies identified tactile allodynia induced by 3 classes of chemotherapeutic, PX, OXA and BTZ, while thermal hyperalgesia was present in the PX model and hypoalgesia in the BTZ model. Samples of foot skin were collected from some of these animals and have been archived. In future studies I would therefore count IENF density using histological techniques to determine whether IENF loss occurred and the impact of PZ treatment.

PART 5: CORNEAL NERVES CIPN AND M_1R INDUCED PROTECTION/RECOVERY

The peripheral terminal regions of sensory nerves may also be assessed by corneal confocal microscopy. This technique is non-invasive and thus allows iterative assessment of onset and progression of distal degenerative neuropathy in both humans and rodents. In humans, loss of corneal nerves has been described in diabetic peripheral neuropathy and appears to be an early and sensitive indicator of onset of small fiber neuropathy (Quattrini et al., 2007), including CIPN (Argyriou et al., 2020). In humans with CIPN there are reports of abnormal corneal nerve architecture (Petropolous et al., 2020). Following the presence of CIPN, remaining corneal nerves that survived degeneration exhibit distinct morphological patterns indicating small fiber degeneration. Previous research has also shown that C57Bl/6 mice receiving a single intraperitoneal injection of PX of varying concentrations (5mg/kg, 10mg/kg, 20mg/kg) exhibited a dose-related reduction of corneal nerves that correlated with other measures of small fiber neuropathy such as loss of IENF (Ferrari et al., 2013). Further, our recent study that applied the

selective M_1R antagonist MT7 direct to the eye of mice with OXA-CIPN (Saleh et al., 2020) found that increased trigeminal ganglion P-AMPK levels were accompanied by recovery of previously reduced corneal nerve density. I was able to collect images of corneal nerves from some of the studies described in this thesis (Study #3, Study #4) and would perform future analyses to determine the effects of PZ in preventing reduction of corneal nerve density in multiple models of CIPN.

PART 6: LARGE FIBER DYSFUNCTION and M_1R INDUCED PROTECTION/RECOVERY

Along with small nerve fibers being subjected to degeneration by chemotherapeutics, large fibers are also affected as seen with MNCV slowing, which represents Aβ fiber function. Nerve conduction studies that measure compound muscle action potential amplitude and latency of evoked responses are used to assess CIPN in animal models (Fukuda et al., 2017). Chemotherapy treatment in rats induces decreased sciatic nerve fiber diameter as well as nerve conduction slowing (Fukuda et al., 2017; Authier et al., 2000; Persohn et al., 2005; Arrieta et al., 2011). Degree of sciatic nerve degeneration is dependent on many variables: chemotherapeutic administered, route of administration, species strain, and dose concentration (Han & Smith, 2013). Symptoms of PX neurotoxicity in the sciatic nerve of mice range from macrophage-mediated demyelination to no pathological changes (Han & Smith, 2013; Mo et al., 2012; Carozzi et al., 2010). Symptoms of OXA neurotoxicity in the sciatic nerve of mice have shown degeneration of fibers, along with platinum accumulation (Marmiroli et al., 2017). BTZ has little to no impact in MNCV and does not impact the sciatic nerve as seen in study #5 and previous literature.

PZ was shown to impact MNCV slowing in both PX and OXA treated mice. Since symptoms of neurotoxicity vary between chemotherapeutics, it is plausible that PZ's mechanism of action may protect against axonopathy and or demyelination. PZ prevented MNCV slowing in PX neurotoxicity, and whereas in OXA-CIPN PZ partially reversed MNCV slowing. PZ's mechanism of action in this case could be that it promoted regeneration of nerve fibers since CIPN pathology did develop following OXA treatment. For future experiments, sciatic nerve tissue will be analyzed through histology to determine if PZ's effects are through preventing axonopathy, demyelination or promoting nerve fiber regeneration.

PART 7: SUMMARY

My research examined whether M_1R antagonism with PZ is a viable therapy for CIPN in different classes of chemotherapeutics, and whether route of administration could affect its efficacy. PZ was found to prevent or reverse certain indices of neuropathy and neuropathic pain in each drug class (see Table 1 below). My findings support the advancement of PZ as a systemic or topical therapy for CIPN towards future clinical trials, while in vitro experiments will guide further understanding of the molecular mechanisms that underlie PZ's mechanism of action when treating CIPN.

TABLE 1: **Summary of symptoms that developed in varied classes of CIPN, and whether PZ intervention had an effect toward attenuating, preventing, or reversing symptoms:**

MODEL	STUDY	Onset of allodynia (Vehicle + CIPN)	PZ pre-treatment effect	Resolution of allodynia (Vehicle + CIPN)	PZ intervention effect	Onset of thermal dysfunction (Vehicle + CIPN)	PZ pre-treatment effect	Resolution of thermal dysfunction (Vehicle + CIPN)	PZ Intervention Effect	Onset of MNCV slowing (Vehicle + CIPN)	PZ pre-treatment effect	Resolution of MNCV slowing (Vehicle + CIPN)	PZ Intervention Effect
PX - CIPN	1	Yes	No	No	Yes	Yes, Heat hyperalgesia	ND	No	Yes	Yes	ND	No	Yes
PX - CIPN	2	Yes	ND	Yes	No	ND	ND	ND	ND	Yes	ND	No	Yes
PX - CIPN	3	Yes	ND	No	Yes	ND	ND	ND	ND	Yes	ND	No	Yes
OXA - CIPN	4	Yes	No	Yes	No	ND	ND	ND	ND	Yes	ND	Yes	Partial
BTZ - CIPN	5	Yes	ND	Yes	Yes	Yes, Heat hypoalgesia	No	Yes	No	No	No	No	No

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