Tissue hypoxia, oxidative stress and topical analgesics in the treatment of complex regional pain syndrome and peripheral neuropathic pain

Oli Abate Fulas

Integrated Program in Neuroscience

Faculty of Medicine

McGill University

August 2020

A thesis submitted to McGill University in partial fulfillment

of the requirements of the degree of Doctor of Philosophy

ABSTRACT

Peripheral neuropathic pain (PNP) and complex regional pain syndrome (CRPS) are chronic pain conditions whose pharmacological treatments are partial in efficacy and cause adverse systemic effects. This work is aimed at developing more effective and better-tolerated analgesics for PNP and CRPS through the targeting of peripheral tissue hypoxia and oxidative stress using topical agents. Both are processes that are prominent inducers of peripheral sensitization and can be directly impacted by topically administered treatments whose systemic effects are minimal.

Initial studies assessed the analgesic effect of a new topical formulation made by combining the vasoactive agent meldonium and the antioxidant N-acetyl cysteine. The treatment alleviated mechanical hypersensitivity in rat models of PNP and CRPS by enhancing nitric oxide-mediated tissue oxygenation. Thus, topical treatment reversed the reduction in tissue oxygenation observed by percutaneous oxygen saturation measurements on the hind paws of chronic post-ischemic pain (CPIP) rat models of CRPS. The anti-allodynic dose of topical meldonium-NAC also produced a significant increase in the nitric oxide levels of plantar muscle of rats with CPIP. In line with this, pre-treatment with a nitric oxide synthase inhibitor attenuated the topical anti-allodynic effects of meldonium-NAC.

In subsequent studies, the repertoire of similarly acting topical combinations was expanded and the method of formulating the combinations refined with the goal of simplifying the drugs' development and hastening clinical translation. To this end, mechanochemistry was used to synthesize novel anti-hypoxic-antioxidant salts and co-crystals by pairing pentoxifylline with protocatechuic acid, clonidine with α-lipoic acid and linsidomine with caffeic acid. These three novel products displayed enhanced efficacy and/or potency in alleviating mechanical hypersensitivity after topical administration to CPIP rats. Follow up mechanism of action studies were performed on a prototype of the new products, a co-crystal between pentoxifylline and protocatechuic acid (pentx-pca). Percutaneous tissue oxygen saturation (SaO_2) measurements taken from the hind paw of CPIP rats revealed that anti-allodynic doses of topical pentx-pca increased local tissue SaO_2. Moreover, assessment of oxygen-dependent mitochondrial function using a triphenyl tetrazolium chloride (TTC) assay showed topical treatment with pentx-pca

significantly alleviated mitochondrial dysfunction in the plantar muscle of CPIP rats. The time point-dependent resolution of plantar muscle mitochondrial dysfunction, that occurred in the CPIP rats at six weeks post-procedure, paralleled the loss of the anti-allodynic response to topical treatment with pentx-pca.

The last section of this thesis presents a phase-II clinical study protocol set to assess the analgesic efficacy of an anti-hypoxic topical combination of clonidine and pentoxifylline as evidence for the potential and feasibility of this approach. The study was designed as a randomized, double-blind and placebo-controlled investigation. It is a single-center, 5-week long cross-over study in which 2-week long treatments with the topical combination and placebo will be administered to the patients with an intervening 1-week washout period. Treatment outcomes are to be comprehensively measured in all patients with a combination of daily assessments of spontaneous pain intensity and sensory tests of dynamic mechanical allodynia and punctate hyperalgesia administered during study visits.

To improve the pharmacological treatment of PNP and CRPS, this work proposes new anti-hypoxic-antioxidant topical analgesics formulations, including newly synthesized salts and co-crystals, that produced potent anti-allodynic effects by alleviating peripheral tissue ischemia/hypoxia and its metabolic consequences. It also establishes the manner by which these treatments can be developed into investigator-driven clinical trials that will aid in their translation.

RÉSUMÉ

Les traitements pharmacologiques de la douleur neuropathique périphérique (DNP) et du syndrome de douleur régionale complexe (SDRC) sont peu efficaces et source d'effets systémiques adverses. Le présent projet vise à développer des agents analgésiques efficaces et tolérables pour ces deux conditions en ciblant les mécanismes de l'hypoxie tissulaire et du stress oxydatif qui sont d'importants agents de sensibilisation nociceptive périphérique et de surcroît susceptibles à des traitements topiques aux effets systémiques minimes.

Les premières expériences démontrèrent les effets analgésiques d'une nouvelle préparation topique combinant l'agent vasoactif meldonium et l'antioxidant N-acétylcystéine (NAC), qui atténua l'allodynie mécanique dans un modèle du SDRC chez le rat en accroissant l'oxygénation tissulaire dépendante de l'action de l'oxyde nitrique. La mesure de la saturation d'oxygène dans le sang permis de démontrer que l'application topique de la préparation prévenait ainsi la réduction de l'oxygénation tissulaire du membre traité après induction de l'allodynie de la patte par un épisode d'ischémie. De plus, les doses topiques de meldonium-NAC ayant des effets analgésiques produisirent un accroissement significatif des niveaux d'oxyde nitrique de la musculature plantaire des pattes traitées. Conformément à ces résultats, une autre expérience démontra que les effets anti-allodyniques de la préparation topique de meldonium-NAC étaient atténués par l'administration d'un agent inhibiteur de la synthase de l'oxyde nitrique.

Les expériences suivantes eurent pour but d'élargir la gamme de combinaisons topiques efficaces et de simplifier la méthode de leur préparation pour faciliter leur application clinique. De nouveaux sels et co-cristaux ayant des propriétés anti-hypoxiques et anti-oxydatives furent produits par procédé mécanochimique en associant la pentoxifylline à l'acide protocatéchique, la clonidine à l'acide alpha-lipoïque et la linsidomine à l'acide caféique. Comparée à l'utilisation individuelle de leurs composants, l'application topique de chacun de ces trois nouveaux agents produisit une activité accrue de l'effet anti-allodynique observé sur le modèle de douleur post-hypoxique du SDRC chez le rat. Des expériences portant sur la caractérisation des mécanismes d'action d'un de ces nouveaux produits furent ensuite réalisées avec un co-cristal de la pentoxifylline et de l'acide protocatéchique (pentx-pca). La mesure de la saturation d'oxygène sanguin (SaO2) permis d'établir que l'application topique de pentx-pca à des doses anti-allodyniques sur la patte post-hypoxique du rat en avait accru l'oxygénation tissulaire. De plus,

l'évaluation des fonctions mitochondriales dépendantes de l'oxygénation par la méthode du chlorure de tétrazolium triphényle (TTC) permis d'établir que l'application topique de pentx-pca avait réduit la dysfonction mitochondriale des muscles plantaires qui caractérise ce modèle de douleur post-hypoxique du SDRC. On observa aussi sur ce modèle que le rétablissement de la dysfontion mitochondriale post-hypoxique était complet six semaines après son induction et, parallèlement, que l'activité anti-allodynique de la pentx-pca avait disparu au bout de ce laps de temps.

La dernière section du présent travail décrit un protocole d'étude clinique de phase-II qui a pour but d'évaluer la faisabilité et l'efficacité analgésique d'un agent topique anti-hypoxique combinant la pentoxifylline et la clonidine dans une étude à répartition aléatoire, contrôlée avec placebo et à double insu. L'étude à site unique de type cross-over s'échelonne sur 5 semaines durant lesquelles deux périodes de traitement de deux semaines chacune utilisant la préparation topique et un placebo seront administrées en ordre aléatoire et séparées par une période d'élimination d'une durée d'une semaine. Les résultats cliniques seront évalués à partir du journal quotidien de l'intensité de la douleur obtenu de chaque patient ainsi que par des mesures de l'allodynie mécanique et de l'hyperalgésie ponctuelle effectuées lors de cinq visites programmées en clinique.

Le présent travail propose d'améliorer le traitement pharmacologique de la DNP et du SDRC par l'emploi de nouvelles préparations analgésiques topiques, incluant de nouvelles synthèses de sels et de co-cristaux possédant des effets anti-hypoxiques et anti-oxydatifs pouvant soulager les conséquences métaboliques de l'hypoxie cellulaire et de l'ischémie périphérique. De surcroît, le présent travail constitut un exemple de comment développer ces nouveaux traitements par la recherche clinique menée directement à l'initiative des chercheurs dans le but d'en favoriser l'application plus rapidement.

ACKNOWLEDGEMENTS

Above all, I would like to honor and glorify my Lord and savior JESUS CHRIST who has sustained me through the past few years. “Through Him all things were made; without Him nothing was made that has been made.” John 1:3.

My heartfelt gratitude and appreciation go to my supervisor Professor Terence J Coderre who kindly and patiently guided me through the years of my training. I will remain grateful for his mentorship and support. I would also like to thank the members of my advisory committee, Drs Laura Stone, Ji Zhang and Mark Ware for their discussions, advice and encouragement.

I am indebted to Andre Laferriere for his valuable advice, excellent technical support and resourcefulness that made the completion of this thesis possible. I am not only grateful for his professional contribution to my work but also his kindness and friendship. I would also like to acknowledge and thank him for writing the French translation of my abstract.

I am immensely grateful to Dr Robin S. Stein for her kind willingness to support my projects with her expertise. I am also thankful for her guidance in helping me find professors in the Chemistry Department whose collaboration greatly aided the completion of my projects. I would like to thank Professor Tomislav Friščić for agreeing to a collaboration with my supervisor and expanding the breadth of my graduate training. I am thankful to members of the Friščić group for accommodating me. I am especially grateful to Ghada Ayoub for her training and supervision as I strived to learn new skills that were foreign to my field. I would also like to thank Professor Scott Bohle for his kind collaboration and his generosity with both his time and resources.

I would like to acknowledge and thank the Louise and Alan Edwards Foundation for their generous funding of the first three years of my training. They have also granted financial support that made a significant portion of my projects possible. My utmost respect and gratitude go to the foundation.

Lastly, I would like to extend my love and appreciation to my family, especially my parents, Baharnesh Yadete and Abate Fulas. Their unconditional love, support and prayers have carried me throughout my life. Their commitment and sacrifice have made my achievements in life possible. I will forever remain grateful for having them as my parents. I would also like to thank my husband, Habte, for his continued love, patience and understanding through the ups and downs of the past few years.

CONTENTS

List of abbreviations

ANOVA: analysis of variance

API: active pharmaceutical ingredient

ASICS: acid-sensing ion channels

ATP: adenosine triphosphate

AUC: area under the curve

BOLD: blood oxygen level dependent

CCI: chronic constriction injury of the sciatic nerve

CIPN: chemotherapy-induced painful neuropathy

clon: clonidine

CNS: central nervous system

CPIP: chronic post-ischemia pain

CRPS: complex regional pain syndrome

CSF: cerebrospinal fluid

DBF: Doppler blood flow

DMA: dynamic mechanical allodynia

DMSO: dimethyl sulfoxide

DPPH: 2,2-diphenyl-1-picrylhydrazyl (*N*-phenyl-*N*-[(2,4,6-trinitrophenyl)-λ^2-azanyl]aniline)

DRG: dorsal root ganglion

DSC: differential scanning calorimetry

EPR: electron paramagnetic resonance

EtOH: ethanol

FDA: *US* Food and Drug Administration

Fig : figure

fMRI: functional magnetic resonance imaging

FTIR-ATR: Fourier-transform infrared attenuated total reflectance

GABA: gamma-amino butyric acid

GCP: Good Clinical Practice

GRAS: generally regarded as safe

Hala: α-lipoic acid

HBO_2T: hyperbaric oxygen treatment

Hcafa: caffeic acid

($Hclon^+$)(ala): salt of clonidine and α-lipoic acid

($Hclon^+Cl^-$): clonidine hydrochloride

HIF-1: hypoxia-inducible factor-1

($Hlin^+$)($cafa^-$): salt of linsidomine and caffeic acid

($Hlin^+Cl^-$): linsidomine chloride

Hpca or pca: protocatechuic acid

i.p.: intraperitoneal

IASP: International Association for the Study of Pain

IHC: International Council for Harmonization

IRI: ischemia reperfusion injury

LAG: liquid-assisted grinding

LDF: laser Doppler flowmetry

LED: light emitting diode

lin: linsidomine

L-NAME: *Nω*-nitro-L-arginine methyl ester

MNIC: mononitrosyl iron dithiocarbamate

MUHC: McGill University Health Center

NAC: N-acetyl cysteine

(Na^+cafa^-): sodium caffeate

Na-DETC: sodium diethyldithio-carbamate trihydrate

NaOH: sodium hydroxide

NIRS: near-infrared spectroscopy

NMR: nuclear magnetic resonance

NO: nitric oxide

NOS: nitric oxide synthase

NSAIDs: nonsteroidal anti-inflammatory drugs

PDE: phosphodiesterase

PEG: polyethylene glycol

pentx: pentoxifylline

(pentx)(Hpca) or pentx-pca: co-crystal of pentoxifylline and protocatechuic acid

PHA: punctate Hyperalgesia

PNP: peripheral neuropathic pain

PTFE: polytetrafluoroethylene

PWT: paw withdrawal threshold

PXRD: powder x-ray diffraction

RBC: red blood cell

RCT: randomized controlled trials

REB: research ethics board

RNS: reactive nitrogen species

ROS: reactive oxygen species

RRID: research resource identifier

SaO_2: oxygen saturation

SCXRD: single crystal x-ray diffraction

SI: supporting information

SNRI: serotonin-norepinephrine reuptake inhibitors

SNS: sympathetic nervous system

SOD: superoxide dismutase

ssNMR: solid-state nuclear magnetic resonance

TCA: tricyclic antidepressants

TGA: thermogravimetric analysis

THF: tetrahydrofuran

TRP: transient receptor potential

TRPV1: transient receptor potential vanilloid 1

TTC: triphenyl tetrazolium chloride

VAS: visual analog scale

V/V: volume/volume

W/V: weight/volume

W/W: weight/weight

List of tables & figures

1 INTRODUCTION

1.1 Neuropathic pain & CRPS: *epidemiologic considerations*

Chronic pain, defined as pain that lasts or recurs for longer than 3 months, is a condition that has a global prevalence of 10-25 % [1, 2]. It is one of the main causes of human suffering and disability that follows various traumatic injuries, stroke, toxic exposures, and viral and metabolic diseases [2]. In addition to impairing individuals' quality of life and productivity, it is costly on a larger scale especially to the health care system [3].

Nearly 18 % of chronic pain patients suffer from neuropathic pain [4]. This number is projected to rise significantly with the increase in the aging population, and the prevalence of diabetes mellites and survival after cancer treatments [5]. Up to 70 % of patients on standard chemotherapy regimens and 20 % of diabetic patients suffer from painful peripheral neuropathy [6, 7]. Complex regional pain syndrome (CRPS), on the other hand, has a yearly incidence between 5.5 and 26.2 cases per 100,000 [8]. Both neuropathic pain and CRPS are more common in females and patients above 50 years of age [5, 9].

1.2 Etiology & symptomatic characterization of peripheral neuropathic pain (PNP) & CRPS

CRPS is a primary chronic pain disorder that occurs as a complication of traumatic injury to the limbs that ranges from minor contusions and sprains to more extensive damage due to fracture, crush injury, or surgery [10, 11]. It most often occurs after fractures, with an incidence of 7 % in the 4 months following limb fractures [12, 13]. The condition is divided into CRPS-1 and CRPS-2 based on the absence or presence of clinically-detectable peripheral nerve damage, respectively [12]. Despite this common diagnostic classification, the two subtypes are largely indistinguishable with regards to signs and symptoms, pathophysiology and treatment responses [14].

Patients with CRPS complain of painful limbs with allodynia (perception of innocuous stimuli as painful) and hyperalgesia (perception of uncomfortable or slightly painful stimuli as disproportionately more painful) [9]. There is usually associated edema, alterations in skin color, temperature and sweating; and there are also changes in the pattern of hair, nail and skin growth [9, 15]. Further examination, particularly after a longer duration of pathology, may reveal reduced

limb strength, tremor and dystonia [15]. A clinical diagnosis of CRPS is commonly given following the Budapest criteria that constitute sensory, motor, sudomotor, vasomotor and/or trophic findings, and exclusion of alternative diagnoses [12].

The symptoms of CRPS widely vary and are often noted to exhibit a pattern with a temporal progression [9]. Commonly, in patients that present within months of an inciting event, affected limbs appear warm, red and edematous and are labeled 'warm CRPS'. Presentation in the later years of the disease course is usually associated with a cold, cyanotic and sweaty extremity, informally categorized as 'cold CRPS' [16, 17]. Further progression sometimes yields motor abnormalities like dystonia and tremor, which is observed much later and likely caused by disuse due to pain that is disabling. Although CRPS of any duration can have any of the above presenting features, acute CRPS (of duration less or equal to a year) mostly presents as warm CRPS, whereas chronic CRPS typically has features of cold CRPS [9].

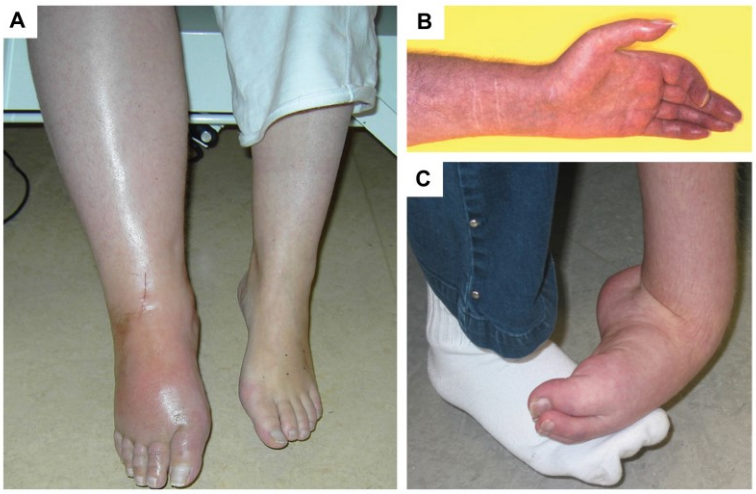

Figure 1. Limbs of patients with CRPS.

(**A**) 'Warm' CRPS features in acute CRPS with hyperemia and edema. (**B**) Chronic, 'cold', CRPS with cyanosis of fingers, increased hair and nail growth. (**C**) CRPS-induced dystonia of the left ankle joint. (Reprinted from The Lancet Neurology, Vol. 10, Marinus, J, et al., Clinical features and pathophysiology of complex regional pain syndrome, 637-648., Copyright (2011), with permission from Elsevier.[18])

On the other hand, neuropathic pain is chronic pain that develops as a direct consequence of damage to the somatosensory nervous system [19]. The anatomic location of the lesion, with specific involvement of the brain and spinal cord, or alternatively peripheral nerves, is the basis

for its classification into central and peripheral neuropathic pain [20]. The majority of the neuropathic pain population suffers from peripheral neuropathic pain (PNP) [21]. As such, the work presented in this thesis is focused on PNP.

Damage to the peripheral nervous system can result from a heterogeneous list of etiologies. Some of these etiologies produce generalized and symmetrical damage to the peripheral nerves, while others cause a more focal involvement, and a select few manifest in both patterns [5]. Focal damage results in pain and dysfunction localized to the innervation boundaries of the affected peripheral nerve. Alternatively, generalized painful neuropathy often symmetrically affects distal peripheral nerve endings in a length-dependent manner, with a distal to proximal progression [5]. Common causes of generalized painful peripheral neuropathy include exposure to chemotherapeutic agents, alcohol and environmental toxins, metabolic dysfunctions like uremia, nutritional deficiencies, immune and inflammatory disorders, and inherited neuropathies and channelopathies [5, 22]. In contrast, post-herpetic neuralgia, post-traumatic neuropathy, post-surgical neuropathy, cervical and lumbar radiculopathy and trigeminal neuralgia are some of the common forms of focal painful peripheral neuropathy [5, 23]. Diabetes mellitus, HIV and leprosy can result in a distribution of painful peripheral neuropathy that is either focal, generalized, or a combination of the two [5, 23].

Regardless of the etiology, the quality of symptoms and physical findings exhibited by patients with PNP has some uniformity [24]. Subjective complaints commonly include pain of burning, tingling and electric shock-like quality [5, 25]. Thermal and mechanical allodynia and hyperalgesia are also common, with or without a concurrent sensation of numbness [5, 25]. A compilation of these symptoms into a patient-reported questionnaire is used as a screening tool for diagnosis. The DN4 or painDETEC are examples of simple to use questionnaires, which differentiate neuropathic pain from non-neuropathic chronic pain with high specificity and sensitivity [26, 27]. However, diagnostic certainty is achieved only after performing an objective neurological assessment to identify the area affected and confirm that it is neuroanatomically congruent with the patient's complaints [5, 28]. This is usually done at the bedside with quantitative sensory testing that evaluates responses to touch, pinprick, pressure, cold, heat and vibration [5, 28, 29]. When warranted, a definite diagnosis of neuropathic pain can be made following neurophysiological tests like nerve conduction velocity measurement, laser-evoked

potentials and skin biopsy that assesses either the responsiveness or abundance of nociceptors in the superficial layers of the skin [5, 29].

As for all chronic pain conditions, both neuropathic pain and CRPS patients report disturbances in their sleep, appetite and mood, with many succumbing to depression and anxiety [9, 14, 30, 31]. These consequences of chronic pain are more pronounced in patients with chronic neuropathic pain than those with non-neuropathic pain [31]. Added to the burden of suffering from persistent pain, these impairments of physical, emotional and mental well-being greatly compromise the quality of life of these patients with CRPS and neuropathic pain [30].

1.3 Proposed mechanisms of PNP & CRPS

CRPS and PNP are both the outcome of a maladaptive response to tissue injury, with CRPS commonly involving a limb in its entirety, while PNP is localized to the distribution of a peripheral nerve [18, 32]. The pathophysiologic processes underlying both conditions are multifactorial with changes at both the injury site and remotely in the CNS [18, 33]. Evidence indicates the involvement of dysfunctions in the neural, immune and vascular systems, with interactions that have yet to be clearly defined [5, 9]. Altered neural sensory processing is due to sensitization in peripheral sensory nerves and upstream pathways within the central nervous system (CNS), with the added role of hypothesized sympathetic nervous system (SNS) dysfunction in CRPS [9, 32]. Immune dysregulation with ramped up proinflammatory and diminished anti-inflammatory activity is observed at the local site of injury and the CNS for both CRPS and PNP [34, 35]. Local vascular impairment with resultant ischemic-hypoxia of the affected limb, in CRPS, and the injured peripheral nerve, in PNP, is also a significant contributory factor [36-38]. The roles played by these peripheral processes are interdependent and not mutually exclusive, but are henceforth individually discussed for clarity of description.

1.3.1 Peripheral neural mechanisms in the pathophysiology of PNP & CRPS

1.3.1.1 Pathophysiologic contribution of changes in the peripheral nerves to PNP & CRPS

In PNP, the causative insult produces a varying extent of damage to the nerve fibers, and changes to their structure and function, including regenerative growth and maladaptive molecular changes that yield hyperexcitability [5]. While clinically identifiable nerve injury is lacking in CRPS-1, reduced epidermal neurite densities, as evidence for focal small-fiber neuropathy, has

been reported [39]. Moreover, signs of nerve fiber loss and regenerative response of variable extent have been observed from histological studies on amputated limbs of CRPS patients [40].

In both PNP and CRPS, primary afferent fibers exhibit a state of hyperexcitability with increased background firing, increased responses to painful stimuli, and decreased thresholds for thermal and mechanical stimuli [5, 9, 41]. While a similar pattern of outcomes is observed in both conditions, the mechanistic details are better worked out for PNP.

Injured peripheral nerves in PNP show both structural and functional alterations of their terminals and axonal projections impacting nociceptive transduction and transmission [33]. Transient receptor potential (TRP) channels, in sensory afferent terminals, are responsible for transducing mechanical, thermal and chemical stimuli [42]. In PNP, there is upregulation of TRP channels along with functional alterations, characterized by an increase in magnitude and duration of excitability [33, 42]. Moreover, there are reports of increased expression and trafficking of voltage-gated sodium and calcium channels in injured nerve terminals, axons, and cell bodies in the dorsal root ganglia (DRG) [43-45]. Following nerve injury, the $\alpha 2\delta$ subunits of voltage-gated calcium channels get preferentially upregulated [44]. These subunits facilitate the insertion of voltage-gated calcium channels at neurotransmitter release sites facilitating more efficient use of the calcium influx that follows an action potential to drive exocytosis [46]. The sodium channels also exhibit intrinsic functional changes like rapid activation and increased current density [33]. This increase in the electrogenic properties of the neuronal membrane causes enhanced subthreshold oscillations that augment the neurons' tendency to fire impulses resulting in ectopic activity [43]. There is a concurrent decrease in the expression of inhibitory potassium channels after nerve injury [47].

These changes in damaged peripheral nerves summate to form the neural basis for peripheral sensitization, where irritable nociceptors result in the pain and hypersensitivity seen PNP and CRPS patients [5, 9]. Predictably, the pathology is not confined to the peripheral nerves, rather the ectopic firing and faulty signal transmission in the injured afferent sensory nerves readily trigger more maladaptive changes in second and higher-order neurons eventually rendering the entire pain circuitry hyperresponsive [5].

1.3.1.2 Sympathetic nervous system changes in CRPS

Dysfunction in sympathetic nervous system activity was initially considered central to the genesis of CRPS, which prompted the first naming of the condition as “reflex sympathetic dystrophy” [48]. Early theories proposed that injury-induced afferent sensory input triggered a heightened and sustained sympathetic outflow that resulted in pain, vasoconstriction and edema, along with the other symptoms and signs of CRPS [48, 49]. This theory was mainly reinforced by reports of pain relief following sympathetic blocks and sympathectomies performed on CRPS patients [48]. However, subsequent studies failed to establish the pathophysiologic role and therapeutic targeting of the SNS in CRPS [50]. Sympathetic activity was demonstrated to be blunted rather than enhanced in CRPS, with lower serum catecholamine levels in CRPS affected limbs compared to the unaffected limb [51]. In line with this, experimental manipulations of cutaneous sympathetic activity with thermal suits failed to alter the diminished venous norepinephrine levels in both warm and cold CRPS patients [52]. The experimental sympathetic stimulation also did not produce the expected change in skin blood flow and vasoconstriction in these patients [52]. Moreover, intraneural recordings from the skin of CRPS patients, with evidence of vasoconstriction, failed to show enhanced sympathetic activity [50, 53]. The efficacy of sympatholytic block for CRPS has also been found to be inconsistent [54]. Alternate explanations have since been proposed for features of CRPS previously attributed to SNS dysfunction [9, 50].

Although sympathetic dysregulation on its own fails to fully explain CRPS, it is still believed to contribute to the disease pathophysiology in at least a subset of CRPS patients [55]. Upregulated expression of α_1-adrenoceptors in dermal nerve fibers and keratinocytes of CRPS affected patients have been reported along with enhanced responsiveness to locally or systemically released norepinephrine during SNS activity [56-58]. This compensatory upregulation of peripheral adrenergic receptors is induced by the reduced levels of circulating norepinephrine in the acute phases of CRPS [13]. Later on in the disease, the abundantly expressed adrenergic receptors cause excessive vasoconstriction and sweating resulting in the cold and cyanotic appearance of the affected limb [13].

The staining intensity of adrenoceptors in epidermal cells has been positively associated with pain intensity in CRPS patients [57]. In one study, the intradermal injection of the α_1-

adrenoceptor agonist phenylephrine produced prolonged pain and hyperalgesia to pinprick in CRPS patients with enhanced α_1-adrenoceptor expression [59]. This evidence for the adrenergic sensitivity of nociceptors, named sympatho-afferent coupling, is a phenomenon that is also observed after peripheral nerve injury [13, 53].

1.3.2 Peripheral immune mechanisms in the pathophysiology of PNP & CRPS

In PNP, the inflammatory immune response that follows nerve injury progressively impacts all the neurons in the pain processing pathway. Distally located, the degenerating cutaneous nerve terminals of an injured peripheral nerve trigger an innate cellular immune response that increases the release of proinflammatory cytokines [60]. These molecules, in addition to recruiting more inflammatory cells, directly activate and sensitize the nociceptor terminals [61]. At the site of nerve injury, there is an accumulation of circulating neutrophils, monocytes, macrophages and later on lymphocytes [62]. The recruited inflammatory cells are primarily intended for the removal of debris and subsequent regeneration. However, they also secrete a substantial quantity and variety of inflammatory molecules that further alter sensory transduction, inducing hyperresponsiveness and ectopic activity of the nerves [35]. In the DRG, parallel inflammatory cell infiltration and glial cell proliferation occur causing the cellular production of mediators that endogenously act to further enhance the excitability of peripherally-located transduction channels and receptors [60].

The inflammatory processes described above have a direct impact on the development of the PNP phenotype. The reduction of inflammation by injection of recombinant lymphocytes that produce anti-inflammatory cytokines has been shown to reduce nerve-injury induced hypersensitivity in mice [63]. Similarly, the administration of molecules that inhibit glial activation has been shown to reverse and prevent the pain behavior of rats with spinal nerve injury [64]. Moreover, the genetic deletion or pharmaceutical blockade of microglial chemokine receptors that receive inflammatory signals from injured primary afferents has been shown to also prevent and reverse mechanical allodynia in mice with partial sciatic nerve ligation injury [65].

There is also abundant evidence for the role of immune and inflammatory mechanisms in CRPS. Neuropeptides, like bradykinin and substance P, and cytokines that promote inflammation, are increased both locally, in the blister fluid and skin biopsies, and systemically, in the serum and CSF, of patients with CRPS [9, 66-68]. A parallel decrease in anti-inflammatory cytokines also

occurs especially in early CRPS [66]. An altered cellular immune response, with an increased number of pro-inflammatory monocytes and mast cells that possibly contribute to the excess production of inflammatory cytokines, has also been reported in CRPS patients [69, 70]. The increased production of cytokines, inflammatory neuropeptides and growth factors in keratinocytes and sensory afferent nerve terminals of CRPS skin both maintain local inflammation and increase the levels of inflammatory mediators [18, 71]. The raised levels of inflammatory mediators locally increase vascular caliber and permeability resulting in the redness, warmth and edema seen in CRPS affected limbs [72, 73]. In the long run, exposure to these substances has been shown to produce trophic effects on the skin, sweat glands and hair follicles contributing to the CRPS symptoms profile [9].

There have been reports of a direct correlation between the extent of mechanical hyperalgesia exhibited by CRPS patients and the level of inflammatory cytokines in blister fluid obtained from their affected limbs [74]. Furthermore, mice injected with immunoglobulins obtained from CRPS patients exhibit CRPS-like hyperalgesia, edema and motor changes [75].

1.3.3 Vascular mechanisms of PNP & CRPS

1.3.3.1 The microvasculature & vascular supply of the peripheral nerve

The microvasculature is a term used to refer to the network of arterioles, capillaries and venules that lie between the arterial and venous vessels of the body. It plays a central role in the regulation of blood flow and the perfusion pressure necessary for nutrient and oxygen delivery to tissues [76]. Circulating hormones, locally-derived mediators, cellular, neural and physical factors act on the microvasculature to determine its tone and patency for the regional distribution of blood [77].

Peripheral nerves receive their blood supply from adjacent arteries through segmental arterioles and capillaries named *vasa nervorum* [78]. These are several small blood vessels that run longitudinally along the epineurium or the outermost connective tissue surrounding peripheral nerves (Figure 2) [79]. The epineural vessels give out branches at regular intervals to penetrate the perineurium or the protective sheath encasing nerve fascicles, and the endoneurium or the thinner layer of connective tissue around nerve axons. Sympathetic tone influences the walls of the epineural arterioles via noradrenergic nerve fibers branching from nearby sympathetic ganglia. The vessels in the endoneurium mostly consist of capillaries that provide blood supply to

individual nerve fibers. Coordination between epineural vascular supply and endoneurial blood flow sustains oxygen and nutrient delivery to peripheral nerve fibers [80].

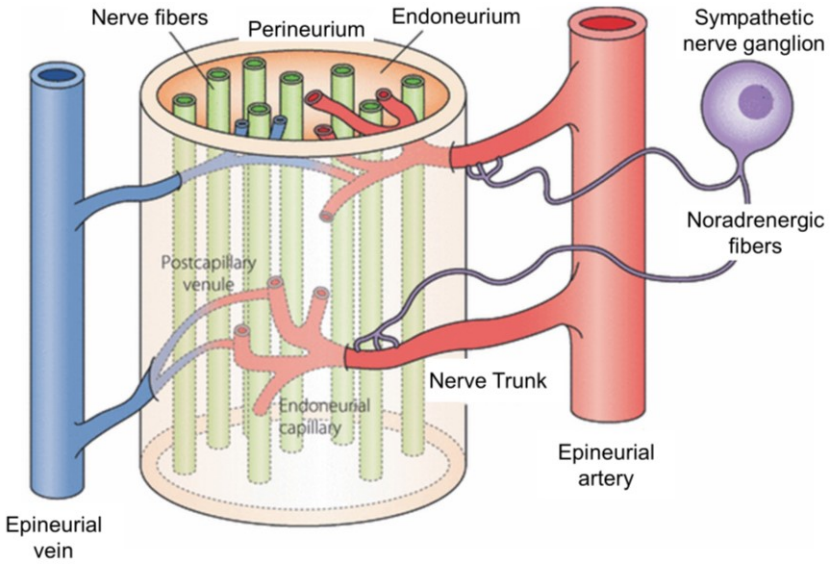

Figure 2. Simplified scheme of the vascular supply of the peripheral nerve.

(Reprinted from Journal of Diabetes Investigation, Vol. 2, Yagihashi, S, et al., Mechanism of diabetic neuropathy: Where are we now and where to go? 637-648., Copyright (2010), with permission from John Wiley & Sons. [79])

1.3.3.2 The vascular endothelium

The walls of all blood vessels, including the *vasa nervorum* and endoneurial capillaries, are lined by the endothelium: a thin layer of squamous epithelial cells, apposed on an extracellular matrix that forms the basement membrane [81]. Endothelial cells are critical determinants of microvascular blood flow [82]. The cells synthesize and release vasodilatory molecules including nitric oxide (NO) and prostacyclin, as well as the vasoconstrictor peptide endothelin [82]. NO is generated by the endothelial cells from L-arginine using endothelial NO synthase [83]. In response to shear stress, endothelial cells use the enzyme cyclooxygenase to produce prostacyclin from arachidonic acid [84]. Both NO and prostacyclin produce vasodilation by diffusing into the underlying vascular smooth muscle cells and altering cyclic mononucleotides levels, causing the cells to relax [85]. Endothelin is a large polypeptide synthesized by the endothelium, and one of its fragments, endothelin-1, causes vasoconstriction by acting on its vascular smooth muscle cell receptors [82]. Local vascular tone is regulated by the summated effects of endothelial NO and endothelin-1, and norepinephrine-mediated sympathetic output [86].

1.3.3.3 Vascular changes in PNP

Numerous studies have assessed the involvement of the vasculature in peripheral neuropathies that cause chronic pain, particularly in the cases of post-traumatic, diabetic and chemotherapy-induced painful neuropathies. A common theme in the reported findings is maladaptive changes to the *vasa nervorum* leading to peripheral nerve ischemic-hypoxia. Abnormal activity in the distal endings of affected nerves can be established and maintained by an accumulating extent and duration of vascular pathology.

1.3.3.3.1 Vascular changes in post-traumatic peripheral neuropathy

Several clinical and preclinical studies have reported pathologic microvascular changes in post-traumatic PNP. Thickening of endoneurial micro-vessels has been observed from the histologic assessment of biopsies from patients with painful chronic entrapment of the superficial radial nerves [87]. Similarly, endoneurial edema and increased endoneurial pressure, resulting in ischemic nerve injury, has been shown in patients with carpal tunnel syndrome, a condition diagnosed in patients who have pain as a consequence of median nerve compression in the wrist [88]. These changes are also seen in diabetic neuropathy, where plasma extravasation into the endoneurial space causes increased interstitial pressure around the nerve and the formation of thrombus and deposition of fibrin [89].

Endoneurial vessel narrowing with protrusion and proliferation of endothelial cells has been seen in rat models of post-traumatic neuropathic pain induced by loosely constrictive ligatures placed around the sciatic nerve [90]. Similarly, endoneurial ischemic-hypoxia is observed in the first few hours post-injury and persists for up to 4 weeks after partial ligation of the sciatic nerve in mice [37]. The ligation injury caused the growth of fibrotic tissue in the endoneurial space impeding diffusion of oxygen and nutrients through the endoneurium. Ischemic- hypoxia of the nerve was worsened by increased metabolic demands in the injured nerve due to Schwann cell proliferation and inflammatory cell infiltration [37].

1.3.3.3.2 Vascular changes in painful diabetic neuropathy

In diabetic peripheral neuropathy, structural alterations in the *vasa nervorum* are preceded by earlier functional changes caused by the metabolic insults of diabetes that cause an imbalance between vasodilation and vasoconstriction. In particular, there are deficits in the vasodilators NO and prostacyclin in the vascular endothelium of tissues affected by diabetes [91].

The microvascular impairment in diabetic neuropathy affects both the peripheral nerve and the skin it innervates [89, 92]. Local microvascular sympathetic dysfunction causes increased cutaneous arteriovenous shunting and reduced dermal nutritive blood flow in the skin of patients with painful diabetic neuropathy [93]. The extent of microvascular dysfunction in these patients is correlated with the visual analog scale (VAS) scores of their pain.

Histologic studies have revealed thickening of the capillary basement membrane and endothelial cell layer with consequent arteriovenous shunting in sural nerve biopsies from patients with diabetic neuropathy. This microvasculature pathology was shown to correlate with the neurophysiological and neuropathological measures of neuropathic severity in the patients [91, 94]. The arteriovenous shunting in the *vasa nervorum*, where the blood fails to flow in the nerve capillary beds, is also a particular feature of acute painful neuropathy of rapid glycemic control, also called insulin neuritis [95]. In this condition, adaptation to nerve hypoxia occurs with the growth of new vessels on the surface of the affected nerves.

A reduction in endoneurial oxygen tension, as measured by *in vivo* microelectrode and spectrophotometric studies, occurs in patients with diabetic sensory neuropathy [96, 97]. This has, in turn, appeared to precipitate a vicious cycle of microvascular changes with endoneurial hypoxia, and reduced diffusion capacity of oxygen through defective micro-vessels further propagating maladaptive basement membrane thickenings and endothelial cell hyperplasia [91].

The microvascular changes in rodent models of diabetic neuropathy are of similar nature to what is observed in diseased humans. Decreased peripheral nerve perfusion, caused by diminished vasodilatory responses to endothelial NO, and increased vasoconstrictive responses to norepinephrine and endothelin-1, is found in rats with early-stage Streptozotocin-induced diabetes [98, 99]. Interestingly, the endoneurial hypoxia caused by the perfusion deficits, shown in these studies, has been observed to correlate significantly with mechanical and thermal hypersensitivity exhibited by the neuropathic rats [91, 98].

1.3.3.3.3 Vascular changes in chemotherapy-induced painful neuropathy (CIPN)

Many of the chemotherapeutic agents in wide clinical use cause dose-limiting painful peripheral neuropathy [7]. The anti-mitotic effects of these drugs, intended for retarding neoplastic proliferation, are accompanied by antiangiogenic effects that damage the host's microvasculature including the *vasa nervorum* [100]. Endothelial apoptosis within the *vasa nervorum* and eventual

decline in the density of *vasa nervorum* has been observed in rodent models of chemotherapy-induced neuropathy [101, 102]. This resulted in the reduction of nerve blood flow and perfusion as measured by *in vivo* Doppler flow imaging. Ischemic injury to peripheral nerves produces a focal axonal degeneration, and this appears to be a major contributor to the overall pathology of CIPN [101, 102].

1.3.3.4 Vascular changes in CRPS

Impaired endothelial function and local vascular reflexes produce exaggerated arteriolar vasoconstriction and reduced vasodilatory responses in the microvasculature of CRPS affected tissue [103, 104]. In line with this, diminished levels of vasodilatory NO, along with increased vasoconstrictive endothelin-1, have been found in blister fluid taken from CRPS-1 affected limbs [105]. Moreover, acetylcholine-induced endothelium-dependent vasodilation, as measured by laser Doppler flowmetry, was observed to be significantly impaired in CRPS-1 affected limbs [106]. As acetylcholine is a known stimulator of endothelial NO production, experimental assessment of endothelium-dependent vasodilation can be performed following its administration [107].

The ischemic environment caused by the decreased tissue perfusion in CRPS promotes the generation of free radicals that results in a vicious cycle of endothelial dysfunction, sustained hypoxia and production of more free radicals [106, 108]. This set of events forms the basis for the chronic post-ischemic pain (CPIP) rodent model of CRPS [49, 109] (discussed in section 1.5.2.1). CPIP rodent paws bear a stark phenotypic resemblance to the limbs of CRPS patients and exhibit evoked nociceptive behavior of similar quality to the human CRPS-1 syndrome [109].

Superimposed on these functional microvascular impairments, is the eventual occurrence of structural changes in the microvasculature that further worsen tissue hypoxia. The integrity of the microvasculature endothelial lining is damaged in CRPS-1 affected limbs as evidenced by endothelial cell shrinkage and clogging of the capillary lumen [108, 110]. The basal membrane of the microvasculature has also been reported to exhibit multi-lamination and thickening causing further impairment of blood flow due to luminal narrowing [110].

Impaired microvascular function and tissue hypoxia are observed in both ‘warm’ and ‘cold’ CRPS despite the vast differences in the appearance of the limbs of these patients (see Figure 1). Sympathetic dysfunction has been shown to cause enhanced blood flow in large and medium

vessels with concurrent arteriovenous shunting sparing little flow for the perfusion of tissue capillaries in months-long ‘warm’ CRPS [111]. On the other hand, a global reduction of arterial blood flow along with maladaptive changes in both the function and structure of the microvasculature has been observed in limbs with ‘cold’ CRPS, where disease duration usually surpasses a year [112].

Moreover, intracutaneous capillary hemoglobin oxygenation is reduced in the affected limbs of patients with acute CRPS as compared to measurements from their unaffected limbs and that of healthy controls [38]. Post-occlusive reactive hyperemic responses are also impaired in CRPS affected limbs indicating lower microvascular reserve [104]. Both findings suggest there is a lowering of nutritive blood flow in early CRPS. The absence of nutritive blood flow in the warm and hyperemic limbs of such patients is due to increased flow through arteriovenous shunts. This AV shunting along with high venous oxygen saturation has been observed from radiographic studies in CRPS affected limbs indicating poor capillary flow and oxygen extraction [111].

The downstream effects of ischemic-hypoxia, like anaerobic glycolysis, have been shown to result in a reduction in adenosine triphosphate (ATP) levels and accumulation of lactate, not only in the skin but also the deeper-lying muscle of CRPS patients [113, 114]. Declines in tissue pH and ATP levels have been reported from spectroscopic measurements taken from lower leg skeletal muscle of patients with CRPS, implying impaired high energy phosphate metabolism caused by cellular hypoxia [113].

A direct link between tissue acidosis and pain symptoms has been suggested in CRPS. Studies have shown that low pH infusion-induced acidification of the skin and muscle produced increased pain in CRPS patients, which they described to be of identical quality to that of their primary disease [115]. Tissue acidosis activates nociceptive peripheral nerves. A buildup of lactate and excess protons can directly activate the acid-sensing ion channels (ASICs) on peripheral nociceptors [116]. Additionally, lactate accumulation has been shown to promote the chelation of extracellular divalent ions resulting in enhanced permeability and activity of ASICs on nociceptors [117, 118]. A pervasive state of tissue acidosis, as it is observed in CRPS, can by itself result in sustained hyperactivity of peripheral nociceptors paving way for long term-downstream changes that result in chronic pain symptoms [49].

1.3.4 Oxidative Stress in the mechanisms of PNP & CRPS

1.3.4.1 The normal balance of the pro-oxidant-antioxidant processes in the body

Reactive oxygen species (ROS) are a group of highly reactive chemical species derived from molecular oxygen. They form as byproducts of normal, as well as pathological, cellular metabolism. The three major ROS of physiologic relevance are superoxide anions, the hydroxyl radical and hydrogen peroxide [119]. ATP synthesis by the mitochondria entails the transfer of electrons through respiratory chain enzymes, and at the final step, the free electrons reduce molecular oxygen to water, in parallel with ATP production. 1-4% of the molecular oxygen that acquires these free electrons is incompletely reduced and converts into superoxide [120]. Superoxide is the most common ROS and serves as a substrate for the enzymatic and nonenzymatic synthesis of other free radicals. Superoxide dismutase, an enzyme that exists in many isoforms, converts superoxide to another less reactive ROS, hydrogen peroxide. Hydrogen peroxide can, in the presence of transition metals like copper and iron, convert into hydroxyl, a highly reactive ROS [120]. Other ROS include peroxyl radicals and hypochlorous acid, the latter formed by the addition of chloride to hydrogen peroxide [119]. Reactive nitrogen species (RNS) are another group of free radicals mostly produced by the reaction of nitric oxide with ROS [121]. Environmental oxidants within cigarette smoke, ionizing radiation and heavy metals produce damage by activating endogenous mechanisms that generate ROS/RNS [119]. Both ROS and RNS modify proteins and DNA resulting in organelle dysfunction, particularly mitochondrial dysfunction. The mitochondria, which are the main cellular site of ATP synthesis via oxidative phosphorylation, are not only a source of ROS/RNS but also are the first organelles to suffer the damage [120].

Antioxidants are substances that retard or prevent the oxidation of substrates by ROS and other oxidants [122]. The antioxidant system of the body is composed of integrated enzymatic and non-enzymatic antioxidants. Examples of enzymatic antioxidants include superoxide dismutase, catalase and glutathione peroxidase. Vitamin A, C and E, b-carotene, and glutathione constitute the non-enzymatic antioxidant defense system [119]. An instance of the two systems working together includes the case in which glutathione peroxidase, using glutathione as a co-factor, converts hydrogen peroxide into water [123].

Oxidative stress is a state defined by a shift in the oxidant/antioxidant balance, in favor of endogenous and environmental oxidants, that overwhelms antioxidant systems of the body [119].

Oxidative stress contributes to the genesis of numerous pathologic conditions, including CRPS and PNP.

1.3.4.2 Oxidative Stress in PNP & CRPS

In CRPS, deep tissue ischemic-hypoxia and ensuing inflammation in the affected limb produces a pro-oxidant state [36, 49]. In PNP, the oxidative stress stems from the underlying microvascular dysfunction mechanisms of the peripheral nerve injury, be it initiated by post-traumatic inflammation or the toxic effects of hyperglycemia and chemotherapeutic agents [120, 124, 125]. Oxidative stress can contribute to the genesis of pain in PNP and CRPS through the direct activation and sensitizing effects of free radicals on peripheral nociceptors [126-128]. Moreover, free radicals act indirectly by injuring the microvasculature, inducing ischemic-hypoxia and causing inflammatory responses, which summate to sustain the sensitized state of peripheral nociceptors (See Figure 3) [49, 129].

1.3.4.2.1 Oxidative Stress in PNP

Peripheral nerves have a high phospholipid content and an axoplasm with abundant mitochondria. When these conditions are added to their weak cellular antioxidant defense systems, like lower levels of the enzymes SOD and catalase, it makes them particularly susceptible to damage from oxidative stress [124, 125]. Additionally, the vascular barriers of peripheral nerves, unlike neurons in the CNS, are permeable to damaging neurotoxins, including ROS. Furthermore, since peripheral nerves lack lymphatic drainage or a protective fluid bath like the CSF, toxic substances formed close by can accumulate within the extracellular matrix propagating damage along the nerve [130].

In several etiologies that cause painful peripheral neuropathies, there is evidence for a direct contribution of oxidative damage to the affected nerves in the pain pathophysiology. For instance, in chemotherapy-induced painful neuropathy, the anti-neoplastic drugs have been shown to adduct to and damage axonal-mitochondrial DNA. This induces both dysfunctions of the electron transport chain and the generation of ROS that cause further mitochondrial damage and inflammation [125]. The excess ROS formed by these events has been demonstrated to directly affect peripheral nerve function by oxidizing and sensitizing transduction ion channels [127, 128]. ROS-induced damage to mitochondrial enzymes, and ensuing bioenergetic dysfunction leading to ATP deficits, have also been shown to affect neuronal excitability [126]. Furthermore,

inflammatory mediators released in response to ROS accumulation can further propagate nociceptor sensitization [129]. Also, ROS-induced structural damage to the cellular phospholipids and microtubules produces demyelination and nerve degeneration [125].

It is the prolonged state of hyperglycemia that increases the formation of ROS in diabetic neuropathy. This pathophysiology occurs through different mechanisms, including auto-oxidation of glucose and its metabolites, oxidation of lipids, nerve ischemia and mitochondrial dysfunction [91, 131]. Once the excess ROS overwhelms the antioxidant capacity of the peripheral nerves, downstream effects like what was described for chemotherapy-induced painful neuropathy are triggered. The oxidative damage to the neuronal membrane and organelles, especially the mitochondrial, result in neuronal energy deficits with consequent alterations in nerve excitability and conduction [132].

Similarly, endoneurial oxidative stress is a pathologic feature of post-traumatic neuropathic pain. This is evidenced by the enhanced lipid peroxidation and depleted antioxidant substrates and enzymes, like glutathione and mitochondrial SOD, observed after transection and constriction injury to the sciatic nerve [124, 133]. Furthermore, systemic antioxidant therapies that attenuate these processes reduce hypersensitivity in rats with post-traumatic painful neuropathy [124].

1.3.4.2.2 Oxidative Stress in CRPS

Oxidative stress is directly involved in the pathophysiology of CRPS. Evidence for this can be drawn from studies that have examined the oxidative status of tissue samples from CRPS limbs, as well as in blood and saliva of CRPS patients [134, 135]. Elevated levels of markers for lipid peroxidation with a rise in antioxidant enzymes like SOD and peroxidase have been measured from the serum and salivary samples of CRPS-1 patients [135]. The elevated levels of antioxidant enzymes in blood and saliva were suggested to be due to compensatory overproduction of these molecules in other body tissues not directly affected by the CRPS pathology.

The source of oxidative stress in CRPS is postulated to be multifactorial, resulting from overproduction of ROS by activated inflammatory cells and microvascular dysfunction. These then cause prolonged ischemia with fluctuating reperfusion resulting in recurring ischemia-reperfusion injury (IRI) [49, 136]. Tissue hypoxia and IRI are known to result in mitochondrial dysfunction which can propagate the oxidative stress by producing more ROS, resulting in an inflammatory/ischemic vicious circle [137]. Evidence of oxidative stress, like depleted SOD

levels, along with damaged mitochondrial proteins, and reduced capacity for ATP production, has been observed in mitochondrial isolates from muscle tissue of amputated CRPS-1 limbs [134]. Such a reduction in muscular high energy phosphate metabolism is also a feature reported from *in vivo* spectroscopic studies on the intact affected limbs of CRPS patients [113]. Successful CRPS prevention and treatment have been reported after the prophylactic and therapeutic use of multiple antioxidants and free radical scavengers like vitamin C and N-acetyl cysteine (See section 1.4.4 for details) [138, 139]. This is indirect evidence for the role played by oxidative stress and its downstream cascade of events in the pathophysiology CRPS.

Findings from rodent models of CRPS also implicate oxidative stress in the pathophysiology of the disease (See details in section 1.5.2). A causative role of ROS in CRPS is suggested by CRPS-like symptoms generated by the continuous intra-arterial infusion of a free radical donor: tert-butylhydroperoxide (tert-BuOOH) in rats [140]. The tert-BuOOH infusion caused increased skin temperature, redness and swelling in the treated hind limb of the rats, whereas similar changes were absent in saline-infused limbs of control rats. Moreover, the rats exhibited unilateral mechanical and heat hypersensitivity for two weeks that later appeared in the contralateral paw as well [140]. Another rodent model of CRPS developed a few years later, involved the induction of an IRI after 3 hours of hind paw ischemia under anesthesia followed by reperfusion. This procedure in rats and mice produced initial hyperemia and edema that gave way into a cold and scaly-skinned hind paw, with mechanical and thermal hypersensitivity that lasted for weeks [109]. Microvascular dysfunction with evidence of lipid peroxidation was observed in the injured hind paw muscle of the rats [141]. In both rodent models, pain behavior improved with antioxidant treatments [109, 140].

1.3.5 A summary of the interactive roles played by microvascular dysfunction, tissue hypoxia & oxidative stress in the genesis of pain in PNP & CRPS

Tissue injury, localized to the peripheral nerve in PNP and involving the entire limb in CRPS, is the trigger for a series of maladaptive responses that eventually precipitate chronic pain [5, 9]. While the two conditions differ in their clinical presentation, the interactive role played by local inflammatory, vascular and metabolic processes resulting in the genesis of pain remains inherently similar in both.

The causative tissue injury, in PNP and CRPS, first triggers an exaggerated inflammatory response involving a myriad of cellular and chemical elements. The inflammation not only propagates further tissue damage but also results in the production of excess ROS that causes injury to the microvascular endothelium [37, 49, 91]. This injury causes both functional and structural alterations to the microvasculature that impair tissue perfusion and oxygenation [76]. The ROS-induced microvascular dysfunction affects the vascular bed of the entire limb in CRPS, while in PNP the *vasa nervorum* of injured peripheral nerves is most affected. A key mechanism of this dysfunction common to both PNP and CRPS is the depletion of endothelium-derived NO by excess ROS resulting in the production of RNS [36, 38, 142, 143]. Diminished levels of NO in the microvasculature causes vasospasms due to a no longer counteracted vasoconstrictive sympathetic tone [142]. Over time, chronic exposure of the microvasculature to ROS results in structural changes that alter vascular patency causing persistent tissue ischemic-hypoxia [76]. Evidence for such microvascular changes, along with the consequent tissue and peripheral nerve hypoxia, are abundant in peripheral nerves and limbs PNP and CRPS patients respectively [37, 38, 108, 143].

The pathophysiologic interaction between oxidative stress and ischemic-hypoxia is bidirectional in PNP and CRPS. While excess ROS due to injury-induced inflammation causes microvascular damage and tissue hypoxia, the lack of proper tissue oxygenation results in mitochondrial dysfunction with impaired aerobic respiration and further production of ROS, adding to the oxidative stress [114]. Furthermore, tissue hypoxia triggers the upregulation of transcription factors that increase the production of pro-inflammatory mediators that activate more ROS-producing inflammatory cells [36].

Each aspect of the interactive inflammatory, vascular and metabolic processes underlying PNP and CRPS impacts the activity of peripheral nociceptors contributing to the genesis of pain. The number and activation profile of transduction ion channels on peripheral nociceptors are directly impacted by inflammatory mediators and ROS [32, 127, 128]. Microvascular compromise and tissue hypoxia cause mitochondrial dysfunction-driven ATP deficit and lactic acidosis that results in the formation of excess protons inducing nociceptor activation and sensitization [114, 115, 144].

A brief summary of what has so far been discovered about the local pathologic processes that summate to cause the sensitization of peripheral nociceptors in PNP and CRPS is presented in

Figure 3. There are similarly intricate maladaptive processes that occur at the level of the spinal dorsal horn, the ascending and descending sensory pathways, and the subcortical and cortical structures involved in pain processing. Peripheral input from nociceptors is known to be crucial to the initiation and maintenance of the maladaptive changes in these upstream CNS regions. It is, therefore, worthwhile to identify and target interventions on the peripheral processes described above to alleviate the pain of PNP and CRPS.

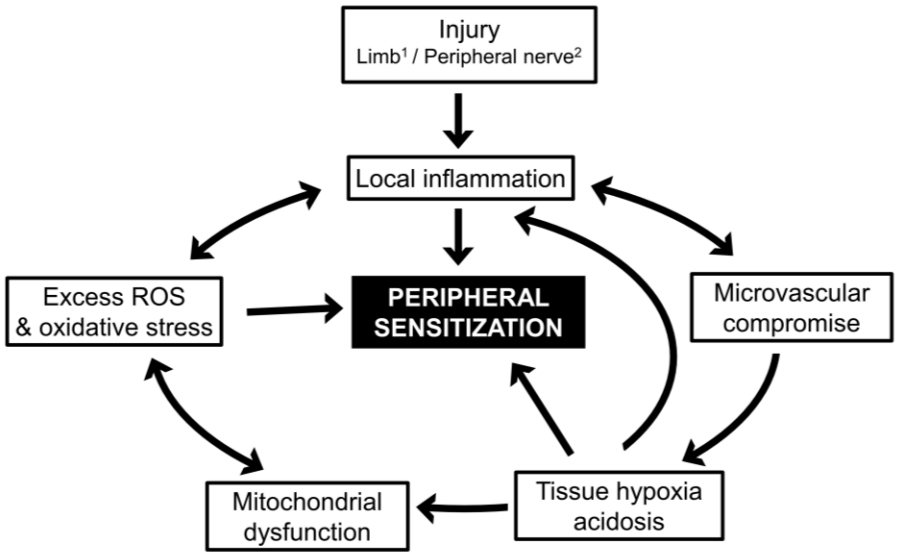

Figure 3. Schematic summary of proposed peripheral mechanisms involved in the genesis of pain in PNP and CRPS. (Inciting injury is sustained by the entire limb in CRPS[1] and the peripheral nerves in PNP [2].

1.4 Pharmacological treatment of PNP & CRPS

As in other types of chronic pain, the clinical management of PNP and CRPS is multimodal and requires the integration of physical therapy, occupational therapy and psychological interventions with pharmacological treatment [5, 9]. This section, however, will only address the pharmacological treatment of these conditions.

Most of the analgesics prescribed for PNP are also extended for use in the treatment of CRPS. There is abundant clinical data on the efficacy of analgesics used for the treatment of PNP but only a few randomized controlled trials (RCTs) have studied the efficacy of the same drugs in CRPS patients [9, 145]. A summary description of the pharmacological treatments that are currently the standard of care for PNP and CRPS are given in Table 1.

1.4.1 Current standard of care for the pharmacological treatment of PNP

Recommendations for the pharmacological treatment of PNP, proposed by the International Association for the Study of Pain (IASP), are based on the ratings of the quality and strength of clinical evidence [146]. The drugs are ranked as first-, second- and third-line treatment options sequenced based on this rating. First-line agents confirmed to be effective in PNP of different etiologies are a subset of repurposed antidepressant and anticonvulsant drugs. The antidepressants effective for the treatment of PNP are tricyclic antidepressants (TCAs) and serotonin-norepinephrine reuptake inhibitors (SNRIs) both of which are postulated to alleviate pain by enhancing central inhibitory control [146, 147]. While both TCAs and SNRIs inhibit the reuptake of serotonin and norepinephrine, TCAs have additional antagonistic effects on adrenergic, opioid, N-methyl-D-Aspartate (NMDA), cholinergic and histaminergic receptors [148]. Their side effects include sedation, dizziness and orthostatic hypotension, with the TCA's causing additional disturbances of heart rhythm requiring baseline cardiac studies before initiation of treatment [146].

The gabapentinoid anticonvulsant drugs are also first-line treatments. They act by binding to the $\alpha_2\delta$ subunit of voltage-gated calcium channels in the DRG and dorsal horn spinal neurons to reduce the presynaptic calcium influx required for excitatory neurotransmitter and neuropeptide release [149, 150]. Upregulation of the $\alpha_2\delta$ subunit expression occurs after nerve injury and gabapentinoids also act by blocking the trafficking of the subunits from DRG cell bodies to their terminals in the periphery and the dorsal horn [44]. Gabapentionids are also reported to enhance descending cortical inhibition and reduce the facilitation of pain signaling [150]. All these effects of gabapentinoids summate to decrease the sensitization of the CNS pain circuitry [147]. Like the rest of the first-line agents, gabapentinoids cause sedation, impaired concentration, confusion, memory loss, dizziness and gait disturbance [150]

In contrast to the first-line agents, the second-line agents are mainly local blockers of peripheral nerve activity [146]. Lidocaine and capsaicin are administered as patches or creams locally applied directly to the peripheral source of pain. Lidocaine acts by blocking sodium channels and is particularly recommended for postherpetic neuralgia [151]. Capsaicin acts by initially over-activating and then desensitizing transient receptor potential vanilloid 1 (TRPV1) transduction ion channels. Its efficacy has been shown in painful diabetic and other neuropathies

[152]. The side effects of these local treatments are local skin irritation but data on long-term effect of the drugs on epidermal nerve fibers is lacking [146, 152]. The weak opioid, tramadol, is also listed as a second-line treatment for PNP [146]. It comes with a risk of abuse and causes sedation and confusion especially in older patients [145].

There is only weak evidence for the benefit of third-line drugs recommended for the treatment of PNP [146]. These include strong opioids like morphine and oxycodone. These opioids also pose a risk of abuse, in addition to morbidity and mortality due to overdose [153]. The other third-line agent is subcutaneous Botulinum toxin type-A: a toxin that blocks peripheral nerve transmitter release and insertion of transduction ion channels into nociceptor membranes [154, 155]. Because its application is painful, it is done in a hospital setting under light anesthesia, restricting its use to refractory cases of localized PNP [145, 146].

1.4.2 Current standard of care for the pharmacological treatment of CRPS

Contrary to PNP, CRPS lacks global guidelines with recommendations for its uniform pharmacological management [9]. In the few national guidelines available, the analgesic anticonvulsants are indicated for the alleviation of pain and hypersensitivity. This is based on some clinical evidence of pain reduction in CRPS patients after treatment with the anticonvulsant gabapentin [156-158]. In contrast to what has been shown in PNP, there is no evidence for the analgesic efficacy of antidepressants in CRPS, and the guidelines recommend its use only for treating concomitant depression and anxiety [158]. The NMDA receptor antagonist ketamine has also been shown to reduce pain in CRPS patients but its narrow therapeutic index and potential for abuse limit its use [157, 158]. Opioid analgesics and transdermal lidocaine are frequently used in CRPS despite no objective evidence for efficacy in this patient population [9].

Although definitive evidence is still lacking for the therapeutic benefit of sympathetic blockade, it is a common treatment given to CRPS patients presenting with an acutely inflamed limb and evidence of sympathetic hyperactivity [9, 139, 159]. Sympatholysis is typically achieved either by performing a fluoroscopy-guided stellate and/or lumbar ganglion blocks using local anesthetics or by performing a regional block using intravenous guanethidine, a sympatholytic drug [9]. Reported adverse effects of sympathetic ganglion blocks include pain at the injection sites, transient motor weakness, blurred vision, nausea, vomiting, hoarseness, difficulties of breathing and swallowing, drowsiness and dizziness [160].

In the acute 'warm' phase of CRPS, systemic administration of corticosteroids has been shown to provide effective analgesia [10]. However, its extensive side effects like hyperglycemia, hypertension, immunosuppression, myopathy and osteoporosis preclude long-term use [161]. Although there have been reports of short-term pain reduction with nonsteroidal anti-inflammatory drugs (NSAIDs), the evidence remains insufficient [157]. Other treatments that impact tissue inflammation like the free radical scavengers, N-acetyl cysteine and dimethyl sulfoxide (DMSO), are reported to reduce pain in CRPS but are not in routine clinical use [9]. Other new and emerging groups of drugs include bone resorption inhibitors like bisphosphonates and calcitonin that alleviate the nociceptive bone pain that results from immobilization- and CRPS-induced bone loss; and vasoactive agents; like calcium channels blockers, nitrates and phosphodiesterase inhibitors are intended to improve perfusion of CRPS-affected limbs [162].

Table 1. Current recommendations for pharmacological treatment of PNP and CRPS

Drug (Group/ *Specific names*)	**Known/proposed mechanisms**	**Common adverse effects**	**Level of recommendation**	
			in PNP	**in CRPS**
ANTIDEPRESSANTS **TCAs** *Amitriptyline* *Clomipramine* *Imipramine* *Nortriptyline*	Increase central inhibitory control *Inhibition of monoamine reuptake, Na^+ channel & cholinergic blockade*	*Sedation, anticholinergic effects, arrhythmia*	1st line [146]	Standard [9]
SNRIs *Duloxetine* *Venlafaxine*	*5HT & NE reuptake inhibition*	*Nausea, abdominal pain, constipation, hypertension*	1st line [146]	Standard [9]
ANTICONVULSANTS *Gabapentin* *Pregabalin*	Decrease central sensitization *Reduce the activity of voltage-gated Ca^{2+} channels*	*Sedation, dizziness, peripheral edema, weight gain*	1st line [146]	Standard [9]
OPIOIDS **Weak** *Tramadol* **Strong** *Morphine* **Oxycodone, Methadone*	Central modulation of pain perception *Mu opioid receptor agonist* **Kappa receptor agonist*	*Sedation, dizziness, nausea, vomiting, constipation, dizziness, dependence*	2nd line [146] 3rd line [146]	Standard [9]
LOCAL TREATMENTS **Plasters/patches** *Lidocaine* *Capsaicin* **Local injection** *Botulinum toxin type A*	Inhibit peripheral sensitization *Na^+ channel blockade* *TRPV1 desensitization* *Reduce transmitter release and signal transduction*	*Local erythema, pruritis, rash, an initial increase in pain (only with capsaicin)* *Injection site pain*	2nd line [146] 3rd line [146]	Standard [9] Emerging [9]

Sympathetic blockade with local anesthetics	*Inhibition of sympathetic output*	*Sedation, dizziness, nausea, vomiting, difficulty breathing & swallowing, motor weakness*	N/A	Standard [9]
Oral corticosteroids	*inhibit inflammation*	*Immunosuppression, metabolic derangements*	N/A	Standard [9]
ROS scavengers *Topical DMSO* *Oral N-acetyl cysteine*	*reduce microvascular dysfunction & inflammation*	*None reported*	N/A	Uncommon [9]
Bone resorption inhibitors *Bisphosphonates* *Calcitonin*	*Slow bone resorption and reduce nociceptive bone pain*	*Pathologic fracture, jaw osteonecrosis*	N/A	Emerging [9]

Key TCA: tricyclic antidepressants, SNRI: serotonin-norepinephrine inhibitors, 5HT: serotonin, NE: norepinephrine, DMSO: dimethyl sulfoxide, TRPV1: transient receptor potential vanilloid, Ca^{2+}: calcium, Na^{+}: sodium, N/A: not applicable.

1.4.3 Shortcomings of the current standard of care in the pharmacological treatment of PNP & CRPS

Although the above selection of analgesic pharmacological agents is available for the treatment of PNP and CRPS, their efficacy upon routine clinical use is unsatisfactory. The first-line oral agents are reported to provide pain relief in only 30-40 % of PNP patients treated [152]. Evidence suggests only one-third of patients with painful diabetic neuropathy report a 50% reduction in pain intensity after being treated with the current systemic therapies [163].

The oral analgesics in current clinical use not only have inadequate efficacy but are associated with adverse effects that make them intolerable for most PNP and CRPS patients (see Table 1). To reiterate some of the serious adverse effects, the oral anticonvulsants, antidepressants and opioid drug groups cause sedation and dizziness which increases the risk of falls and injury especially in older patients. This reduces treatment compliance since it is difficult to work or conduct routine daily activities with these symptoms. Moreover, the TCAs are arrhythmogenic and especially dangerous if used in patients with cardiac disease. Their anticholinergic effects also make them unusable in patients with glaucoma and prostatic adenoma. One of the SNRIs, duloxetine, can worsen hepatic disorders, and venlafaxine can not be used in cardiac disease and hypertension because it raises blood pressure. All the opioids pose the risk of dependence and abuse in addition to gastrointestinal disturbances like nausea, vomiting and constipation.

These adverse effects are especially more frequent when the drugs are used at higher doses and in combination, as is commonly required to achieve pain control in PNP and CRPS. Another

factor contributing to the prevalent poor safety profile of these drugs is their frequent use in patients of older age, and those with comorbid medical conditions that impair drug metabolism and clearance [164, 165]. Such a lack of tolerability, on its own, serves to limit efficacy as the occurrence of side effects impedes proper dose titration to therapeutic levels.

With the gradually accumulating knowledge on the different pathophysiologic mechanisms involved in PNP and CRPS, there are greater prospects for the development of new mechanistically directed agents that provide analgesia with greater efficacy and/or safety [154]. Also, with the multiple peripheral disease processes identified in PNP and CRPS, more localized treatments been proposed as this approach avoids the undesired side effects of systemic treatments [152].

1.4.4 Pharmacological treatments of PNP & CRPS that target peripheral mechanisms

1.4.4.1 Mechanism-based approach in the treatment of pain in PNP & CRPS

Clinical implementation of the current guidelines for pharmacological treatment for PNP largely follows a cause-based and disease-specific pattern [45]. This is also reflected in the design of clinical drug trials for PNP where greater emphasis is placed on treating a selection of patients characterized by a given initiating etiology more so than targeting underlying pathologic mechanisms [166, 167]. Mechanism-based pain treatments yield better outcomes, and this has been observed in better-understood pain pathologies like inflammatory arthritis [168]. The converse is true when distinctly identified pathophysiology is lacking, as in fibromyalgia, where treatments are often not mechanism-based and yield little success [45]. Although PNP and CRPS do not have their mechanisms completely elucidated, different aspects of the contributing pathologies have been described enough to devise mechanistically-targeted treatments and avoid a regimented approach solely based on the general recommendations from evidence-based medicine [169]. In the few instances of clinical trials, where mechanistic patient categorization was employed to better target therapy in PNP, a more meaningful response to treatment has been reported. A good example is a study that tested the analgesic effects of topical clonidine in patients with painful diabetic neuropathy where patients were stratified upon enrolment based on the level of skin nociceptor sensitivity using their response to low dose topical capsaicin [170]. Treatment with topical clonidine produced significant analgesic efficacy in the patient group with functional nociceptors but not in those with skin denervation.

The pathophysiologic mechanisms of pain in PNP and CRPS involve maladaptive changes that interactively involve the peripheral and central nervous systems [5, 9]. Since inciting injury occurs in the periphery in both conditions, increased input from the peripheral sensory nerves triggers and drives pathologic changes that occur centrally [171]. Studies point towards peripheral hyperactivity in the initiation and maintenance of pain in the majority of chronic pain conditions [172]. Sensitization of peripheral sensory nerves, in turn, is a summated outcome of inflammatory, vascular and metabolic pathologic processes that occur within and around the affected peripheral nerve [33]. It is therefore conceivable that therapies directed to alter these contributary peripheral mechanisms will have a wholesome impact in alleviating the pain symptoms and signs of PNP and CRPS.

When pathological processes are peripherally located, mostly in and around the terminals of primary afferents embedded in the skin, the therapeutic agents are best delivered by targeting the drug locally [173]. Unlike routes that require systemic absorption and distribution, the topical route evades gastrointestinal metabolism and plasma protein binding, ensuring a steady and locally-effective drug concentration [152]. Additionally, with the negligible systemic absorption that occurs after topical application, undesired side effects that plague most oral analgesics are largely avoided [173]. As the pain pathology is localized to defined regions of the body in 60% of neuropathic pain, and in most CRPS cases, the topical route of analgesic administration has viable applicability for these conditions [174].

The topical agents that impact peripheral processes of PNP and CRPS, both those that are experimental and in current clinical use, fall into two categories. The first category includes drugs that directly impact electrical activity within the primary afferent nerve terminals and the second category includes drugs that alter the microenvironment of the sensory nerves to indirectly modulate their afferent activity.

1.4.4.2 Analgesics with direct impact on primary afferent neuronal activity in PNP & CRPS

Lidocaine and capsaicin are the two topically-administered analgesics currently recommended for clinical use for both PNP and CRPS [146, 158]. Both drugs directly influence the activity of ion channels involved in the transduction and transmission of sensory stimuli received by primary afferent sensory terminals. Although lidocaine is primarily known for its

inhibition of voltage-gated sodium channels, the drug also blocks potassium and TRPV transduction ion channels [175, 176]. These actions of lidocaine halt the generation and propagation of ectopic signals generated by injured peripheral nerves effectively producing analgesia [173]. Capsaicin, on the other hand, is an agonist of the TRPV-1 channels that are abundant on nociceptor terminals [177]. These channels, with a high-intensity stimulation, undergo use-dependent desensitization and internalization that nearly silences nociceptive terminals and the pain signals they generate [178].

The other topical analgesics having a direct peripheral neuronal impact are topical anticonvulsants. These agents are still largely investigational and are reported to calm hyperactive sensory neuronal activity while sparing normal nociceptor function [173]. Some of these agents, like topical phenytoin and ambroxol, produce a use-dependent reduction of sodium channel activity and have shown efficacy in case-series reports of PNP patients [179, 180]. Likewise, topical gabapentin, which affects peripheral nerves by reducing calcium currents and nerve injury-induced calcium channel trafficking, has also been demonstrated to reduce pain in both PNP and CRPS [181].

The NMDA receptor antagonist, ketamine, has significant side effects that normally preclude its systemic use, but its topical formulations produce negligible plasma levels of the drug and its metabolites [182]. As peripheral nociceptors express NMDA receptors, topical ketamine has been investigated at different concentrations for the treatment of both PNP and CRPS, producing some evidence for its efficacy [183-187]. These results are from open-label and retrospective studies and need verification with RCTs.

Other agents whose topical administration can potentially have a direct impact on the hyperactivity of peripheral nociceptors include opioids, cannabinoids, GABA agonists and antidepressants [173]. Most of the topical actions of these drugs is via their respective receptors on the peripheral nociceptor membranes [188, 189]. Topical antidepressants exert their peripheral effect through multiple receptors including opioid, cholinergic, histaminergic and adenosine receptors [190]. Case reports that tested topical amitriptyline in PNP have shown analgesic effects, though follow-up clinical trials have failed to replicate the results [191-193]. Another antidepressant, doxepin, has also exhibited topical analgesic effects in CRPS and PNP patients [194, 195]. Similarly, the GABA-B receptor agonist baclofen, in combination with other agents,

has shown analgesic effects in neuropathic pain [196]. Moreover, in an open-label study on patients with post-herpetic neuralgia, the topical administration of a cannabinoid agonist has produced pain relief [197]. The clinical applicability of all these agents as topical analgesics for CRPS and PNP requires more evidence for their efficacy from better designed large-scale RCT studies.

There are also clinical studies that report the analgesic effect of topical treatments formulated from two or more agents that impact peripheral nerve sensitization. Among these is the topical combination of amitriptyline and ketamine that has demonstrated analgesic efficacy in post-herpetic neuralgia, although trials with mixed etiologies of PNP failed to replicate the positive outcome [198, 199]. A similar topical formulation, with the addition of baclofen, has demonstrated analgesic benefit in a double-blind placebo-controlled trial in patients with CIPN [200]. Likewise, an RCT on the topical combination of doxepin and capsaicin has demonstrated the treatment alleviates PNP with an earlier onset of effects as compared to its individual constituents [201].

1.4.4.3 Analgesics with indirect impact on primary afferent activity in PNP & CRPS

1.4.4.3.1 Drugs that impact peripheral inflammation in PNP & CRPS

In CRPS affected limbs and peripheral nerves with PNP, tissue injury produces an inflammatory soup of mediators released by immune and non-immune cells that both initiate and maintain primary afferent sensitization [34, 202]. Topical agents that target these processes have been shown to have the potential to produce analgesia in these syndromes.

Non-steroidal anti-inflammatory drugs (NSAIDs) are a group of drugs that inhibit a ubiquitous enzyme, cyclooxygenase, responsible for the production of lipid-derived inflammatory mediators named prostanoids. These mediators are produced during inflammation and can act as direct sensitizers of nociceptors [203]. Though not as extensively investigated in PNP and CRPS, abundant clinical evidence exists for the analgesic efficacy of topical NSAIDs in chronic inflammatory musculoskeletal pain [204]. Topical aspirin has been observed to produce analgesic effects in patients with the acute pain of shingles as well as post-herpetic neuralgia in a double-blind placebo-controlled clinical study [205]. In a follow-up study by the same group, oral aspirin was compared with its topical version in the same population and only the topical formulation produced pain relief; there was an 80% greater analgesic effect after topical administration as compared to oral dosing [206]. Furthermore, in a more recent study, topical diclofenac was found

to be analgesic in both CRPS and some specific types of PNP [207]. NSAIDs including ibuprofen, ketoprofen and indomethacin have also been topically formulated with other agents and produce analgesic effects in PNP [190].

Another group of potent anti-inflammatory drugs is corticosteroids. The anti-inflammatory effects of these drugs are wide-reaching and impact both humoral and cell-mediated inflammatory responses [208]. This is achieved through a global reduction in the expression of pro-inflammatory enzymes and cytokines. Though the analgesic benefits of corticosteroids are primarily through their anti-inflammatory effects in CRPS, they have also been shown to directly inhibit ectopic and enhanced peripheral sensory nerve activity [209]. Systemic steroids are routinely used in the clinics for the treatment of acute CRPS, so their topical use may be beneficial [158].

Local targeting of inflammatory responses via intraarticular administration of corticosteroids effectively alleviates persistent pain due to inflammatory and noninflammatory joint disorders [210, 211]. Similarly, local corticosteroid injections are analgesic and beneficial to the recovery of function in patients with PNP due to carpal tunnel syndrome, a painful compressive neuropathy of the median nerve [212]. Topical steroids are also reported to decrease pain behavior in rodents with post-traumatic PNP [209, 213].

1.4.4.3.2 Drugs that impact peripheral vascular supply and tissue oxygenation in PNP & CRPS

Local microvascular compromise with consequent tissue hypoxia is a common underlying mechanism for both PNP and CRPS (see section 1.3.3). Ischemic-hypoxia contributes to the genesis of pain by affecting the entire limb tissue in the case of CRPS and the peripheral nerve in PNP. This is supported by abundant preclinical and some clinical evidence on the analgesic potential of locally targeting ischemic-hypoxia in both conditions. Most of the vasodilatory agents shown to have analgesic efficacy in PNP and CRPS work by enhancing NO-mediated vasodilation or through blockade of sympathetic vasoconstriction, thereby reducing ischemic-hypoxia.

Local administration of peripheral adrenergic blockers like prazosin, yohimbine and clonidine has been shown to dose-dependently reduce nociceptive behaviors in the CPIP rodent models of CRPS [214, 215]. Similarly, topical clonidine has reduced hyperalgesia in rats with chronic constriction injury of the sciatic nerve [216]. Clonidine, a sympatholytic drug that reduces vasoconstriction by inhibiting the release of norepinephrine from post-ganglionic sympathetic

terminals has also been effective in clinical trials [170, 217]. Particularly, transdermal clonidine has been reported to alleviate hyperalgesia in CRPS patients [218]. Moreover, a topical clonidine gel was effective at reducing pain in an RCT of patients with painful diabetic neuropathy [170]. A systematic review of clinical studies evaluating the analgesic effect of topical clonidine in PNP has also indicated moderate to low evidence for the use of the drug in painful diabetic neuropathy [219].

Analgesic agents that cause NO-mediated vasodilation act by donating NO or fortifying downstream NO signaling pathways. The NO donors isosorbide dinitrate and glyceryl trinitrate, applied as topical sprays, were analgesic in two RCTs on patients with painful diabetic neuropathy [220, 221]. Similarly, a topical ointment formulation of isosorbide dinitrate has produced pain alleviation in a pilot study on a small group of CRPS patients [103].

Phosphodiesterase (PDE) inhibitors prevent the degradation of cyclic mononucleotides that act as second messengers in the NO-signaling pathway. Pentoxifylline, a non-specific PDE inhibitor, has been demonstrated to produce both analgesia and enhanced microvascular blood flow in a rodent model of CRPS. [222]. Similarly, oral pentoxifylline has reduced pain and increased skin blood flow in clinical studies on patients with diabetic neuropathy [223, 224]. Other PDE inhibitors, tadalafil and sildenafil, have also been shown to alleviate pain behavior in rodents with diabetic- and chemotherapy-induced painful neuropathy [225-227].

Further evidence for the therapeutic viability of targeting both tissue and endoneurial hypoxia is provided by reports of the synergistic analgesic effects of topical combinations of vasodilatory agents. Drugs that are NO-mediated vasodilators and sympathetic blockers have been formulated into topical formulations that produce analgesic effects in rodent models of both CRPS and PNP [215, 222]. Some of these combinations include the peripheral adrenergic blockers clonidine and apraclonidine mixed with either the NO-donor linsidomine or the PDE inhibitor pentoxifylline and its active metabolite lisofylline. This approach holds great potential for successful clinical translation as robust analgesic effects were reported after the use of a topical combination of clonidine and pentoxifylline in healthy human volunteers with an experimentally-induced surrogate of PNP [228].

The key role played by local tissue and endoneurial oxygenation in PNP and CRPS is also supported by the fact that non-pharmacological therapies that alleviate hypoxia produce analgesic

effects. Hyperbaric oxygen therapy, which entails administration of 100 % oxygen at greater than atmospheric pressure to achieve a net gain in tissue oxygen concentration, is an emerging treatment for PNP and CRPS [229]. Multiple studies have shown that hyperbaric oxygen treatment improves nerve blood flow, and decreases edema and cellular damage, thereby alleviating the hypersensitivity exhibited in rats with nerve injuries [144, 230, 231]. Additional clinical evidence exists for CRPS, where a small RCT has shown that two weeks of hyperbaric oxygen treatment reduces pain and improves the range of joint movement in patients with CRPS [232].

The evidence detailed above indicates that alleviating tissue hypoxia through pharmacological or non-pharmacological means is a viable approach to achieving analgesia in PNP and CRPS. This is possibly due to the expected beneficial effects of tissue oxygenation on mitochondrial function and energy production, inflammation and oxidative stress, all of which can reduce peripheral nociceptor activity that drives the pain of PNP and CRPS (See Figure 3).

1.4.4.3.3 Drugs that impact oxidative stress in PNP & CRPS

Excess ROS and oxidative stress contribute to the pathophysiology of PNP and CRPS in multiple ways. ROS sensitize peripheral nociceptors directly, exacerbate tissue inflammation, and cause microvascular injury and dysfunction. Both local and systemic administrations of antioxidants have been demonstrated to alleviate pain and improve the overall pathology of PNP and CRPS. In preclinical studies, the free radical scavengers N-acetyl cysteine and Tempol have been shown to produce a significant reduction of hyperalgesia in CPIP rats [109, 222]. Human studies parallel these results. Oral N-acetyl cysteine and topical DMSO have been reported to improve pain and disability measures in two RCTs on CRPS patients [16, 233]. Moreover, the prophylactic use of vitamin C in limb-fracture patients has been shown to reduce the incidence of CRPS [138, 139].

Preclinical studies using rodent models of PNP, of different etiologies, also indicate antioxidant therapy alleviates hypersensitivities. The administration of a xanthine oxidase inhibitor, allopurinol, and plant-derived antioxidant, resveratrol, has been shown to alleviate mechanical and thermal hypersensitivity, and improve nerve blood flow and conductive function in rodent models of diabetic neuropathy [234, 235]. Similarly, the antioxidant, N-acetyl cysteine has been reported to produce a significant reduction in mechanical and thermal hypersensitivity, as well as of markers of oxidative stress in the nerve-injured rats [124]. The administration of the

free radical scavenger, phenyl N-tert-butylnitrone has been shown to dose-dependently alleviate mechanical hypersensitivity in rodents with CIPN following paclitaxel administration [236]. Curcumin, an herbal extract antioxidant, also reduced mechanical and thermal hypersensitivity and improved peripheral sensory nerve conduction after oral administration to rodents with oxaliplatin-induced painful neuropathy [129]. Other medicinal herbs containing antioxidant compounds like flavonoids and polyphenols have also shown promising effects in ameliorating oxidative stress and hypersensitivity in CIPN [237].

Furthermore, there is promising evidence for the clinical efficacy of antioxidants in alleviating pain in PNP. The systemic administration of the endogenous antioxidant, α-lipoic acid, significantly reduces pain, burning and numbness in patients with painful diabetic neuropathy according to a meta-analysis of four RCTs on the effect of α-lipoic acid in this PNP [238]. Likewise, small group-sized clinical trials imply antioxidants like vitamin E, the vitamin B-6 extract mangafodipir and omega-3 lipids alleviate and prevent chemotherapy-induced painful neuropathy [239].

1.5 Rodent models of PNP & CRPS

The current mechanistic understanding and therapeutic recommendations for PNP and CRPS have required basic pre-clinical explorations using different rodent models. The models are designed to reflect the specific injuries and diseases that underlie the pain syndromes observed in human patients [240]. Though far from perfect, it is the representation of phenotypic features and disease mechanisms obtained from these rodent models that provides the foundational data used as a stepping-stone for more advanced human studies.

1.5.1 Rodent models of PNP

The existing rodent models of PNP are generated by inducing traumatic, metabolic and toxic peripheral nerve injuries of comparable nature to the human pathology. Most of the commonly used traumatic rodent models of PNP involve injury to the sciatic nerve, its distal branches, or spinal roots of origin. The rodent sciatic nerve is the major hind extremity peripheral nerve and has roots originating from the lumbar segment of the spinal cord at L4 and L5 with minor contributions from L3 and L6 [241]. It is composed of sensory, motor and autonomic fibers. After running parallel to the femur until the knee joint, the nerve divides non-symmetrically into

its tibial, common peroneal and sural branches. The three branches make small interconnected muscular, articular and sensory branches that innervate the various structures of the leg [242].

Some of the sciatic nerve based, post-traumatic PNP rodent models involve traumatic injury to the sciatic nerve trunk. These include models made by transection of the entire nerve trunk, applying loose ligatures around the nerve trunk (chronic constriction injury/CCI), or a partial tight ligation of $1/3^{rd}$ to half of the nerve [243, 244]. Comparably common models are prepared by more proximal injuries to the spinal roots of the sciatic nerve as in the spinal root ligation model, where the L5/L6 spinal roots are tightly ligated close to their exit through the spinal foramina [245]. More distal injuries to the three branches of the sciatic nerve have also been used to generate widely used post-traumatic PNP rodent models. In the spared nerve injury model, two of the three branches of the sciatic nerve are transected at their point of trifurcation commonly leaving the dominantly sensory sural branch intact [246]. Sciatic-nerve injury based mononeuropathy in rodents has also been produced using alternate means. These include sciatic nerve injury induced by photochemically-induced ischemia and cryoneurolysis [247, 248].

The commonly employed non-traumatic models of human PNP entail the systemic administration of agents that induce toxic and/or metabolic insults to the peripheral nerves of animals. In chemotherapy-induced painful neuropathy, long-lasting sensory neuropathy is typically produced in rodents by the administration of several antineoplastic agents that are known to have peripheral neurotoxicity in humans [249]. Some of the chemotherapeutic drugs used include vincristine, platinum-derived products (oxaliplatin, cisplatin and carboplatin), and taxanes (paclitaxel and docetaxel), which are administered to rodents using varying treatment regimens [250].

To model diabetic neuropathy, the anti-cancer/antibiotic agent streptozotocin is parenterally administered to rats for three days inducing pancreatic β-cell destruction and hyperglycemia. The resulting metabolic derangement causes symmetrical painful neuropathy along with other systemic signs of physical weakness, gastrointestinal and genitourinary symptoms [249]. An alternate rodent model of diabetic neuropathy has since been generated by deleting the leptin gene, a gene responsible for the regulation of food intake and energy expenditure [251].

Two rodent models of postherpetic neuralgia are available, one generated following the chronic infection with the varicella-zoster virus and another through the administration of

resiniferatoxin, a compound that depletes capsaicin-sensitive afferents [252, 253]. To model human alcohol-induced neuropathy, an alcohol-containing diet is administered to rats in incremental doses, for several weeks [250].

The work discussed in this thesis employed the use of two PNP models: the rat CCI model of post-traumatic PNP, and the rat paclitaxel-induced painful neuropathy model of CIPN. Hence, the two rat models are discussed in greater detail below.

1.5.1.1 The CCI rat model of PNP

Chronic constriction of the sciatic nerve (CCI) is a rodent model of post-traumatic painful mononeuropathy generated by surgically exposing one of the sciatic nerves near the distal thigh and applying four loose chromic gut ligatures around the nerve just proximal to its point of trifurcation [244]. CCI rats exhibit nocifensive behavior including guarding, excessive limping, and licking of the ipsilateral hind paw between 7 days to 7 weeks after surgery, with a peak in these features observed in the second week. Additionally, the rats show hypersensitivities to non-noxious mechanical and thermal stimuli, as well as thermal hypersensitivity that peak at 10-14 days post-procedure. The rat CCI model is considered reliable and easily reproducible, and so is a frequently utilized model of post-traumatic PNP. It also exhibits face validity by producing several of the pain phenotypes of PNP [254, 255]. Its limitation occurs due to possible variabilities in the tension of the ligatures put around the sciatic nerve that can result in inconsistencies in the extent of injury to the nerve, with overly tight ligatures leading to autotomy or self-mutilation of the hind paw digits [255].

Constriction injury of the sciatic nerve, the CCI model, is followed by intraneural edema, a focal decline in the nerve's blood supply, and degeneration [250]. Both inflammatory and neuronal mechanisms are proposed to contribute to the pain pathology in the model [256, 257]. Examination of the injured nerve in CCI has revealed damage to both myelinated and non-myelinated sensory fibers with a decline in nerve conduction. Accompanying vascular changes reported include endoneurial vessel distension with the gradual enlargement of endothelial cells, arteriolar wall thickening and luminal narrowing [90]. Moreover, CCI also produces reduced blood flow in the injured nerve with epineurial and endoneurial vascular stasis and edema [258]. Likewise, a rise in the expression of proinflammatory cytokines in the injured nerve has been observed in the rats that positively correlated with the onset of hyperalgesia, [259].

CCI is a good model of post-traumatic painful mononeuropathy as it replicates the mechanism of injury of entrapment neuropathies like carpal tunnel syndrome, a prevalent condition affecting nearly 8% in the general population [260, 261]. This rodent model, therefore, makes possible the exploration of disease mechanisms and pharmacological therapies for better understanding and treatment of one of the commonest forms of post-traumatic PNP [260, 261].

1.5.1.2 The rat paclitaxel-induced painful neuropathy model of PNP

Paclitaxel is a chemotherapeutic agent in clinical use for the treatment of ovarian, breast and non-small cell lung cancers [262]. It works by binding to the microtubules of cancerous cells and suppressing spindle-microtubule dynamics, halting cell division and inducing apoptosis [263]. It has known dose-limiting toxicity to peripheral nerves causing a painful symmetric sensory neuropathy characterized by burning pain, tingling and numbness along with thermal and mechanical allodynia in a glove/stocking-like distribution [264]. These symptoms can persist for months to years following completion of cancer therapy [262]. Data on the nerve pathology in humans is scarce. A length-dependent, distal neuropathy with fiber loss, axonal atrophy and secondary demyelination has been observed from the sural nerve biopsies obtained from a patient treated with a long course of paclitaxel [265].

As compared to the other common anti-cancer agents that cause neurotoxicity, the use of paclitaxel in animals is an ideal prototype for modeling and studying the details of pain pathology in chemotherapy-induced neuropathy. This is because paclitaxel-induced peripheral neuropathy has one of the highest incidences, averaging at about 70%, after its administration [266]. Moreover, the type of neuropathy paclitaxel causes is mostly peripheral and sensory, unlike the neuropathy of platinum compounds which also produce somatic and visceral motor nerve injury, and that of vinca alkaloids where autonomic neuropathy is also a feature [267].

Several studies have reported varying doses and routes of administration of paclitaxel to generate painful neuropathy in rats [268-271]. A widely used regimen that was also used in this thesis entails four intraperitoneal injections of paclitaxel given on alternate days [268]. The prolonged intermittent administration mimics the pattern commonly used in cancer treatment cycles [272]. With this treatment, the rats exhibit bilateral hind paw and tail hypersensitivity to both thermal and mechanical stimuli without concurrent defects in motor function or systemic toxicities [268]. The sensory features appear as early as five days after completion of treatment and last for up to four weeks.

The peripheral pathophysiologic changes described in rat paclitaxel-induced neuropathy have vascular, metabolic and structural aspects. Affected sensory fibers exhibit significant endoneurial edema as well as swollen and vacuolated mitochondria [262]. The resolution of these mitochondrial changes occurs coincidently with the resolution of pain behavior in the rats [262]. Furthermore, impaired high energy phosphate metabolism has been observed from sciatic nerves of rats with paclitaxel-induced painful neuropathy [273]. The painful symptoms of paclitaxel-treated animals are assumed to depend on mitochondrial dysfunction due to oxidative stress from excess ROS that overwhelms endogenous antioxidant systems [274]. Vascular changes have also been described in paclitaxel-induced neuropathy, characterized by attenuated peripheral nerve blood flow and decreased number of *vasa nervorum,* both of which appeared to reverse with angiogenic therapy that also improves the physiology of the affected nerve [102]. Finally, paclitaxel-treated rats have degeneration of distal sensory peripheral nerve arbors in the epidermis but not in the proximal axons of their nerve trunks [275].

1.5.2 Rodent models of CRPS-1

The common rodent models used to study CRPS utilize traumatic procedures that have been reported to precede the occurrence of CRPS in human patients. Tibial fracture and immobilization, and limb immobilization on its own are procedures used to model CRPS that occurs during the fracture and healing process [276, 277]. In the chronic post-ischemic pain (CPIP) model of CRPS, the injury takes the form of tourniquet application to induce prolonged hind limb ischemia followed by reperfusion [109]. A detailed description of the CPIP rat model of CRPS-1 is given in section 1.5.2.1 as it was extensively used in the experiments outlined in this thesis.

In the tibial fracture and immobilization model, the distal tibia is fractured with plyers and the hind limb wrapped in casting tape immobilizing the hip, knee and ankle in the flexed position of a normal standing gait [277]. After cast removal at 4 weeks post-injury, the rats show hind paw skin warmth, edema and mechanical allodynia. The rats also exhibit local inflammation and periarticular bone loss [277, 278]. The analgesic effects of systemic treatment with free radical scavengers suggest a role for oxidative stress in this model [279]. However, the rats showed mechanical but not thermal hypersensitivity and were not observed for evidence of spontaneous nocifensive behavior. Moreover, the inflammatory signs exhibited on the injured limbs appear inconsistently, with erythema and edema in only 41% of the animals [277]. These data suggest

that, although the injury used for this model is a common cause of CRPS, the resulting phenotype and pathophysiologic features reported do not completely reflect the human syndrome.

Limb immobilization, on its own, has also been shown to elicit local inflammation and a pain syndrome in rats, and this has been used to model some aspects of CRPS. In this model of chronic post-cast pain, a plaster cast is wrapped from the trunk to the middle of one of the hind paws for two weeks [276]. The rats exhibit calf muscle atrophy and local inflammatory changes like swelling, erythema and hyperemia of the hind limb. Moreover, signs of spontaneous pain behavior and mechanical hyperalgesia have been reported for up to ten weeks [276]. The proposed mechanism of disease in this model was a prolonged ischemia-reperfusion injury in the hind limb. In a slight variation of the chronic post-cast pain model of CRPS, rats were cast-immobilized for four weeks resulting in local inflammatory changes and pain behavior similar to what is described above but lasting for a shorter duration [277]. The role of immobilization in inducing a transient CRPS-like condition has been demonstrated in healthy volunteers after an experimental 4-week long cast immobilization of the forearm that resulted in cold and mechanical hyperalgesia with skin temperature differences [280].

There are also more mechanistically specialized rodent models for CRPS. Intraarterial infusion of a free radical donor, tert-BuOOH, into the rat hind limb has been used to induce CRPS-like features, emphasizing the role of oxidative stress [140]. In this model, a 24-hr long intra-arterial infusion of tert-BuOOH was performed in the rat hind limb. Following the infusion, the rats showed plantar skin warmth, erythema and edema, while saline infusion in sham controls had no such effect. Between 5 and 28 days following the infusion, the rats exhibited mechanical and thermal hypersensitivity along with some spontaneous pain behavior. The hind limb muscle of the rats showed cellular degeneration with leukocyte infiltration and build-up of interstitial fluid [140].

Pain hypersensitivity seen in CRPS has also been reproduced in rats using a needle stick injury to the tibial nerve [281]. In this sciatic nerve need-stick injury rat model of CRPS-1, the sciatic nerve was exposed at its trifurcation and the tibial branch pricked once with a needle of specific caliber [281]. The rats exhibited mechanical allodynia and hyperalgesia and cold allodynia for two weeks post-procedure. Histologic examination of the injured tibial nerve revealed degenerating fibers within the sciatic nerve.

1.5.2.1 Chronic post-ischemic pain (CPIP)

The CPIP rat model of CRPS-1 is induced by three hours of hind paw ischemia in anesthetized animals using a tight-fitting O-ring tourniquet around the ankle followed by reperfusion [109]. During the ischemic period, the hind paw is cyanotic with a near-complete cessation of blood flow. After removal of the tourniquet and reperfusion, the hind paw exhibits hyperemia, warmth and edema that remains up to 24 hours post-reperfusion. In the following 72 hours, the hind paw becomes dry and shiny and the rats exhibit short-term spontaneous pain behavior like licking, shaking and paw favoring, followed by mechanical allodynia and hyperalgesia and cold allodynia lasting for up to 4 weeks post-procedure. The hind limb appearance in the CPIP rats after reperfusion and their subsequent pain behavior resembles the progression of symptoms commonly observed in CRPS patients [109]. (An image of CPIP hind paw appearance is presented in section 4.5.2.).

The mechanism underlying the persistent mechanical and thermal hypersensitivity in CPIP rats has been attributed to changes in the injured paw tissue vasculature, inflammatory and oxidative statuses, and cutaneous innervation [141]. The ischemia-reperfusion injury (IRI) in CPIP rats results in the generation of excess ROS and this is evidenced by enhanced lipid peroxidation seen in the muscle of the CPIP hind paw [109, 141]. One of the downstream effects of excess ROS is an injury to the microvascular wall that causes vasospasm and capillary impairment reducing or completely impeding blood flow [282]. Such dysfunction of the microvasculature causes fluctuations in capillary blood flow that result in localized areas of IRI-induced recurrent microvascular injury. A significant feature of the microvascular dysfunction in the CPIP hind paw tissue is endothelial dysfunction with an enhanced vasoconstrictive response to the endothelium-derived vasoconstrictive molecule endothelin and the sympathetic mediator norepinephrine [214, 283]. Both molecules, in addition to eliciting enhanced vasoconstrictive responses, also enhance pain behavior in CPIP animals.

Another consequence of the oxidative stress, due to IRI in the CPIP hind paw, is increased pro-inflammatory cytokines and overall inflammatory responses [141]. The CPIP hind paw not only exhibits deep tissue ischemia but is also laden with lactate and inflammatory mediators that cause nociceptor sensitization and pain hypersensitivity [49, 141]. Further exacerbation of pain behavior has been observed in the rats following exercise that causes a build-up of muscle lactate.

Moreover, ischemic-hypoxia in the peripheral nerve fibers with a consequent spontaneous ectopic discharge has been reported to result from the microvascular injury to endoneurial vessels in the CPIP hind paw [49]. Where endoneurial capillary dysfunction is possibly at its worst is in the distal cutaneous nerve branches, and small-fiber degeneration has been observed with a significant reduction of intraepidermal nerve fibers in the CPIP hind paw skin [141].

Nearly all of these pathologic features reported in CPIP can be matched with alterations of similar significance reported in clinical studies of CRPS patients [38, 39, 69, 104, 114, 135]. Moreover, the response profile of CPIP rats to standard analgesic drugs and systemic steroids matches what has been reported for patients with CRPS [284]. This evidence has prompted the choice to use this model in the experiments reported in this thesis.

1.6 The use of evoked pain outcome measures for the assessment of topical analgesic efficacy in PNP & CRPS

The chronic pain features that characterize PNP and CRPS have similarities, mostly described by patients as spontaneous pain with burning, tingling, shock-like and dull-aching qualities [5, 9]. Physical examination reveals further symptoms and signs like allodynia and/or hyperalgesia to mechanical and/or thermal stimuli. Animal models used in preclinical studies on PNP and CRPS are designed to feature the signs and symptoms of patients with these conditions. The preclinical data collected on analgesic drug development for PNP and CRPS should constitute appropriate pain outcome measures to ensure translatability to clinical application. Finding an accurately quantifiable behavioral response in rodents that is comparable to the pain experience in humans has been challenging [285]. This is because pain is a subjective experience that is verbally reported in humans and non-communicating subjects like rodents lack the means of allowing its direct measurement. Therefore, the unpleasantness of pain in rodents needs to be inferred indirectly from their pain-like behaviors such as withdrawal, increased grooming of affected area and vocalization upon sensory stimulation.

The nociceptive outcome measures used in rodent models of PNP and CRPS can be classified into evoked and non-evoked, based on the presence or absence of external stimuli eliciting a withdrawal response by the animals [240]. The evoked outcome measures quantify the avoidant responses to graded mechanical or thermal stimuli applied to a defined area of the body [285]. On the other hand, the non-evoked outcome measures assess pain-induced changes in

different aspects of spontaneous behaviors that are used to gain insight into the emotional and affective aspects of the pain experience [285].

Evoked measures of mechanical hypersensitivity can be obtained after applying mechanical stimuli that are static or dynamic. The manual von Frey test entails the use of a monofilament applied perpendicularly to the plantar hind paw, until it buckles, delivering a pre-calculated force for a few seconds or until the animal responds by a brisk paw withdrawal, shaking or licking of the paw [285]. Other approaches to assess mechanical hypersensitivity include the electronic von Frey test and the paw pressure test that involves the application of increasing force, using a pointed probe tip, to a restrained rodent till the animal vocalizes or withdraws its paw [286].

Thermal stimuli have also been used to measure evoked thermal hypersensitivity. Some of the methods used in rodents include the hot-plate test, radiant heat test (Hargreaves test), thermal probe test, and the acetone evaporation test [285]. In the hot-plate test, the latency until the animal elevates or licks a hind paw or jumps is measured after being placed on a constantly heated (~ 54^{o}C) metal surface [287]. In a variant of this test, the temperature of the metal surface is steadily raised to noxious levels until response from the rodent is elicited [288]. In Hargreaves test, a radiant or infrared heat source is used to stimulate the plantar surface of the rodent's hind paw and a withdrawal latency is recorded [289]. The thermal probe test is a recent test developed with mice, where a steadily heating small metal probe is applied to the hind paw until it withdraws [290]. In the acetone evaporation test, acetone is sprayed on the hind paw of a rodent placed on a mesh floor [291]. This elicits cooling of the skin to temperatures of 15-21^{o}C and cold hypersensitivity is recorded by quantifying the number, duration and/or severity of response [285]. These mechanical and thermal evoked pain outcome measures are widely reported in pain studies assessing the effect of topical analgesics. They are robust, reliable, and give consistent measurements with relative ease and simple instrumentation [292].

Topically administered analgesics are localized treatments given to achieve clinically effective concentrations only at the site of application with little or no systemic absorption [190]. It is therefore conceivable that the pain-relieving effects of topical treatments originate at the circumscribed area of hypersensitivity that has been treated. To accurately assess the direct effect of such localized treatments, tests used as outcome measures need to be spatially directed to the

site of treatment. This makes the evoked pain outcome measures suitable for pre-clinical assessment of the effect of topical analgesics.

Evoked pain outcome measures have recently been successfully used in rat models of PNP and CRPS to assess the efficacy of topical analgesics that have now progressed into experimental clinical use [216, 293-295]. Topical gabapentin is an emerging treatment for PNP which was initially investigated for analgesic effects in the rat model of painful diabetic neuropathy using evoked tests of mechanical and thermal hypersensitivity [295]. The data acquired from this study laid the groundwork for the progress of the topical agent for subsequent clinical use and implementation [294]. Similarly, preclinical assessment with evoked outcome measures of mechanical and thermal hypersensitivity in rat models preceded the current clinical use of topical clonidine for painful diabetic neuropathy and CRPS [215, 216, 219, 293]. These instances are good evidence for the valid utility of evoked pain outcome measures in the initial assessment of topical analgesic effects in rodent models of PNP and CRPS.

The sensory-discriminative aspect of pain that can be effectively assessed with evoked nociceptive outcome measures provides valuable information on one of the dimensions of the overall pain experience [240]. It has also been proposed that the spontaneous pain complaints of chronic pain conditions like PNP and CRPS contain the summated effects of the allodynia and hyperalgesia induced by the internal-physiologic and external stimulations of the activities of daily life [296]. Given this interdependence between spontaneous and evoked pain sensations and the practicality of using evoked outcome measures in topical analgesic studies, these outcome measures have been used in the work reported in this thesis.

1.7 Rationale and specific aims

The current pharmacological treatment for PNP and CRPS is centered on oral antidepressants and anticonvulsants that provide suboptimal pain relief and cause dose-limiting side effects. There is a great need for more effective and more tolerable pain-relieving therapies. This need can only be met by critical assessment and targeting of the complex peripheral and central pathophysiologic processes that underlie chronic pain in PNP and CRPS. The role played by peripheral input from hyperactive and irritable sensory nerve fibers in initiating and maintaining the pathologic upstream processing and perception of pain is well established. This peripheral sensitization, featured in both PNP and CRPS, occurs as a consequence of dysregulated injury-

induced inflammatory, vascular, metabolic and oxidative tissue reparative responses of the body. The targeting of these maladaptive responses has the potential to curb the gain of function in the sensory nerves and alleviate pain. Moreover, the peripheral location of these processes allows the administration of treatments localized to the site of pathology and avoiding undesired systemic effects.

This thesis describes work aimed at testing and demonstrating the analgesic efficacy of topical agents that alleviate tissue hypoxia and oxidative stress in rat models of PNP and CRPS. Tissue hypoxia and oxidative stress were selected as therapeutic targets because of their interactive and wide-reaching role in triggering sensory nerve hyperactivity both directly, and indirectly through the induction of inflammatory responses. Moreover, the simultaneous targeting of these two processes to achieve analgesia in PNP and CRPS has not been well explored. Local administration of vasoactive drugs in combination with antioxidants has the potential to dampen peripheral sensitization and reduce pain in PNP and CRPS. With the combination of these agents formulated into topical treatments, fast delivery of optimally therapeutic local drug concentrations can be achieved while avoiding the occurrence of systemic side effects.

Pharmacological development of drug combinations as plain drug mixtures requires the completion of efficacy and safety studies done at different doses for the individual constituent drugs and their combination. This expensive endeavor reduces the probability of clinical translation of such formulations. To address this issue, a section of this thesis focused on refining the preparation of the vasoactive-antioxidant drug combinations. The approach adopted was the synthesis of salts and co-crystals by pairing selected vasodilatory anti-hypoxic drugs with plant-derived potent antioxidants using mechanochemical green chemistry methods. Salts and co-crystals are weakly bonded multi-component compounds, that merge into discrete solid forms of fixed stoichiometric ratio. They can be singularly dosed in their pharmacological investigation, avoiding the need for studies of constituents and their combinations at different doses. This immensely reduces expenditure in resources and time. Additionally, salts and co-crystals have a unique molecular organization that has the potential to yield enhanced physicochemical properties translating into improved solubility, stability and half-life. Also, they are considered novel and patentable products even when made from two drugs already on the market. This increases the prospects of these products attracting investors for further study and extensive clinical trials necessary for eventual clinical use.

To summarize, in search of safe and effective analgesic for PNP and CRPS, the work presented in this thesis takes the approach of locally targeting poor tissue oxygenation and oxidative stress using topical drug combinations. To promote clinical translation and potentially creating more effective drug combination, the pairing of anti-hypoxic and antioxidant agents through salt and co-crystal synthesis was tested.

The following are specific aims targeted by the constituent projects of the thesis. The work described in **Manuscript 1** tested the efficacy of the topical combination of the vasodilatory drug, meldonium, and the antioxidant, N-acetyl cysteine, in alleviating mechanical allodynia in rat models of PNP and CRPS. Follow up experiments evaluated the effect of the topical treatment on NO-mediated tissue oxygenation in the CRPS rat model. The work presented in **Manuscript 2** demonstrates the successful mechanochemical synthesis and validation of analgesic salts and co-crystal made from pairings of vasodilatory drugs with antioxidant compounds. The novel salts and co-crystal have subsequently been evaluated for anti-allodynic efficacy and potency in CRPS rats in comparison to their parent constituent agents. In **Manuscript 3**, a prototype of the novel salts and co-crystal was assessed for its effects of enhancing local tissue oxygenation in CRPS rats. Finally, **Manuscript 4** presents a clinical trial protocol that is in current use for a phase-II study testing the analgesic effects of the vasodilator drugs clonidine and pentoxifylline, as a topical combination, in post-traumatic neuropathic pain patients.

2. MANUSCRIPT 1: Topical combination of meldonium & N-acetyl cysteine relieves allodynia in rat models of CRPS-1 & peripheral neuropathic pain by enhancing NO-mediated tissue oxygenation

2.1 Contribution of Authors

This work has been published previously as: Fulas OA, Laferriere A, Stein RS, Bohle DS, Coderre TJ. Topical combination of meldonium and N-acetyl cysteine relieves allodynia in rat models of CRPS-1 and peripheral neuropathic pain by enhancing NO-mediated tissue oxygenation. J Neurochem. 2020:152;570-584.

Chapters 2.2 to 2.6 are used with permission from Fulas *et al*. 2020, of which I am the first author.

Prof Terence J Coderre conceived the idea for this project. I designed the experiments with inputs from Prof Coderre and Andre Laferriere. I performed the CCI surgeries and formulated the topical analgesic drugs. Andre Laferriere and I prepared the CPIP and CIPN rat models, administered the drugs and collected behavioral data. Prof D. Scott Bohle oversaw the electron paramagnetic spectroscopy (EPR) experiments. I synthesized the EPR MNIC standard and grew its single crystals. Prof Bohle acquired and solved the single-crystal structure data. Robin S. Stein operated the EPR spectroscope and acquired the raw experimental data. I prepared the EPR experiment samples, processed and analyzed the collected raw data. This manuscript text was drafted by me and revised by Profs. Coderre and Bohle.

2.2 Abstract

Local microvascular dysfunction and consequent tissue ischemia/hypoxia contribute to the symptoms of CRPS and PNP. As NO is a key regulator of microvascular blood flow, compounds that increase it are potentially therapeutic for these pain conditions. This led us to hypothesize that the topical administration of drugs that modulate local tissue NO levels can alleviate the pain of CRPS and peripheral neuropathic pain. We investigated the anti-allodynic effect of a combination of two NO-modulating drugs: meldonium and N-acetylcysteine (NAC). An equimolar topical formulation of the two drugs was tested on CPIP, a rat model of CRPS, CCI of the sciatic nerve and CIPN, rat models of PNP. Topical meldonium-NAC produced significant anti-allodynia in CPIP, CCI and CIPN rats. Moreover, repeated application of topical meldonium-NAC produced an increase in the duration of anti-allodynia in the CPIP and CCI rats. While pre-treatment with an NO synthase inhibitor attenuated the anti-allodynic effects of meldonium-NAC, 30-minute hyperbaric oxygen treatment combined with a non-effective dose of meldonium-NAC produced significant anti-allodynic effects in CPIP rats. Both experiments implicated NO in the drug combination's anti-allodynic effects. To ascertain the role played by changes in local tissue NO, we performed a quantification of plantar muscle NO in CPIP rats after hind paw topical treatment with meldonium-NAC and revealed significantly increased plantar muscle NO levels in drug-treated rats. The drug combination also reversed the reduction in tissue oxygenation normally observed in CPIP hind paws. In addition to introducing a novel topical treatment for mechanical allodynia in CRPS and peripheral neuropathic pain, this work showcases the analgesic potential of locally targeting microvascular dysfunction and tissue ischemia/hypoxia in these conditions, with emphasis on the role of NO.

2.3 Introduction

Vascular compromise and resulting tissue ischemia/hypoxia of varying extent has been described in the pathogenesis of different chronic pain conditions, including peripheral neuropathic pain and complex regional pain syndrome (CRPS). Among the processes identified are structural and functional vaso-occlusive changes in the micro-vessels of affected peripheral nerves and surrounding tissue [1, 2].

In the limbs of CRPS patients, there is enhanced production and responsiveness to vasoconstrictive catecholamines and endothelin-1, along with diminished production of vasodilatory molecules like nitric oxide (NO) [3, 4]. Moreover, these patients demonstrate significant reductions in acetylcholine-induced endothelial NO-dependent vasodilatory responses within their affected extremities [5]. The resultant impairment in nutritive blood flow with consequent tissue hypoxia and acidosis likely contributes to increased pain and hypersensitivity in CRPS [6, 7].

Likewise, the pathophysiology of painful peripheral neuropathies caused by various etiologies has been reported to involve impaired blood flow and ischemic hypoxia of affected peripheral nerves [1]. In post-traumatic, diabetic and chemotherapy-induced peripheral neuropathy, studies show endoneurial micro-vessels are reduced in quantity and exhibit pathologic endothelial wall thickening and luminal narrowing [8-10]. The diseased nerves that are rendered hypoxic exhibit metabolic dysfunction that likely contributes to their pain-inducing abnormal activity [1, 9, 11].

Given this evidence, interventions that target microvascular flow and tissue oxygenation have great potential to enhance therapeutic regimens devised for CRPS and neuropathic pain. It has already been demonstrated that hyperbaric oxygen and genetic-angiogenic therapies that restore peripheral nerve oxygenation prevent and reverse pain hypersensitivity in animal models and in patients with these conditions [10, 12, 13]. A different approach to achieving the same goal with potentially faster clinical translation is the development of safe pharmacological formulations that act on key molecules that regulate microvascular flow like NO. NO, mainly produced by microvascular endothelium, is a vital signaling molecule essential for vasodilation, prevention of smooth muscle cell proliferation, inhibition of platelet activation and leukocyte adhesion. These effects add together to increase microvascular patency and to enhance tissue oxygenation [14].

Defective endothelium-dependent NO-mediated vasodilation has been reported in the affected tissue of both CRPS and peripheral neuropathic pain patients [5, 15].

Despite its crucial role in maintaining microvascular patency and tissue oxygenation, the abundant availability of NO does not guarantee these effects. The oxidative status of the microenvironment in a tissue determines how much of the NO remains in its native form to exert its desired vasodilatory changes [16]. This is because NO is a highly reactive molecule that readily reacts with several compounds including oxygen free radicals yielding in the formation of toxic reactive nitrogen species (RNS) [17]. Therefore, the therapeutic use of NO to target the microvascular dysfunction in disease conditions of increased oxidative stress requires concomitant measures to decrease the abundance of oxygen free radical to prevent NO quenching and RNS generation. Increased tissue level of ROS has been reported in the pathogenesis of both CRPS and peripheral neuropathic pain via its direct cellular toxicity and secondary inflammatory and vascular consequences [18-20].

In this study, we investigated the analgesic effect of vascular NO boosting compounds, meldonium and N-acetyl cysteine (NAC). Meldonium is an anti-ischemic drug in clinical use for the treatment of ischemic cardiovascular and neurological diseases in Eastern Europe [21-24]. A carnitine-mediated inhibitor of fatty acid β-oxidation with long-term use, it is reported to improve glucose transport, uptake and utilization [25]. Meldonium also has an acute, carnitine-independent effect of increasing vascular NO levels [21, 23, 24, 26]. N-acetyl cysteine (NAC) is a precursor of the potent antioxidant glutathione that also stimulates endothelial NO production and sustains its level by neutralizing NO-consuming oxidative free radicals [27-31]. By replenishing glutathione, it alleviates oxidative stress in different tissues including vascular endothelial and smooth muscle cells [32].

We studied the analgesic effects of an equimolar topical preparation of meldonium and NAC in rat models of CRPS-1 and neuropathic pain. We further investigated the effect of tissue NO modulation on the analgesia achieved by this topical combination by adding systemic administration of the NO synthase inhibitor *Nω*-nitro-L-arginine methyl ester (L-NAME). We did a spectroscopic measurement of the change in local tissue oxygenation brought about by topical treatment with an analgesic dose of meldonium-NAC, and separately investigated the effect of increasing tissue oxygenation by hyperoxia on the potency of the topical analgesia. We also performed a relative quantification of the change in plantar muscle NO level and local tissue

oxygenation achieved after hind paw treatment with the topical formulation on a rat model of CRPS-1. These experiments attempted to provide mechanistic evidence for the hypothesis that factors that influence tissue microvascular flow and oxygenation like NO contribute to CRPS and neuropathic pain and are potential therapeutic targets.

2.4 Methods

All experimental manipulations in experimental animals were approved by the McGill University Animal Ethics committee in conformity to CCAC regulations (protocol application # 20177877). A total of 97 rats were used in these experiments and all were housed two per cage in 45cm*24cm*20cm cages with micro-filter tops (Ancare crop, Bellmore, NY cat # R20HT). Animals were housed in a temperature (21-25^{o}C) and humidity-controlled room on a 12 h light/dark cycle beginning at 7:00 AM with ad libitum access to rodent chow and water. The study described in this manuscript was not pre-registered. The timeline of each experiment along with the number of rats used in each experiment is as depicted in Fig 4.

2.4.1 Chronic post-ischemic pain (CPIP)

CPIP rats were prepared by inducing prolonged hind paw ischemia and subsequent reperfusion, as previously described by Coderre et al., 2004 [33]. In short, male Long Evans (RRID:RGD_2308852) or Sprague Dawley rats (RRID:RGD_10395233) (the latter used for experiments with hyperbaric oxygen treatment) (300-400 g; Charles River, QC, Canada) were anesthetized over a 3-hour period with a bolus (55 mg/kg, intraperitoneally [i.p.]) followed by maintenance i.p. infusion (0.15 mL/hour) of sodium pentobarbital (Ceva Santé Animale, Libourne, France) for 2 hours. One mL of Dextrose in water (40% w/v) was administered intraperitoneally every hour of the occlusive period, as hyperglycemia enhances CPIP mechanical allodynia [34]. Following induction of anesthesia, a Nitrile 70 Durometer O-ring (O-rings West, Seattle, WA cat # 76245-368) with an internal diameter of 5.5-mm was slipped around the rat's left hind limb proximal to the ankle joint to effect a complete blockage of arterial blood flow. The ring was left in place for 3 hours, and the rats recovered from anesthesia 30 to 60 minutes following reperfusion. The CPIP procedures were performed at 8-12 H of each day. Anti-allodynia trials with topical treatments were performed 7-14 days after the CPIP procedure. An apriori plan was put in place to exclude rats that failed to exhibit mechanical allodynia at 7 days post-procedure. Six to eight rats per group were used in behavioral experiments assessing the acute topical analgesic effect of

meldonium, NAC and meldonium-NAC. In the experiments testing the effect of long-term repeated treatment with topical meldonium-NAC, 6 and 7 CPIP rats were assigned to meldonium-NAC and vehicle treatment groups, respectively, after a simple randomization procedure.

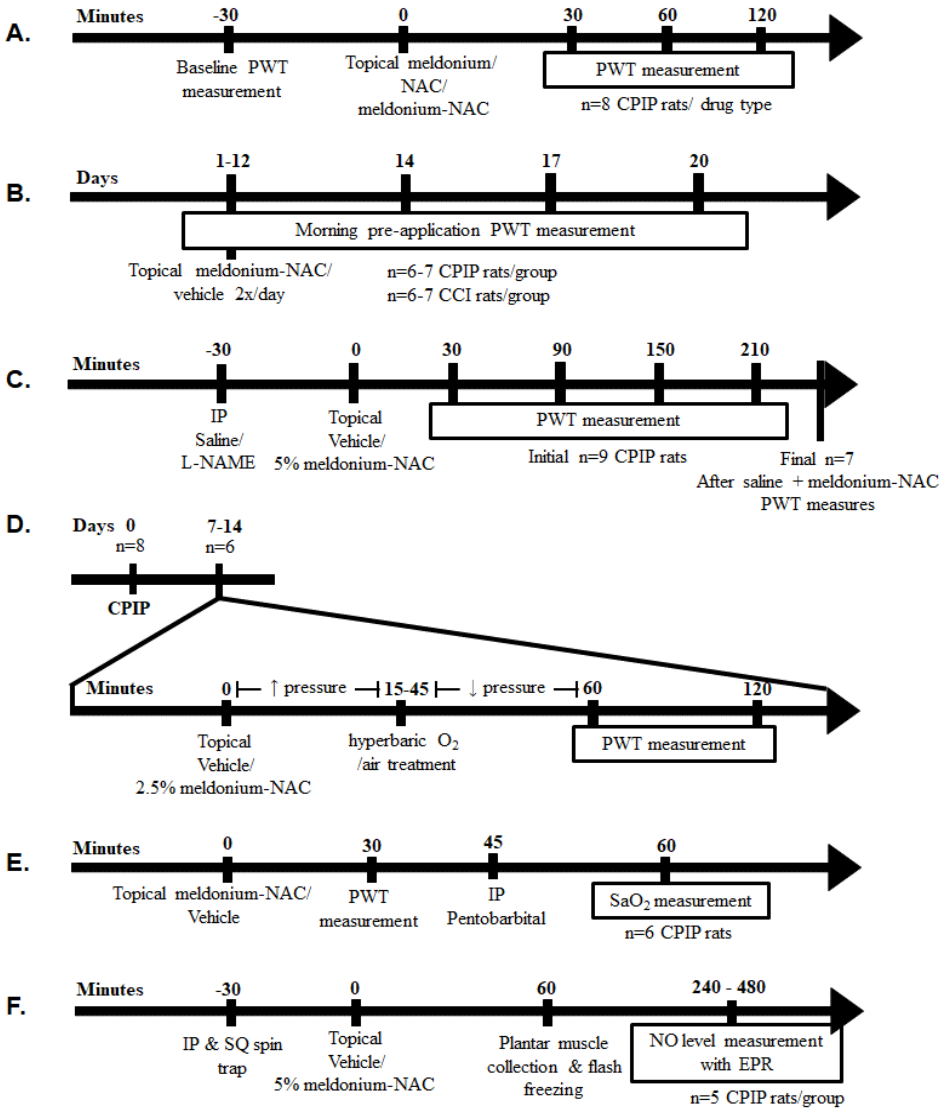

Figure 4. Timeline and design of experiments.

(**A**) Effect of topical meldonium (n=8 rats), NAC (n=8 rats) and meldonium-NAC (n=8 rats) in the CPIP model of CRPS. (**B**) Effects of long-term repeated treatment with topical meldonium-NAC on CPIP (n= 13 rats, 6 drug/7 vehicle) and CCI (n= 13 rats, 6 drug/7 vehicle) models of CRPS and peripheral neuropathic pain. (**C**) Effects of pairing treatments with topical meldonium-NAC and the nitric oxide synthase inhibitor, L-NAME on CPIP rats (Total n=9 reduced to n=7 after excluding saline + meldonium-NAC unresponsive rats at the end of experiment) (**D**) Effect of combining a sub-therapeutic dose of topical meldonium-NAC with hyperbaric oxygen in CPIP rats. (Total n=8 rats reduced to n=6 rats after excluding 2 that were non-responsive to the CPIP

procedure, before the start of treatment protocols) (**E**) Effect of topical meldonium-NAC on plantar tissue oxygen saturation (n=6 rats) (**F**) Experiment assessing change in plantar muscle NO level post-topical treatment with meldonium-NAC in CPIP rats (n=5 rats in vehicle and n=5 rats in meldonium-NAC treatment groups).

2.4.2 Sciatic nerve chronic constriction injury (CCI) model

Male Long Evans rats (200-250 g; Charles River, QC, Canada) were used. Constriction injury of the left sciatic nerve (CCI) was performed according to the protocol of Bennett et al., 1988 [35]. The surgeries were carried out at 8-16 H during the day. The rats were anesthetized with isoflurane, the skin on the lateral aspect of the thigh was incised and the fascia and muscle bluntly dissected to expose the common sciatic nerve just above its trifurcation point. Four loose ligatures were then placed with a 4-0 chromic gut suture around the nerve with approximately 1-mm spacing. The wound was closed in layers with 3-0 silk sutures. Finally, topical lidocaine-bupivacaine was applied to the skin as a postoperative analgesic, and the animals were left to recover. Anti-allodynia trials with topical treatment were performed 7-14 days after CCI surgery. A group of 6 rats was used in behavioral experiments assessing the topical analgesic effect of meldonium-NAC. In the experiments testing the effect of long-term repeated treatment with topical meldonium-NAC, 6 and 7 CCI rats were assigned to meldonium-NAC and vehicle treatment groups, respectively, after a simple randomization procedure.

2.4.3 Chemotherapy-induced painful neuropathy (CIPN) model

Male Long Evans rats (250–300 g) were injected i.p. with the chemotherapeutic agent paclitaxel (Taxol; Bristol-Myers Squibb, E. Syracuse, NY cat # 1097). Paclitaxel was prepared as a 10 mg/mL stock solution in 50% Cremophor EL (EMD Chemicals, Gibbstown, NJ cat # 61791-12-6) and 50% absolute ethanol vehicle. The stock solution was diluted to 1 mg/ml in saline and injected (1.5 mg/kg) i.p. on the morning (8-10 H) of 5 alternate days (days 0, 2, 4, 6 and 8) following a procedure previously described with some modifications [36, 37]. Anti-allodynic trials with topical treatments were performed 6 weeks after the paclitaxel treatment regimen. A group of 8 rats was used in behavioral experiments assessing the topical analgesic effect of meldonium-NAC.

2.4.4 Drug preparation

Meldonium dihydrate was obtained from Toronto Research Chemicals, Toronto, ON (cat # M233350). *N*-acetyl cysteine (cat # A-7250), *Nω*-nitro-L-arginine methyl ester hydrochloride

(L-NAME) (cat # N5751), sodium diethyldithio-carbamate trihydrate (Na-DETC) (cat # 22868), ferrous sulfate heptahydrate (cat # F8633) and sodium citrate tribasic dihydrate (cat # S279-500) were all obtained from Sigma-Aldrich, St. Louis, MO.

The meldonium-NAC combination was made of equimolar quantities of meldonium (146.19 g/mol) and NAC (163.2 g/mol) . To prepare 5% weight/weight (W/W) meldonium-NAC, meldonium dihydrate (2.36%, W/W) was heated at 70^{o}C for 5 mins and an equimolar quantity of NAC (2.63%, W/W) dissolved in milli-Q water was added. The mixture was then dried by a combination of heating at 70-80^{o}C in an incubator and vacuum desiccation.

All topical formulations were prepared as ointments using a composite, water-soluble polyethylene glycol (PEG) base system consisting of 40% carbowax (PEG 3350) and 60% PEG 400 (both from Sigma Aldrich, St. Louis, MO, cat # P3640, P3265 respectively). The required amounts of the active ingredients were weighed and added to the molten base and mixed well. The vehicle treatment consisted of the same water-miscible base ointment without the active drugs. Following a preliminary screen, the 5% W/W dose of meldonium-NAC effectively relieved mechanical allodynia in the animal models and so was used in subsequent experiments.

2.4.5 Mechanical hypersensitivity testing

As a measure of mechanical hypersensitivity, paw withdrawal threshold (PWT), was tested on the plantar surface of the injured left hind paw of the rats. No blinding procedures or sample size calculations were performed. Sample size was determined based on prior projects done in our laboratory that examined the analgesic effect of topical combinations of vasodilator drugs on CPIP, CCI & CIPN rat models [36]. All behavioral experiments were done between 8 and 14 H. The rats were first habituated for 30 minutes in a test chamber. Nylon monofilaments (Semmes-Weinstein von Frey hairs, Stoelting, Wood Dale, IL cat # 58011) were applied in either ascending (after negative response) or descending (after positive response) force. Each filament was applied for 10 s or until a flexion reflex occurred. The minimum stimulus intensity was 0.25 g and the maximum was 15 g. Based on the response pattern, and the force of the final filament (5th stimulus after first direction change), the 50% threshold (grams) was calculated as ($10^{[Xf+ k\delta]}$) /10000, where X_f = value (in log units) of the final von Frey hair used, k = value for the pattern of positive/negative responses and δ=mean difference in log unit between stimuli (here, δ=0.224, for more detail see Chaplan et al., 1994) [38]. Experiments requiring PWT data collection were done on a daily basis

starting from the 7th post-procedure day for the CPIP and CCI rats and after 6 weeks of paclitaxel administration in the CIPN rats.

2.4.6 Long-term repeated topical meldonium-NAC treatment in CPIP and CCI rats

For both the CPIP and CCI experiments, 13 rats each were used. The rats were assigned to vehicle and 5% meldonium-NAC treatment groups (7 & 6 rats each, respectively) using block randomization. Each of the two rats in a housing cage was first randomly assigned to one of the two treatment blocks. PWT values were ranked and the mean pre-treatment PWT calculated separately for each group. A significant difference in mean PWT was corrected by exchanging the rat with the highest PWT measure in one group with the rat with the lowest PWT in the other group until comparable pre-treatment mean PWTs between the two treatment groups was achieved. After being assigned to vehicle and 5% meldonium-NAC treatment groups, allodynic CPIP and CCI rats were topically treated twice daily with vehicle or 5% meldonium-NAC for 12 days. Pre-application daily morning PWTs were then measured to assess if the repeated topical treatment prolonged the anti-allodynic effect of the drug long enough to be carried overnight to the following morning. Serial PWT testing was carried out every 2-5 days during the 12-days course of treatment, and every 3 days for 8 more days after treatment cessation (Fig 4).

2.4.7 L-NAME effects on the analgesic effects of topical treatments in CPIP rats

After measuring pre-drug PWTs, intraperitoneal L-NAME (20 mg/kg in saline, 1 mg/ml or an equivalent volume of saline) was administered. Thirty minutes later, topical 5% (W/W) meldonium-NAC or vehicle was applied to the injured left hind paw of the CPIP rats. Post-drug PWTs were then measured at 30, 90, 150 and 210 minutes after topical application (Fig 4). A group of 7 CPIP rats in total were used in this experiment. The rats received 4 different treatment combinations on alternate days over a period of 8 days. The order of experimental sessions in terms of the i.p. and topical treatments the rats received is as follows: firstly i.p. saline followed by topical vehicle, secondly i.p. saline followed by topical meldonium-NAC, thirdly i.p L-NAME followed by topical meldonium-NAC and lastly i.p. L-NAME followed by topical vehicle. The absence of carryover effects was ensured by obtaining pre-treatment PWT measures before each session. As the experiment was aimed at testing the effect of L-NAME's NOS inhibition on responsiveness to topical meldonium-NAC, only rats that exhibited a post-treatment of PWT of at least 9 g in the i.p. saline/topical meldonium-NAC run were included in subsequent data analysis.

2.4.8 Combination treatment with hyperbaric oxygen & topical meldonium-NAC in CPIP rats

Sprague-Dawley rats were used to carry out these experiments given previous experience in the laboratory indicating Long Evans rats poorly tolerated hyperbaric oxygen treatment. This observation is in line with prior reports of greater lung tidal volumes of Sprague Dawley rats as compared to other strains [39]. After measuring baseline PWTs, topical meldonium-NAC (2.5% W/W) or vehicle was applied to the injured hind paw of Sprague-Dawley rats that underwent the CPIP procedure as previously described. Rats were then placed in 10-liter plexiglass cylinders to obtain treatment with either regular air or hyperbaric oxygen at 2.5 atmospheres (atm) for 30 minutes. Post-topical treatment PWTs were then measured at 60 and 120 minutes (Fig 4).

Hyperbaric oxygen treatment was given in a previously described, custom-made 10-liter chamber that had a 165 cm^2 vented circular cell culture plate containing 230 g of calcium sulfate (Drierite®, Thermo Fisher Scientific, cat # 43064) to sequester moisture and CO_2 [1]. The hyperbaric chamber was filled with 100% O_2 (Praxair, Montreal QC, cat # MO-K), and chamber pressure was slowly increased to 2.5 atm over 10 minutes. The flow rate of O_2 was maintained at 4.72 L/min, and the treatment lasted for 30 min. The hyperbaric chamber was then depressurized over 10 min to atmospheric pressure. In air-treated animals, animals were treated with air at normal atmospheric pressure.

A group of 6 rats in total were used in behavioral experiments assessing the combined analgesic effect of meldonium-NAC and hyperbaric oxygen. In four different sessions, first topical vehicle combined with either air or hyperbaric oxygen was tested followed by topical meldonium-NAC combined with either air or hyperbaric oxygen.

2.4.9 Measurement of plantar tissue oxygen saturation

Using a visible light-based spectroscopic vascular monitoring system (moorVMS-OXY, Moor Instrument Ltd, Devon UK), percent oxygen saturation of hemoglobin (SaO_2) was measured from the plantar side of the rat's hind paw before the O-ring occlusion of the CPIP procedure, 15 minutes after reperfusion and 5-7 days after the CPIP procedure following topical hind paw treatment with vehicle or 5% (W/W meldonium-NAC). To perform the hind paw SaO_2 measurements, a laser probe (7 mm) that transmits low-power white light and collects reflectance light from the tissue and blood cells was apposed to the hind paw of the rats using a loose-fitting elastic bandage (3 cm inner diameter) after light anesthesia with IP pentobarbital (40mg/kg).

Continuous measurements of total hemoglobin, oxygenated hemoglobin, deoxygenated hemoglobin and SaO_2, were recorded at 2 HZ for 30 seconds. A group of 6 rats underwent SaO_2 measurement sessions 60 minutes following topical treatment with vehicle or 5% (W/W) meldonium-NAC on two alternate days. A mean of each rat's 30-second recording was calculated for comparison between sessions.

2.4.10 Measurement of plantar muscle nitric oxide using EPR spectroscopy

2.4.10.1 Synthesis of mononitrosyl iron dithiocarbamate (MNIC)

Chemical synthesis of MNIC used as a standard in this experiment was performed as previously described by Platt, 2010 [40]. Thus, 0.75 g of iron (II) sulfate heptahydrate and 0.225 g of sodium nitrite was mixed into 15 ml of 0.1 M sulfuric acid to which 1.5 g of sodium diethyl dithiocarbamate (sodium DETC) was added. The solution was stirred vigorously and transferred into a separatory funnel and extraction was performed with small volumes of chloroform. The extracted solution was dried with anhydrous $MgSO_4$. The dehydrated filtrate was then dried with a rotary evaporator and recrystallized in a mixture of dichloromethane and hexane.

2.4.10.2 Crystallography

Purified single crystals of MNIC were grown by vapor diffusion from chloroform and hexane at room temperature. Deep red plates of $Fe(NO)(DETC)_2$ form readily and one with dimensions of 0.15 x 0.1 x 0.05 mm was mounted with epoxy on a glass fiber and its diffraction data collected at room temperature. The single-crystal X-ray diffraction measurements were obtained with a BRUKER SMART CCD or BRUKER APEX-II CCD diffractometer using graphite-monochromated Mo radiation (Kα λ=0.71073 Å). SAINT7 was used for integration of the intensity reflections and scaling, and SADABS8 for absorption correction. Intrinsic phasing was used to solve the positions of most heavy atoms with final refinement being accomplished by difference Fourier Methods. One of the diethyl amide substituents was disordered over two sites in a refined 45/55% ratio. The major position is shown in Fig 9A and a view of both sites are shown in Fig S1.2 (see Appendix). The hydrogen atoms were placed at calculated locations but were not refined. Non-hydrogen atoms are located by difference Fourier maps and final solution refinements are solved by full-matrix least-squares method on F^2 of all data, using SHELXTL7 software. The final anisotropic full-matrix least-squares refinement on F^2 with 208 variables converged at R1 = 5.50%. The goodness-of-fit was 0.985. Crystallographic details have been deposited with the Cambridge Crystallographic Data Center, CCSD # 1938170.

2.4.10.3 Sequence of EPR experiments

EPR measurement of plantar muscle NO was performed as previously described by van Faassen et al., 2008 with some modifications (Fig 4) [41]. CPIP rats were first randomly assigned to vehicle and drug treatment groups. The NO spin trap Na-DETC 400 mg/kg dissolved in milli-Q water at 100 mg/ml was administered intraperitoneally and ferrous citrate made by mixing ferrous sulfate 37.5 mg/kg and sodium citrate 187.5 mg/kg was administered subcutaneously into the scruff of the neck. Thirty minutes later, topical 5% (W/W) meldonium-NAC or vehicle (5 rats/group, treatment group assigned by block randomization to ensure comparable pre-treatment PWT as previously described) was applied to the injured paw of the CPIP rats. After 60 minutes of topical treatment, rats were anesthetized with i.p. pentobarbital (55 mg/kg) and plantar muscle samples (70-120 mg) were collected from the hind paws. The muscle samples were immediately placed in EPR tubes and flash-frozen in liquid nitrogen until EPR measurements.

2.4.10.4 EPR acquisition parameters

The EPR spectra were acquired using a Bruker Elexsys 580 X-Band FT/CW EPR console (Rheinstetten, Germany) operating at 9.42 GHz with 5 mW microwave power and power attenuation of 16 dB. The spectra were obtained at 140 K by cooling with a constant flow of liquid nitrogen and the samples in frozen 4 mm diameter quartz EPR tubes (Wilmad-LabGlass, Vineland NJ, cat # W707SQ250M-1). The magnetic field was modulated at 100 kHz with an amplitude of 10 G and a sweep width of 300 G. Time constant and conversion time were set at 1.28 ms and 163.84 ms, respectively. The receiver gain was set to 60 dB. To obtain an acceptable signal-to-noise ratio, 3 scans were averaged. DPPH was used as an EPR standard. Using the Bruker X_{EPR} data acquisition software, baseline correction of raw EPR spectra was performed and single integrals calculated to obtain spectra in terms of absorption intensities. Then, the absorption intensity values of each spectra lying in the MNIC signal *g*-factor range 2.03 - 2.06 were isolated and averaged by treatment group.

2.4.11 Euthanasia of experimental animals

Rats used in the plantar muscle NO level determination with EPR were euthanized with pentobarbital overdose of approximately 120 mg/kg i.p. followed by surgically-induced pneumothorax. Rats used in all remaining experiments were euthanized with CO_2 exposure (5 L/min until fully unconscious followed by 10 L/min until breathing stops) and surgically-induced pneumothorax.

2.4.12 Statistical Analysis

All data analysis was performed using the statistical software Statistica (version 6, StatSoft). Time-course measurements of raw PWTs after vehicle and drug administration were subjected to an analysis of variance (ANOVA) for repeated measures, having tested for normality of data distribution using the Shapiro Wilk (SW) test. Pairwise post-hoc comparisons within groups were performed before and after drug or vehicle treatment using Dunnett's test. Pairwise post-hoc comparisons between groups were performed at each experimental time point using Tukey's HSD test. The data collected to compare the effects of meldonium versus meldonium-NAC at 1 week and 8 weeks post-CPIP did not pass the SW test of normality and were analyzed using the Mann-Whitney U test. No testing for outliers was done.

Cumulative anti-allodynic effects measured over 120-minute time course experiments were assessed by calculating the area under the curve (AUC) of PWT elevations plotted after topical application using the trapezoidal method. Comparisons of different drug doses versus vehicle were performed using repeated-measures ANOVA followed by Tukey's HSD test. The local plantar tissue SaO_2 measurement data was similarly analyzed using a repeated measure ANOVA followed by post-hoc pairwise comparisons using Tukey's HSD test. For the data from the EPR experiment, assessment of the difference between the mean absorption intensities of MNIC signal belonging to the drug-treated group and vehicle-treated group were compared using an independent two-tailed *t*-test.

All time-course behavioral data are presented as grouped box-whisker plots: the box extends from 25^{th} to 75^{th} percentiles and whiskers minimum to maximum. A line across each box indicates the median and a dot is there to show where the mean value lies. Single measure data are displayed as bars of mean values ± SEM with overlapping symbols displaying individual data points.

2.5 Results

2.5.1 Effects of topical meldonium, NAC & meldonium-NAC on the CPIP rat model of CRPS-1

Initial experiments individually assessed meldonium and NAC for possible topical anti-allodynic effects in the CPIP rat model of CRPS (Fig 5). Ointments prepared with varying W/W concentrations of meldonium or NAC were applied to the injured hind paw of CPIP rats and resulting changes on PWT serially measured at 30, 60 and 120 min time points. The main effects of dose ($F_{2,14} = 7.3$, $p < .01$) and time ($F_{3,21} = 5.6$, $p < .01$) and dose × time interaction ($F_{6,42} = 3.9$, $p < .01$) were significant. At a concentration of 2.5% W/W, topical meldonium significantly elevated PWTs of CPIP rats above pre-drug levels at 30 ($p = .0002$) and 60 ($p = .0013$) minutes post-application (Fig 5A). On the other hand, topical NAC failed to significantly alter the pre-drug PWT of the rats at W/W dose of 2.5% and 5% (Fig 5B).

In subsequent experiments, the anti-allodynic effects of meldonium and NAC in combination were assessed in CPIP rats using an equimolar mixture of the compounds. Given the similar molar weight of meldonium (146.2 g/mol) and NAC (163.2 g/mol), the mixture (meldonium-NAC) approximately contained a 1:1 composition by weight of each drug. Significant main effect of dose ($F_{2,14} = 14.4$, $p < .001$) and time ($F_{3,21} = 13.9$, $p < .0001$) were observed and the dose×time interaction term neared significance ($F_{6,42} = 2.1$, $p = .069$). At a W/W dose of 5%, meldonium-NAC (containing 2.4% meldonium and 2.6% NAC) relieved allodynia in CPIP rats for at least 2 hours. Significantly elevated PWTs were measured at 30, 60 and 120 min post-topical application (Fig 5C) ($p = .0003$, $p = .00029$ and $p = .0011$ respectively).

Both topical meldonium and meldonium-NAC produced robust and comparable anti-allodynic effects in CPIP rats at 1-week post-procedure (Fig 5D). However, at 8 weeks after CPIP, topical meldonium's anti-allodynic effect declined significantly ($p = .003$), while meldonium-NAC made with a matching W/W dose of meldonium largely remained anti-allodynic (Fig 5D). At this 8-wks post-CPIP time point, the change in PWT produced by topical meldonium-NAC significantly exceeded that obtained with matching W/W dose of meldonium ($p = .047$).

Topical meldonium at W/W dose of 2.5% significantly elevated the pre-drug PWT of the CPIP rats for 60 mins, while 5% (W/W) meldonium-NAC with comparable content of meldonium had effects that lasted at least twice as long even though the added quantity of NAC exhibited no

anti-allodynic effect of its own. The screening experiments described above indicated that analgesia was potentiated by combining meldonium and NAC. The CPIP rat model, which exhibits a more widespread microvascular dysfunction, was used as a screening step to test the anti-allodynic effects of meldonium and NAC individually, and in combination. Experiments on the other models were performed using the meldonium-NAC combination, after concluding that the drugs in combination exhibited a better anti-allodynic profile.

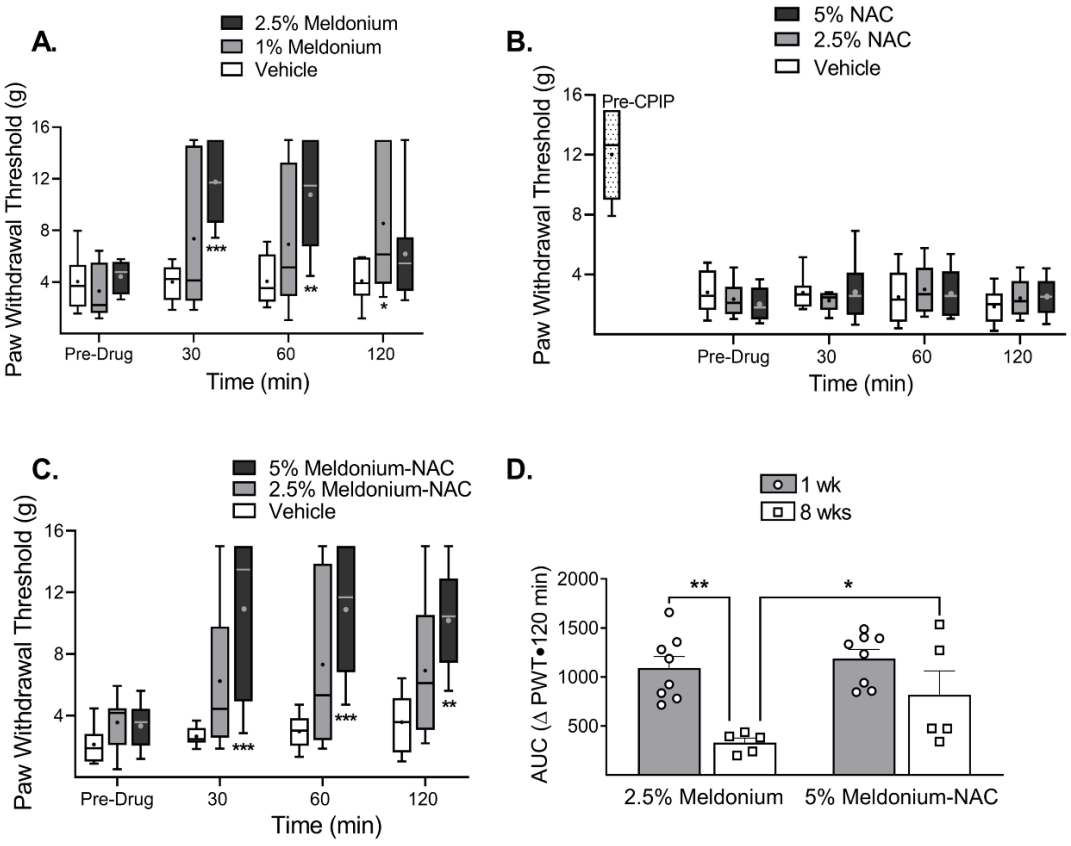

Figure 5. The effect of topical meldonium, N-acetyl cysteine (NAC) and their equimolar mixture (meldonium-NAC) on PWT to von Frey stimulation of ipsilateral hind paw of CPIP rats 7-14 days post-procedure.

Mean PWTs remained significantly elevated for at least 120 mins post-treatment with 5% (W/W) topical meldonium-NAC (n=8 rats) (*$p < .05$, **$p < .01$, ***$p < .001$ compared to respective pre-drug PWT) (**C**). 5% (W/W) topical meldonium produced anti-allodynic effects lasting for 60 mins (n=8 rats) (**A**). 2.5 and 5% (W/W) NAC failed to produce any significant change in PWT (n=8 rats) (**B**). (Dots in the box plots indicate the mean value of the group). (**D**) The anti-allodynic effect produced by topical meldonium waned significantly after 8-weeks (n=8 rats) as compared to 1-week (n=8 rats) post-CPIP, while meldonium-NAC containing matching W/W dose of meldonium retained its anti-allodynic effects (*$p < .05$, **$p < .01$).

2.5.2 Effects of meldonium-NAC on CCI & CIPN rat models of PNP

The topical formulation was tested on rat models of post-traumatic and chemotherapy-induced neuropathic pain (Fig 6). In the CCI rat model of traumatic peripheral neuropathic pain, topical application of meldonium-NAC at doses of 2.5% and 5% (W/W) produced significant increases in PWTs tested at 30, 60 and 120 mins. Main effects of dose ($F_{2,10} = 12$, $p < .01$) and time ($F_{3,15} = 3.5$, $p < .05$) and the dose×time interaction ($F_{6,30} = 2.8$, $p < .05$) were significant.

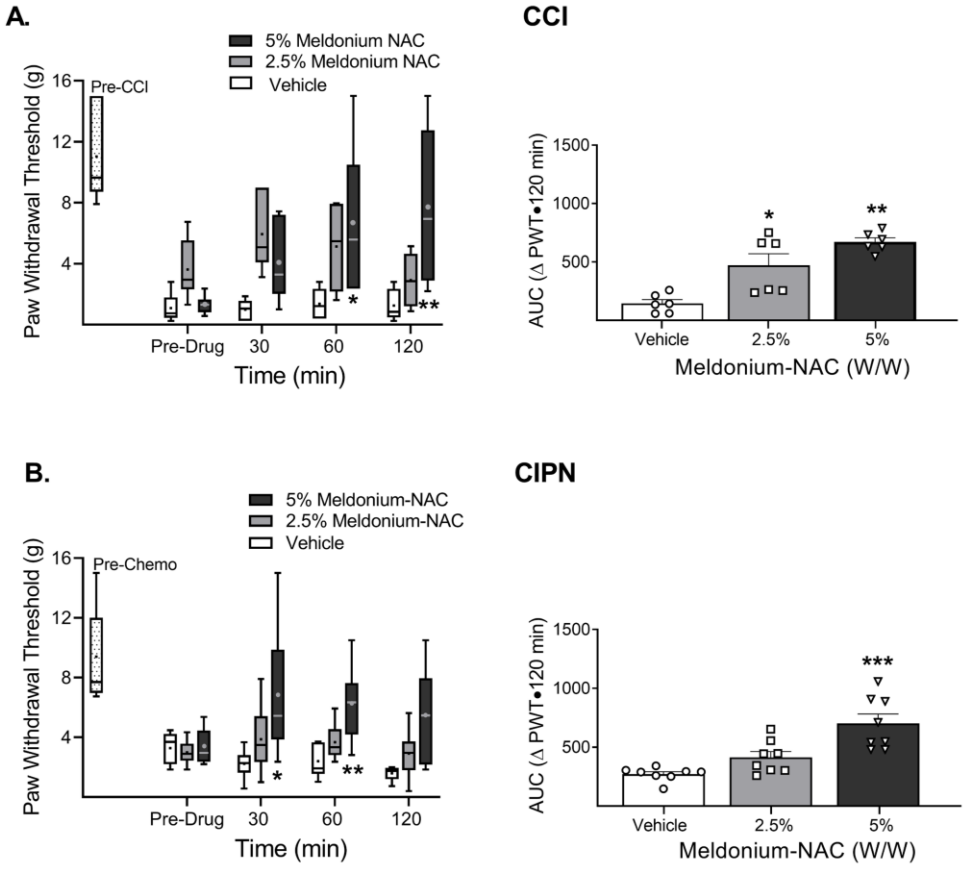

Figure 6. The effect of topical meldonium-NAC on mechanical allodynia in rat CCI and CIPN neuropathic pain models.

Significantly elevated PWTs were achieved after topical treatment with 5% (W/W) meldonium-NAC in both CCI (n=6) (**A**) and (**B**) CIPN (n=8) rats (*$p < .05$, **$p < .01$ and ***$p < .001$ compared to respective pre-drug PWTs; $^{\dagger}p < .05$, $^{\dagger\dagger}p < .01$ and $^{\dagger\dagger\dagger}p < .001$ compared to vehicle AUC values). (Dots in the box plots indicate the mean value of the group).

At the 5% (W/W) dose, meldonium-NAC produced a progressive PWT elevation in CCI rats significant at 60- and 120-mins post-application compared to pre-drug values ($p = .016$ and $p = .0029$ respectively) (Fig 6A). Similarly, allodynic rat models of CIPN exhibited significant

elevations in PWT after topical treatment with 2.5% and 5% (W/W) meldonium, with the main effect of dose being ($F_{2,14} = 17.9$, $p < .001$), and the dose×time interaction ($F_{3,21} = 3.2$, $p < .05$). Topical treatment with 5% (W/W) meldonium-NAC significantly increased pre-drug PWTs at 30- and 60-mins post-application ($p = .00095$ and $p = .008$ respectively) (Fig 6B).

2.5.3 Effects of long-term repeated treatment with topical meldonium-NAC on CPIP & CCI rat models of CRPS & PNP

The topical application of 5% meldonium-NAC produced significant elevations in PWTs that lasted for at least 2 hours in both CPIP and CCI rats. We performed subsequent experiments to assess if the daily repeated application of the drug resulted in longer-lasting relief from allodynia. In both the CPIP and CCI rats, the repeated topical application of 5% meldonium-NAC resulted in a gradual increase in morning pre-application PWTs.

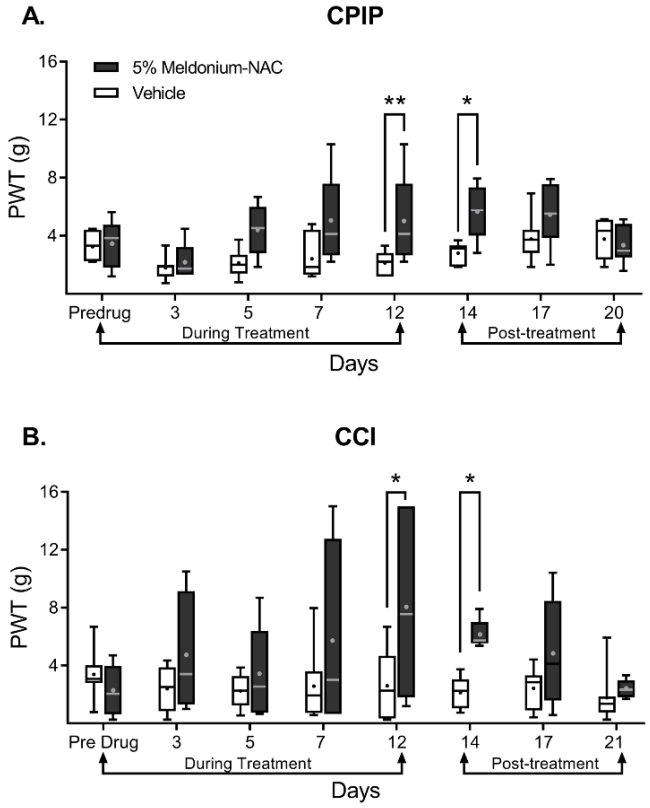

Figure 7. The effect of repeated long-term treatment with 5% (W/W) topical meldonium-NAC on the PWTs of CPIP and CCI rats.

Significantly elevated PWTs that sustained overnight were achieved after 12-day long, two times daily topical treatment with 5% (W/W) meldonium-NAC (*$p < .05$, ***$p < .01$ compared to respective vehicle values, n=6 CPIP/CCI rats for the meldonium-NAC group, n=7 CPIP/CCI rats for the vehicle group, total n=26 rats). (Dots in the box plots indicate the mean value of the group.)

There was a significant treatment×time interaction following the 12 days of treatment in both CPIP ($F_{4,44} = 2.8$, $p < .05$) and CCI ($F_{4,44} = 2.9$, $p < .05$) rats, and this effect extended during the post-treatment follow-up period ($F_{3,33} = 3.3$, $p < .05$ for CPIP and $F_{3,33} = 5.2$, $p < .01$ for CCI). Further analysis indicated significant elevations of PWTs in the meldonium-NAC treated rats as compared to the vehicle-treated rats occurred on the 12th day of topical treatment in both CPIP ($p = .006$) and CCI ($p = .02$) rats (Fig 7). The relative PWT elevations were sustained for 2 more days after treatment completion ($p = .02$ for CPIP and $p = .01$ for CCI rats) Fig 7.

2.5.4 Effects of pairing a nitric oxide synthase inhibitor or hyperbaric oxygen treatments with topical meldonium-NAC on CPIP rats

The following experiments explored the involvement of NO and change in tissue oxygenation in the anti-allodynic effects observed in CPIP rats topically treated with meldonium-NAC. The effect of systemic administration of the NO synthase (NOS) inhibitor L-NAME or saline on rats that were subsequently treated with topical meldonium-NAC or vehicle (PEG) was first assessed. PWT responses were periodically tested for 210 minutes. Significant main effects of treatment ($F_{3,18} = 10.4$, $p < .001$) and time ($F_{4,24} = 6.9$, $p < .001$), as well as a significant treatment×time interaction ($F_{12,72} = 4.1$, $p < .0001$) were observed.

In the trial with topical 5% meldonium-NAC after i.p. saline, previously observed anti-allodynic effects of meldonium-NAC were reproduced with significant PWT elevations after 30, 90 and 150 mins of topical application ($p = .0183$, $p = .000014$ and $p = .000009$ respectively) (Fig 8A). In the following trial, where topical 5% meldonium-NAC was applied after i.p. treatment with L-NAME, the rats exhibited a brief elevation in PWT at 30 mins ($p = .003$) but failed to sustain this change in subsequent measures at 90 and 150 min post-topical application (Fig 8A). Moreover, at these 90- and 150-min time points, the PWTs of the rats pre-treated with i.p. saline showed a significantly greater post-meldonium-NAC anti-allodynic effect as compared to the trial in which i.p. pre-treatment with L-NAME was used ($p = .0047$ and $p = .00097$, respectively). The control experiments with topical vehicle ointment after i.p. L-NAME and topical vehicle ointment after i.p. saline did not result in any significant change in the PWT of the rats.

The finding that NOS inhibition attenuated the anti-allodynic effects produced by topical meldonium-NAC implied the anti-allodynic effects of the drug involved modulation of tissue NO with possible changes in blood flow and tissue oxygenation. This led to a hypothesis that a low

and sub-therapeutic dose of topical meldonium-NAC can be potentiated to produce anti-allodynic effects via 100% oxygen supplementation. To test this, a 2.5% W/W topical preparation of meldonium-NAC that failed to exhibit significant effects on the PWT of CPIP rats was combined with a 30-minute-long treatment with either hyperbaric oxygen at 2.5 atm or free-flowing room air at normal atmospheric pressure for comparison (Fig 8B). Comparing these groups, we observed a significant main effect of treatment, $F_{3,20} = 3.2$, $p < .05$. Neither topical 2.5% meldonium-NAC combined with air, nor topical vehicle with 2.5 atm hyperbaric oxygen produced any significant elevations in PWTs. In contrast, topical 2.5% meldonium-NAC combined with 2.5 atm hyperbaric oxygen resulted in a significant increase in PWTs ($p = .044$) as compared to the control treatment with topical vehicle ointment + air (Fig 8B).

2.5.5 Effect of topical meldonium-NAC on local tissue oxygen saturation

Subsequently, we used a white-light spectroscopic technique, to measure the local plantar tissue oxygenation of CPIP rats after topical treatment with 5% meldonium-NAC as compared to vehicle. As baselines for comparison, the rat's plantar hind paw SaO_2 measurement was first performed during the CPIP procedure: pre-occlusion and post-reperfusion. Compared to the mean baseline plantar SaO_2 value prior to the O-ring occlusion, values were increased by approximately 10% at thirty minutes post-reperfusion (Fig 8C). The effect of topical meldonium-NAC on plantar tissue SaO_2 was assessed 5-7 days post-CPIP procedure when the rats exhibited consistent mechanical allodynia. Significantly lower plantar SaO_2 values were measured in the rats at 5-7 days post-CPIP, as compared to values obtained before the CPIP injury ($p < .001$). Moreover, the post-CPIP plantar SaO_2 of the rats following topical treatment with meldonium-NAC was significantly higher, as compared to values measured with vehicle treatment ($p < .001$) (Fig 8C). The mean post-CPIP plantar SaO_2 of the rats measured after topical meldonium-NAC was only 10% lower than the SaO_2 levels measured during the hyperemic phase of the CPIP injury at 30 mins post-reperfusion (Fig 8C).

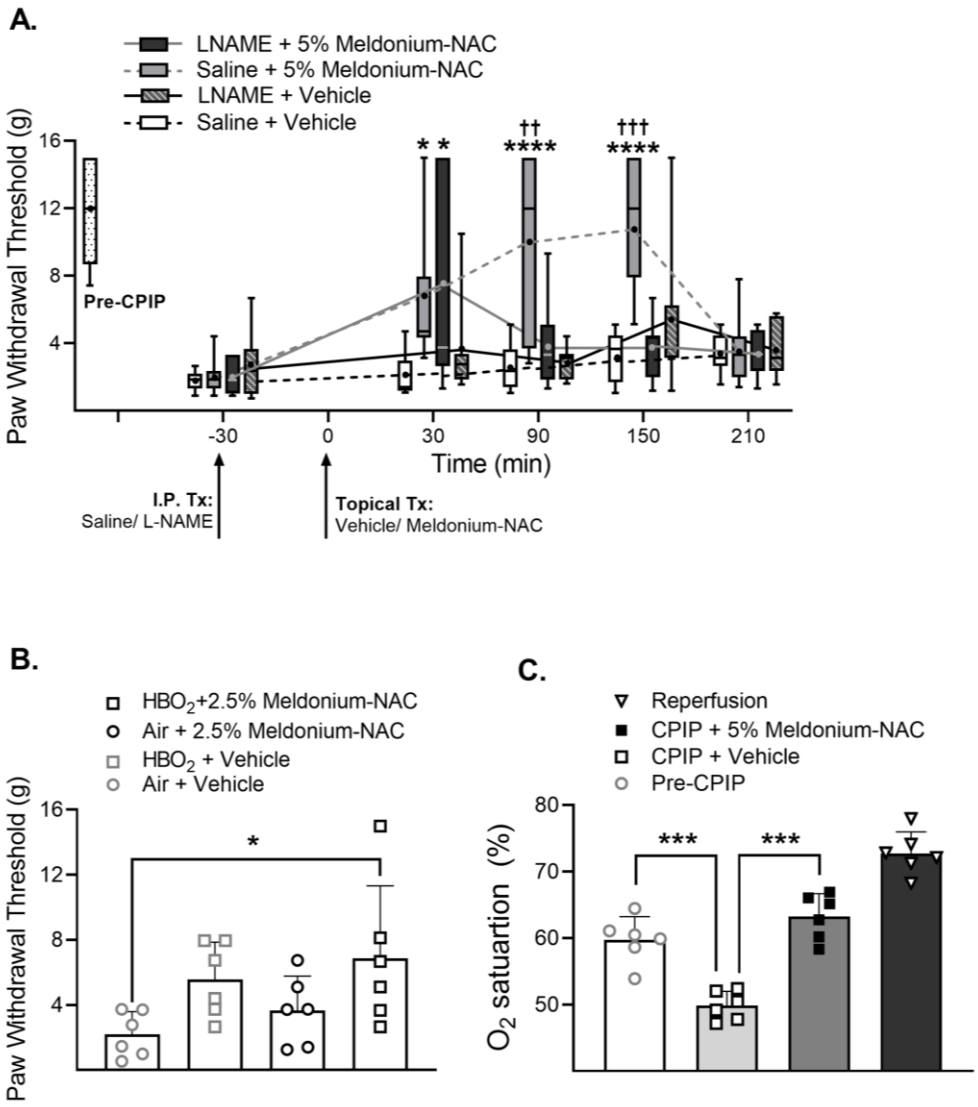

Figure 8. The anti-allodynic effect of topical meldonium-NAC in CPIP rats depended on increases in NO and involved alterations in local tissue oxygenation.

(**A**) Pre-treatment with the NOS- inhibitor L-NAME diminished the anti-allodynic effect achieved by topical 5% (W/W) meldonium-NAC. ($^{*}p < .05$, $^{****}p < .0001$ pre- Vs. post-drug PWTs during the saline → 5% meldonium-NAC session; $^{**}p < .01$ pre- Vs. post-drug PWTs during the L-NAME → 5% meldonium-NAC session; and $^{††}p < .01$, $^{†††}p < .001$ PWTs of the saline → 5% meldonium-NAC session Vs. the L-NAME → 5% meldonium-NAC session at matching time points) (n=8 rats). (**B**) Adding a 30-minutes long 2.5 atm hyperbaric oxygen (HBO_2) treatment to a sub-therapeutic (2.5% W/W) dose of topical meldonium-NAC resulted in significant elevation of PWT ($^{*}p < .05$) (n=6 rats). (**C**) The CPIP procedure significantly reduced oxygen saturation of hemoglobin in the hind paw plantar tissue and (5% W/W) topical meldonium-NAC reverses the decline ($^{***}p < .001$, n=6 rats).

2.5.6 Alterations in plantar muscle NO levels post-topical treatment with meldonium-NAC in CPIP rats

The observation that inhibiting NOS results in reduced anti-allodynic effects of topical meldonium-NAC in CPIP rats suggested the involvement of NO-mediated tissue oxygenation in the analgesia achieved by the drug. An EPR-based experiment quantifying the relative plantar tissue NO levels was used to ascertain the involvement of local tissue NO in the anti-allodynic effects of topical meldonium-NAC. To overcome the inaccuracies imposed by the short half-life and reactivity of NO, an EPR measurement protocol was used that entailed an electron (spin)-trapping procedure [41]. NO trapped by the spin traps, sodium-DETC and iron citrate is expected to produce mononitrosyl iron dithiocarbamate (MNIC). To obtain a reference for the shape and location of the MNIC signal generated by the samples, pure MNIC was first chemically synthesized as described in Platt, 2008 and validated using IR spectroscopy and single-crystal X-ray diffraction (Fig 9A) [40, 42]. Note that the structural model and data presented here correspond to improvement in R from 15.7 % in prior studies to 5.5 % here. An EPR spectrum was then acquired from a solution of the pure MNIC in tetrahydrofuran (THF) at 140 K and the expected triplet EPR signal was observed at $g = 2.045$, $a_N = 13.1$ G (Fig 9B). In a parallel experiment, EPR spectra, also measured at 140 K, were acquired from a flash-frozen plantar muscle of a CPIP rat pre-treated with the iron-dithiocarbamate spin-trap mixture to yield in a MNIC signal of comparable shape and position (Fig 9B). This confirmed successful spin-trapping and EPR detection of NO in the plantar muscle of the CPIP rats in the experiment.

In subsequent experiments, allodynic CPIP rats were generated and randomly assigned to vehicle and 5% meldonium-NAC treatment groups to test the change in plantar tissue NO induced by topical meldonium-NAC. Spin trap compounds were administered 30 min prior to topical treatment with vehicle or 5% meldonium-NAC. After 60 min topical treatment, plantar muscle from the CPIP hind paw was excised and flash frozen for EPR measurement of spin-trapped NO (MNIC) content at 140 K. Baseline corrected derivative EPR spectra were first integrated into absorption spectra and then mean absorption intensity was calculated per the corresponding group. A small but significantly higher mean MNIC absorption intensity was measured from the samples in the meldonium-NAC treated group (3.23 ± 0.08 AU) as compared to vehicle-treated counterparts (2.97 ± 0.07 AU) ($p = .015$) (Fig 9C).

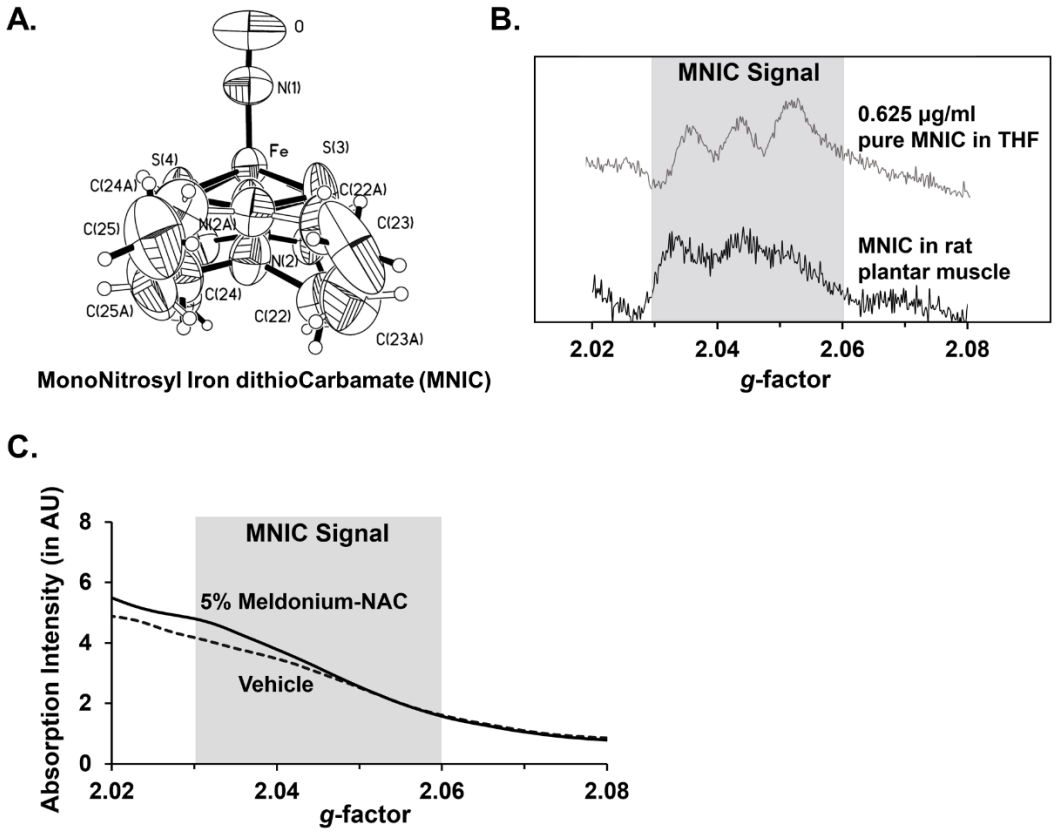

Figure 9. Topical treatment with meldonium-NAC (5% W/W) elevated plantar muscle nitric oxide in CPIP rats.

(A) Crystal structure of one-unit cell of mononitrosyl iron dithiocarbamate (MNIC). **(B)** Sample EPR spectra at 140 K obtained from purified MNIC in tetrahydrofuran (THF) (top) and plantar muscle of a spin-trap treated CPIP rat (bottom). **(C)** Mean traces of absorption intensity of MNIC signal (single integrals of raw EPR spectra) obtained from the plantar muscle of rats treated with topical meldonium-NAC (W/W) versus vehicle (n=5 rats/group).

2.6 Discussion

First-line analgesics for CRPS and neuropathic pain are largely aimed at centrally blunting the input of the sensory system, including the use of systemically administered anticonvulsant and antidepressants. These drugs are often ineffective and cause a myriad of side-effects. Although maladaptive plasticity in the CNS is a significant contributor to chronic pain symptoms in CRPS and neuropathic pain, it is often maintained by pathologic sensory input from peripheral nerves at the site of inciting tissue injury [43]. The topical use of agents that locally suppress such peripheral processes underlying sensory nerve sensitization may thus be an effective and safe means of providing analgesia for these conditions. Sensory alterations often depend on microvascular dysfunction and impaired local flow within multiple tissues for CRPS and confined to the injured

sensory nerve for peripheral neuropathic pain [1, 10, 44-46]. One approach to alleviate this microvascular dysfunction is through pharmacological manipulation of local tissue levels of endogenous vasodilators like nitric oxide. Our data demonstrate the successful topical analgesic use of a NO-modulating topical formulation meldonium-NAC in rat models of CRPS and neuropathic pain.

In CPIP rats, topical meldonium and combined meldonium-NAC treatment produced a robust increase in PWT that was sustained for 2 hours. The agents relieved allodynia with comparable efficacy to vasoactive topical formulations previously investigated and reported by our group [36]. Unique to topical meldonium-NAC is its tendency to produce significant elevation of PWT in the later stage of the CPIP disease spectrum at 8 weeks post-ischemia reperfusion injury. In contrast, the anti-allodynic effects of topical meldonium waned at 2 weeks after the CPIP procedure.

Impaired microcirculation with diminished nutritive blood flow both in superficial and deep tissue is reported in the late stage of CRPS [46, 47]. More so than in the earlier phase of CRPS, endothelial dysfunction, including reduced NO levels, are reported to underlie this pathology in cold CRPS [3, 45, 48]. This is supported by reports of reduced pain in cold CRPS patients treated with the transdermal NO donor isosorbide dinitrate [47]. The sustained anti-allodynic effect of the topical meldonium-NAC in late CPIP could stem from its greater ability to counteract the endothelial dysfunction and low microvascular NO that has been reported. Having independent mechanisms of elevating tissue and vascular NO, meldonium and NAC as a topical combination is deemed to produce a higher boost in local NO levels [26, 29, 49]. Moreover, the added antioxidant effects of NAC can conceivably quench the abundant oxygen free radicals in the CPIP hind paw tissue minimizing the formation of peroxynitrites that both worsen endothelial dysfunction and further dissipate microvascular NO levels [33, 45, 50, 51]. This strong ability of topical meldonium-NAC to counteract endothelial dysfunction is also a possible explanation for its versatile anti-allodynic utility in the CCI and CIPN models of neuropathic pain. Localized to the microvasculature of the injured peripheral nerve, endothelial dysfunction with consequent endoneurial hypoxia contributes to the pain pathology of different types of peripheral neuropathic pain [1, 10, 44].

The onset of anti-allodynic effects seen in the CPIP, CCI and CIPN models varied. The earlier onset of anti-allodynia at 30 minutes post-application seen in the CPIP rats is likely due to

its effects on the widespread microvascular dysfunction involving the skin, muscle and nerves of the affected hind paw [20]. Likewise, in CIPN rats, where the systemic administration of paclitaxel affects the microvasculature of the peripheral nerves in uniformity, extending all the way to intraepidermal nerve fibers, the topically administered meldonium-NAC could readily reach its target to produce anti-allodynic effects within 30 minutes [10]. However, as it has been previously reported in other post-traumatic neuropathic pain models of the sciatic nerve, CCI rats are likely to exhibit focal pathologic changes in the *vasa nervorum* that decline in severity with distance from the site of injury [1]. Thus, the microvasculature pathology in CCI is more proximal than is observed for CIPN or CPIP, potentially increasing the onset of action of the drug.

Tolerance to analgesics with reduced efficacy and duration of action after repeated long term use is an important problem that needs to be remedied in the pharmacotherapy of chronic pain [52-53]. Unlike common analgesics, such as opioids, which exhibit tolerance with repeated daily use, the repeated topical meldonium-NAC treatment exhibits a prolonged duration of action resulting in elevated pre-drug morning PWTs in both CPIP and CCI rat models of CRPS and neuropathic pain. This indicates that persistently alleviating microvascular dysfunction and tissue hypoxia has the potential to incrementally restore sensitized sensory nerves' normal function, resulting in sustained or even improved analgesic activity. Meldonium's added metabolic activity in inhibiting fatty acid β-oxidation, a highly oxygen-consuming process, and instead enhancing glucose utilization could have positively contributed to this effect as well [23].

Although both meldonium and NAC have been reported to alter vascular and tissue NO levels in the setting of other pathologies, the hypothesis that the topical anti-allodynic effects produced by their combination involve those same processes needed to be proven experimentally [23, 26, 29]. We tested that by using both pharmacological and non-pharmacological techniques that showed that NO-mediated alteration of tissue oxygenation indeed contributes to the anti-allodynic effects of the formulation. The systemic pre-administration of the robust non-selective NOS inhibitor L-NAME reversed the anti-allodynic effects of topical meldonium-NAC. These effects were observed after an expected delay needed for the absorption and distribution of the i.p. administered L-NAME dose. While L-NAME's inhibition of NO synthesis had a negative impact on the anti-allodynic effect of meldonium-NAC, adding hyperbaric oxygen to a subtherapeutic dose of the topical treatment produced a significant PWT elevation. Furthermore, our percutaneous

laser-spectroscopy based measurement of local tissue SaO_2 provided more insight into the changes in the plantar tissue micro-environment post-topical hind paw treatment with meldonium-NAC. As predicted, lower SaO_2 values were observed at 5-7 days post-CPIP procedure. Interestingly, topical meldonium-NAC reversed this decline 60 minutes after its administration. The magnitude of change in SaO_2 produced by the topical treatment can be put in perspective by comparing it to the rise in SaO_2 observed at 30 minutes post-reperfusion following the 3-hr occlusion of the CPIP procedure. As a post-occlusive hyperemic response is reflective of the microvascular reserve of the tissue, a comparable magnitude of SaO_2 increase produced by topical meldonium-NAC indicates significant involvement of microvascular alterations in its observed effects [54]. These experiments imply that topical meldonium-NAC's NO-mediated microvascular changes result in improved local tissue oxygenation that relieves mechanical allodynia in CPIP rats.

It needed to be empirically shown that the enhanced plantar tissue oxygenation observed after treatment with topical meldonium-NAC involved local alterations in tissue NO. The use of EPR, enhanced by the administration of a NO-spin trap, was able to detect a modest increase in tissue NO level following the application of the topical formulation. The iron-based spin-trap made of sodium-DETC and iron citrate possessed a high affinity for NO, and the spin-trapped NO formed MNIC: a stable compound that is non-reactive with oxygen. MNIC has a distinctly characteristic three-line EPR signal detectable at non-liquid helium temperatures and this minimized the co-occurrence of potentially contaminating signals generated by ferric heme signals from cytochromes. Despite the small sample size of the experiment, these attributes contributed to the detection of the increase in plantar muscle NO in CPIP rats produced by topical meldonium-NAC, confirming that modulation of local NO level was involved in the anti-allodynic effect of the drug.

In conclusion, the significant anti-allodynic effects of topical meldonium-NAC in rat models of CRPS and peripheral neuropathic pain imply the topical use of agents that modulate local NO to alleviate microvascular dysfunction and tissue hypoxia is a viable means of achieving analgesia in the two chronic pain conditions. Moreover, the prolongation of anti-allodynic effect with the formulation's repeated long-term use indicates the mechanisms targeted by such drugs have potential for partial tissue restoration leading to the achievement of long-lasting analgesia.

2.7 References

1. Lim TK, Shi XQ, Johnson JM, Rone MB, Antel JP, David S, et al. Peripheral Nerve Injury Induces Persistent Vascular Dysfunction and Endoneurial Hypoxia, Contributing to the Genesis of Neuropathic Pain. J Neurosci. 2015;35(8):3346-59.
2. Laferriere A, Abaji R, Tsai CY, Ragavendran JV, Coderre TJ. Topical combinations to treat microvascular dysfunction of chronic postischemia pain. Anesth Analg. 2014;118(4):830-40.
3. Groeneweg JG, Huygen FJ, Heijmans-Antonissen C, Niehof S, Zijlstra FJ. Increased endothelin-1 and diminished nitric oxide levels in blister fluids of patients with intermediate cold type complex regional pain syndrome type 1. BMC Musculoskelet Disord. 2006;7:91.
4. Teasell RW, Arnold JM. Alpha-1 adrenoceptor hyperresponsiveness in three neuropathic pain states: complex regional pain syndrome 1, diabetic peripheral neuropathic pain and central pain states following spinal cord injury. Pain Res Manag. 2004;9(2):89-97.
5. Schattschneider J, Hartung K, Stengel M, Ludwig J, Binder A, Wasner G, et al. Endothelial dysfunction in cold type complex regional pain syndrome. Neurology. 2006;67(4):673-5.
6. Birklein F, Weber M, Ernst M, Riedl B, Neundorfer B, Handwerker HO. Experimental tissue acidosis leads to increased pain in complex regional pain syndrome (CRPS). Pain. 2000;87(2):227-34.
7. Heerschap A, Hollander JAD, Reynen H, Goris RJA. Metabolic changes in reflex sympathetic dystrophy: A 31P NMR spectroscopy study. Muscle Nerve. 1993;16(4):367-73.
8. Malik RA, Tesfaye S, Newrick PG, Walker D, Rajbhandari SM, Siddique I, et al. Sural nerve pathology in diabetic patients with minimal but progressive neuropathy. Diabetologia. 2005;48(3):578-85.
9. Lim TK, Rone MB, Lee S, Antel JP, Zhang J. Mitochondrial and bioenergetic dysfunction in trauma-induced painful peripheral neuropathy. Mol Pain. 2015;11(1):58.
10. Kirchmair R, Tietz AB, Panagiotou E, Walter DH, Silver M, Yoon Y-S, et al. Therapeutic angiogenesis inhibits or rescues chemotherapy-induced peripheral neuropathy: taxol-and thalidomide-induced injury of vasa nervorum is ameliorated by VEGF. Mol Ther. 2007;15(1):69-75.
11. Zheng H, Xiao WH, Bennett GJ. Functional deficits in peripheral nerve mitochondria in rats with paclitaxel- and oxaliplatin-evoked painful peripheral neuropathy. Exp Neurol. 2011;232(2):154-61.
12. Kiralp MZ, Yildiz S, Vural D, Keskin I, Ay H, Dursun H. Effectiveness of hyperbaric oxygen therapy in the treatment of CRPS. J Int Med Res. 2004;32(3):258-62.
13. Schratzberger P, Walter DH, Rittig K, Bahlmann FH, Pola R, Curry C, et al. Reversal of experimental diabetic neuropathy by VEGF gene transfer. J Clin Invest. 2001;107(9):1083-92.
14. Laurindo FRM, Liberman M, Fernandes DC, Leite PF. Chapter 8 - Endothelium-Dependent Vasodilation: Nitric Oxide and Other Mediators. In: Da Luz PL, Libby P, Chagas ACP, Laurindo FRM, eds. Endothelium and Cardiovascular Diseases. 1st ed. London: Academic Press; 2018. p. 97-113.
15. Veves A, Akbari CM, Primavera J, Donaghue VM, Zacharoulis D, Chrzan JS, et al. Endothelial dysfunction and the expression of endothelial nitric oxide synthetase in diabetic neuropathy, vascular disease, and foot ulceration. Diabetes. 1998;47(3):457-63.
16. Bielli A, Scioli MG, Mazzaglia D, Doldo E, Orlandi A. Antioxidants and vascular health. Life Sci. 2015;143:209-16.

17. Hsieh H-J, Liu C-A, Huang B, Tseng AH, Wang DL. Shear-induced endothelial mechanotransduction: the interplay between reactive oxygen species (ROS) and nitric oxide (NO) and the pathophysiological implications. J Biomed Sci. 2014;21(1):3.
18. Estebe JP, Atchabahian A. The nerve: A fragile balance between physiology and pathophysiology. Eur J Anaesthesiol. 2017;34(3):118-26.
19. Tan EC, Janssen AJ, Roestenberg P, van den Heuvel LP, Goris RJ, Rodenburg RJ. Mitochondrial dysfunction in muscle tissue of complex regional pain syndrome type I patients. Eur J Pain. 2011;15(7):708-15.
20. Coderre TJ. Complex regional pain syndrome: what's in a name? J Pain. 2011;12(1):2-12.
21. Zhu Y, Zhang G, Zhao J, Li D, Yan X, Liu J, et al. Efficacy and Safety of Mildronate for Acute Ischemic Stroke: A Randomized, Double-Blind, Active-Controlled Phase II Multicenter Trial. Clin Drug Investig. 2013;33(10):755-60.
22. Svalbe B, Zvejniece L, Vavers E, Pugovics O, Muceniece R, Liepinsh E, et al. Mildronate treatment improves functional recovery following middle cerebral artery occlusion in rats. Behav Brain Res. 2011;222(1):26-32.
23. Sjakste N, Gutcaits A, Kalvinsh I. Mildronate: an antiischemic drug for neurological indications. CNS Drug Rev. 2005;11(2):151-68.
24. Sesti C, Simkhovich BZ, Kalvinsh I, Kloner RA. Mildronate, a novel fatty acid oxidation inhibitor and antianginal agent, reduces myocardial infarct size without affecting hemodynamics. J Cardiovasc Pharmacol. 2006;47(3):493-9.
25. Sokolovska J, Isajevs S, et al. Correction of glycaemia and GLUT1 level by mildronate in rat streptozotocin diabetes mellitus model. Cell Biochem Funct. 2011; 29(1):55-63.
26. Sjakste N, Kleschyov AL, Boucher JL, Baumane L, Dzintare M, Meirena D, et al. Endothelium- and NO-dependent vasorelaxing activities of ϒ-butyrobetaine esters: possible link to the anti-ischemic activities of mildronate. Eur J Pharmacol. 2004;495(1):67-73
27. Bavarsad Shahripour R, Harrigan MR, Alexandrov AV. N-acetylcysteine (NAC) in neurological disorders: mechanisms of action and therapeutic opportunities. Brain Behav. 2014;4(2):108-22.
28. Millea PJ. N-acetylcysteine: multiple clinical applications. Am Fam Physician. 2009;80(3):265-9.
29. Xia Z, Nagareddy PR, Guo Z, Zhang W, McNeill JH. Antioxidant N-acetylcysteine restores systemic nitric oxide availability and corrects depressions in arterial blood pressure and heart rate in diabetic rats. Free Radic Res. 2006;40(2):175-84.
30. Xia Z, Liu M, Wu Y, Sharma V, Luo T, Ouyang J, et al. N-acetylcysteine attenuates TNF-α-induced human vascular endothelial cell apoptosis and restores eNOS expression. Eur J Pharmacol. 2006;550(1-3):134-42.
31. Su W, Zhang Y, Zhang Q, Xu J, Zhan L, Zhu Q, et al. N-acetylcysteine attenuates myocardial dysfunction and postischemic injury by restoring caveolin-3/eNOS signaling in diabetic rats. Cardiovasc Diabetol. 2016;15(1):146.
32. Rodrigues SD, França KC, Dallin FT, Fujihara CK, Nascimento AJ, Pecoits-Filho R, et al. N-acetylcysteine as a potential strategy to attenuate the oxidative stress induced by uremic serum in the vascular system. Life Sci. 2015;121:110-6.
33. Coderre TJ, Xanthos DN, Francis L, Bennett GJ. Chronic post-ischemia pain (CPIP): a novel animal model of complex regional pain syndrome-Type I (CRPS-I; reflex sympathetic dystrophy) produced by prolonged hindpaw ischemia and reperfusion in the rat. Pain. 2004;112(1–2):94-105.

34. Ross-Huot MC, Laferriere A, Gi CM, Khorashadi M, Schricker T, Coderre TJ. Effects of glycemic regulation on chronic postischemia pain. Anesthesiology. 2011;115(3):614-25.
35. Bennett GJ, Xie YK. A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. Pain. 1988;33(1):87-107.
36. Ragavendran JV, Laferriere A, Xiao WH, Bennett GJ, Padi SS, Zhang J, et al. Topical combinations aimed at treating microvascular dysfunction reduce allodynia in rat models of CRPS-I and neuropathic pain. J Pain. 2013;14(1):66-78.
37. Polomano RC, Mannes AJ, Clark US, Bennett GJ. A painful peripheral neuropathy in the rat produced by the chemotherapeutic drug, paclitaxel. Pain. 2001;94(3):293-304.
38. Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL. Quantitative assessment of tactile allodynia in the rat paw. J Neurosci Methods. 1994;53(1):55-63.
39. Strohl KP, Thomas AJ, St Jean P, Schlenker EH, Koletsky RJ, Schork NJ. Ventilation and metabolism among rat strains. J Appl Physiol (1985). 1997;82(1):317-23.
40. Platt AWG. Preparation of nitrosyl complexes of iron and nickel. In: Woollins JD. ed Inorganic experiments. 3rd ed.Weinheim:Wiley-VCH; c2010. p. 155-7.
41. van Faassen EE, Koeners MP, Joles JA, Vanin AF. Detection of basal NO production in rat tissues using iron-dithiocarbamate complexes. Nitric Oxide. 2008;18(4):279-86.
42. Colapietro M, Domenicano A, Scaramuzza L, Vaciago A, Zambonelli L. The crystal and molecular structure of nitrosyliron bis-(NN-diethyldithiocarbamate). Chem Commun (London). 1967(12):583b-4.
43. Meacham K, Shepherd A, Mohapatra DP, Haroutounian S. Neuropathic Pain: Central vs. Peripheral Mechanisms. Curr Pain Headache Rep. 2017;21(6):28.
44. Sommer C, Myers RR. Vascular Pathology in CCL Neuropathy: A Quantitative Temporal Study. Exp Neurol. 1996;141(1):113-9.
45. Kortekaas MC, Niehof SP, Stolker RJ, Huygen FJ. Pathophysiological Mechanisms Involved in Vasomotor Disturbances in Complex Regional Pain Syndrome and Implications for Therapy: A Review. Pain Pract. 2016;16(7):905-14.
46. Koban M, Leis S, Schultze-Mosgau S, Birklein F. Tissue hypoxia in complex regional pain syndrome. Pain. 2003;104(1-2):149-57.
47. Groeneweg G, Niehof S, Wesseldijk F, Huygen F, Zijlstra FJ. Vasodilative effect of lsosorbide dinitrate ointment in complex regional pain syndrome type 1. Clin J Pain. 2008;24(1):89-92.
48. Schattschneider J, Binder A, Siebrecht D, Wasner G, Baron R. Complex regional pain syndromes: the influence of cutaneous and deep somatic sympathetic innervation on pain. Clin J Pain. 2006;22(3):240-4.
49. Girouard H, Chulak C, Wu L, Lejossec M, de Champlain J. N-acetylcysteine improves nitric oxide and alpha-adrenergic pathways in mesenteric beds of spontaneously hypertensive rats. Am J Hypertens. 2003;16(7):577-84.
50. Kwak KH, Han CG, Lee SH, Jeon Y, Park SS, Kim SO, et al. Reactive oxygen species in rats with chronic post-ischemia pain. Acta Anaesthesiol Scand. 2009;53(5):648-56.
51. Mokhtari V, Afsharian P, Shahhoseini M, Kalantar SM, Moini A. A Review on Various Uses of N-Acetyl Cysteine. Cell J. 2017;19(1):11-7.
52. Stein C. Opioids, sensory systems and chronic pain. Eur J Pharmacol. 2013;716(1):179-87.
53. Ballantyne JC, Shin NS. Efficacy of opioids for chronic pain - A review of the evidence. Clin J Pain. 2008;24(6):469-78.
54. Roustit M, Cracowski JL. Non-invasive assessment of skin microvascular function in humans: an insight into methods. Microcirculation. 2012;19(1):47-64.

3. Preamble to MANUSCRIPT 2:

Towards refining anti-hypoxic-antioxidant analgesic formulations for PNP & CRPS

Previous work done in our laboratory has demonstrated the analgesic efficacy of topical combinations of different agents that enhance blood flow and alleviate microvascular dysfunction in rat models of PNP and CRPS [215]. These drug combinations, composed of phosphodiesterase and phosphatidic acid inhibitors, α_2-adrenergic receptor agonists and NO donors, showed analgesic activity that lasted a few hours with no long-term benefits upon repeated use. Moreover, these drug combinations were formulated as plain mixtures of the constituent drugs. The subsequent work on topical meldonium-NAC was performed in the continuing efforts to discover effective topical analgesics for PNP and CRPS by locally targeting similar peripheral processes. As a step up, meldonium-NAC had a more diverse range of mechanisms of action as it contained drugs of anti-hypoxic and antioxidant activity. This is in contrast to the previous drug combinations that were composed of agents with redundant anti-hypoxic action [215]. One feature of topical treatment with meldonium-NAC, not observed with the previous vasoactive drug combinations, is the prolonged antiallodynic effects it exhibited after its repeated administration in CPIP and CCI rats [215, 297]. This could possibly be an outcome of its dual mechanism of action.

Another dimension that was required to advance the development of these topical drug combinations for PNP and CRPS, was refining the formulation techniques used to pair the agents. A step in that direction was attempted during the preparation of meldonium-NAC by exploring the possibility of salt formation between the two compounds. This was done by using the constituent drugs in equimolar quantities and employing the solvent evaporation technique that entailed dissolving substrates in a suitable solvent followed by desiccation with the aim of achieving salt formation [297, 298]. This contrasts with the first vasoactive drug formulations prepared in our laboratory, where plain drug mixtures were made by combining constituents of varying W/W concentrations [215]. Although the salt formation attempted between meldonium and NAC could not be fully validated, it gave way into subsequent efforts that focused on developing refined anti-hypoxic-antioxidant formulations as salts and co-crystals that are compact but easily dissociable.

3.1 Rationale for the synthesis of salt & co-crystal hybrids for the treatment of PNP & CRPS

In designing multimodal therapies for conditions like PNP and CRPS, several drug development issues necessitate a shift from preparing traditional drug combinations to synthesizing salts and co-crystals whenever feasible. Amongst these issues are the difficulty in mitigating the heterogeneity in drug physical properties that occurs with plain drug combinations [299]. Being composed of two compounds that possess distinct characteristics of stability, solubility and bioavailability, traditional drug combinations can potentially vary in the extent and speed with which constituent drugs reach their target [299, 300]. There is also the possibility of unprecedented particle-particle physical and chemical interactions between constituent drugs that can negatively impact the combinations' pharmacokinetics and overall treatment effect [299]. In instances where traditional drug combinations are the only way of formulating multi-component therapies, the pharmacokinetic and pharmacodynamic studies will need to be performed on a trial and error basis, using different doses of the constituent drugs to arrive at a therapeutically acceptable final composition [299]. This process is time, labor and resource-intensive making the development of plain drug combinations unworthwhile to most pharmaceutical companies estimating the probability of their advancement to clinical translation [300]. The pathway to approval through drug regulatory agencies is particularly cumbersome with toxicity, safety and clinical trials requiring assessments of multiple doses of individual drugs as well as their combination. The composition of plain drug combinations from agents that are already in the market and lack of patent rights is also another factor that makes these formulations poorly investable.

The synthesis of multicomponent salts and co-crystals can overcome most of the challenges encountered with developing traditional drug combinations [300]. Salts and co-crystals are solid forms composed of two or more dissociable components arranged in a crystal lattice [301]. With salt and co-crystal formation, a unique set of intermolecular interactions assemble the constituent agents into novel solid forms in a uniform stoichiometric ratio, orderly arranged as regular repeating units [301]. The difference between salts and co-crystals lies in the completeness of transfer of protons between the substrate compounds that then determines the occurrence of an ionic or non-ionic intermolecular interaction in the making of the new solid form [302]. Ionic or non-ionic, the bonds still create a distinct solid form which is considered to be a novel

pharmaceutical agent. Due to these interactions, that are unique for each salt and co-crystal, the products gain new physicochemical properties that can potentially translate into enhanced pharmacological benefits [303]. In addition, the intermolecular interactions within multicomponent salts and co-crystals mold these products into a uniformly built compact solid form [301]. This allows singular dosing during pharmacokinetic and pharmacodynamic studies exponentially reducing the development costs that would have been incurred if the drugs were to be formulated as traditional drug combinations. Moreover, salts and co-crystals are deemed patentable by regulatory bodies [301]. This is because each multicomponent salt and co-crystal is unique in terms of stoichiometric ratio, nature of intermolecular interaction and molecular arrangement in the crystal lattice [301, 304]. All these factors strongly argue for replacing traditional drug combinations with multicomponent salts and co-crystals whenever feasible.

3.1.1 Mechanochemistry as a superior technique for the synthesis of multicomponent analgesic salts & co-crystals

Methods of salt and co-crystal synthesis have evolved in recent years. Conventional approaches entail the use of a suitable solvent media to drive the reactions and subsequent purification steps follow to remove undesired by-products and unreacted substrates [298]. Such solution-based techniques utilize bulk solvents during most of their steps, generating waste that is difficult and expensive to safely dispose [303]. Solution synthesis also lacks efficiency as the completeness of reactions are highly influenced by substrate solubility and stability in the solvent media being used [303, 305]. Moreover, it typically tends to be a slow and expensive multi-step process [298, 306]. These drawbacks have led to the implementation of alternate approaches like mechanochemical synthesis.

In mechanochemical synthesis, mechanical energy is used to drive reactions between compatible substrates to synthesize new products like salts and co-crystals [307]. A typical example of this technique, also used in work described in this thesis, is liquid assisted grinding (LAG), as opposed to dry grinding. In LAG, substrates are placed in a reaction jar with milling balls and high frequency grinding carried out using a milling machine [303]. A very small volume of solvent, in microliters, is added to the substrates as a catalyst to increase the efficiency of mechanochemical reactions [303]. The LAG protocol is specified by several variables including the size and nature of the milling jars and balls used, milling duration and frequency, substrate ratio, and volume and nature of LAG solvents. In contrast to larger solution-based synthesis,

mechanochemical synthesis is quick and highly efficient as it avoids variabilities introduced due to altered reactivity and stability caused by solvent-substrate interactions [307]. It is also a prototypical implementation of green chemistry: a recently advocated approach in synthetic chemistry that is aimed at reducing or eliminating the use and generation of hazardous substances [308]. Mechanochemistry upholds the principles of green chemistry that include preventing rather than cleaning up waste, reducing energy use for synthesis, and maximizing the incorporation of all materials used in a synthetic process into the final product [306, 308]. These considerations were the basis for the use of LAG in the efforts that led to the synthesis of the analgesic salts and co-crystal reported in the following manuscript.

3.1.1.1 Techniques of validation & characterization of new salts and co-crystals

Newly synthesized salts and co-crystal need to be validated and characterized. Commonly used techniques to achieve this include X-ray diffraction, spectroscopy and thermal analysis [309]. A compilation of data obtained from these various techniques is commonly required to ascertain the successful synthesis of novel salts and co-crystals [309]. In X-ray diffraction, the scattering of X-rays from constituent atoms of a molecule creates a diffraction pattern that is informative of their physical arrangement [310]. The technique can, on one hand, be applied on the powder product, also called powder X-ray diffraction (PXRD), as a quick screening step for assessing the formation of new solid forms [298]. On another hand, X-ray diffraction can also be used for a detailed examination of single crystals grown from newly synthesized salts and co-crystals to decipher the arrangement of constituent active pharmaceutical ingredients (APIs) and their intermolecular interactions [298]. There are databases that contain the X-ray diffraction patterns of most crystalline compounds, which can be used as references in efforts to synthesize new salts and co-crystals [311]. Powder X-ray diffraction is a widely applied screening technique of pharmaceutical salt and co-crystal characterization because it is non-destructive, inexpensive, fast, reliable and user-friendly [310]. PXRD was extensively used as a screening step during the initial phases of the analgesic salts and co-crystals synthesis reported in the following manuscript.

In spectroscopy, products are irradiated with electromagnetic energy resulting in absorption, reflection or scattering that generates spectra indicating the detailed structures of organic pharmaceutical complexes [298]. Common spectroscopy methods that are used in the solid-state, to characterize salts and co-crystals, include Fourier-transform infrared spectroscopy (FTIR) and solid-state nuclear magnetic resonance spectroscopy (ssNMR) [298]. With FTIR,

clearly different spectra are expected from co-crystals or salts versus the mixture of components due to the occurrence of hydrogen bonds and ionized chemical groups that form with salts and co-crystals. In addition to shifts in the position and amplitude of spectral patterns from specific functional groups, the FTIR spectra also contains a 'fingerprint' region, with a pattern that can be used to detect co-crystal and salt formation [298]. With ssNMR, the chemical composition of a product is deciphered from the number, position and intensity of signals generated by the magnetic nuclei of specific atoms, commonly carbon, hydrogen and nitrogen [309]. The information acquired from ssNMR can provide sufficient details of molecular structure to discriminate between individual compounds, and salt and co-crystals complexes [298].

Characterization of salts and co-crystals with thermal analysis is performed by using devices that deliver a precise heating and cooling protocol to produces temperature changes within a controlled atmosphere and assess the physical changes a product displays in response [298]. Differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA) are among the common thermal analysis methods used for salt and co-crystal characterization and have been used in the work described in the following manuscript. In DSC, the sample undergoes a series of heating and cooling cycles, and the heat energy supplied to, and released from, a sample is plotted as a function of the temperature of the sample. The plot indicates the temperatures at which samples undergo thermally induced events like melting and crystallization, which can then be used as parameters to determine purity [312]. Whereas, in TGA, a sample is increasingly heated to higher temperatures and the resulting change in weight is measured over time. The TGA plot indicates the temperature at which a decline in sample weight or decomposition commences and this aids in determining the thermal stability of the sample [313]. As every compound has a characteristic set of responses to thermal challenge, these thermal analyses have the capacity to reveal the composition and purity of products like salts and co-crystals [298]. In addition, they provide valuable information on the thermal and oxidative stability of novel products [309].

3.2 The chosen anti-hypoxic-antioxidant pairs for salt & co-crystal synthesis for the topical treatment of PNP & CRPS

The first step of salt and co-crystal synthesis undertaken in the following work was identification of vasoactive agents and antioxidant nutraceuticals to be paired. Agents were selected based on demonstrated anti-hypoxic and antioxidant activity, as well as expected

analgesic potential. In the next step, the list was narrowed based on chemical structure and the existence of compatible chemical groups for salt and co-crystal formation. Simply put, the ideal drug with its salt/co-crystal co-former should contain complementary hydrogen bond or proton, donor and acceptor moieties, that allow salt or co-crystal formation [301].

Our final list of vasoactive agents contained the non-selective phosphodiesterase inhibitor: pentoxifylline, the sympatholytic: clonidine, and the NO donor: linsidomine. Linsidomine is a NO-releasing vasodilatory drug that acts by increasing cellular synthesis of cyclic mononucleotides to cause relaxation in vascular smooth muscle and inhibit aggregation of platelets [314]. It has been used in the form of its prodrug molsidomine for the treatment of ischemic heart disease [315]. The vasoactive action of pentoxifylline occurs through the inhibition of phosphodiesterase enzymes that degrade the cyclic mononucleotides [316]. It increases vascular flow, inhibits platelet aggregation, decreases blood viscosity and enhances red blood cell flexibility [316-318]. Its successful clinical use for the treatment of peripheral arterial disease is well documented [316]. On the other hand, clonidine is a long-used antihypertensive drug that acts by activating presynaptic α_2-adrenergic receptors to causes vasodilation through the reduction of the central and peripheral release of norepinephrine [217]. It also activates α_2-receptors on the endothelium to increase the release of NO, which subsequently induces vasodilation [319]. All of these anti-hypoxic agents have previously demonstrated some analgesic effects in rat models of PNP and CRPS [215].

The antioxidant compounds chosen as co-formers for the salt and co-crystal synthesis were selected nutraceutical phenolic acids: protocatechuic acid, caffeic acid and gallic acid and the potent endogenous lipophilic antioxidant: α-lipoic acid. Phenolic acids are a family of plant-derived free radical scavengers that are carboxylic acids containing an aromatic ring bonded to three hydroxyl groups. Their multiple hydroxyl groups are behind their strong antioxidant activity and their carboxylic acid group, that can readily act as a hydrogen bond or proton donor, makes them suitable salt/co-crystal co-formers. All the phenolic acids mentioned in the following manuscript have been reported to show effective antioxidant and anti-inflammatory activities [320-322]. Protocatechuic acid is abundant in many food plants and is a major metabolite of other ingested polyphenols after breakdown by intestinal microflora [323]. Its antioxidant effects include ROS scavenging, improving mitochondrial function, preventing lipid peroxidation and enhancing transcription of antioxidant enzymes [320]. Its preventive benefits have been described for

neurodegenerative processes, diabetes and aging [320, 323, 324]. Caffeic acid is both an antioxidant and an inhibitor of 5-lipoxygenase, an enzyme used for the synthesis of pro-inflammatory leukotrienes induced by cellular ischemic injury [321, 325]. It has demonstrated both preventive and therapeutic effects on ischemic brain injury through its antioxidant and anti-inflammatory actions [325, 326]. Its specific antioxidant mechanisms include potent inhibition of the formation of hydroxyl radicals, and prevention of lipid peroxidation [327, 328]. Gallic acid is also a potent antioxidant that has been reported to alleviate ischemia-reperfusion injury in different organs including the brain, heart, stomach, liver and kidneys [322, 329, 330].

α-lipoic acid is an endogenous compound that acts as a cofactor for mitochondrial enzymes. It is also found in trace quantities in some foods [331]. It has a plethora of antioxidant activities that occur through the quenching of ROS, regeneration of endogenous antioxidants, chelation of metal ions, reparation of oxidized proteins and inhibition of prooxidant gene expression [332]. α-lipoic acid has been shown to enhance nerve blood flow, reduce oxidative stress and improve distal nerve conduction in experimental diabetic neuropathy [333]. A meta-analysis of clinical trials investigating its use in symptomatic diabetic polyneuropathy has shown that it produces a clinically meaningful alleviation of both positive (pain, burning, paresthesia) and negative (anesthesia, numbness) neuropathic symptoms [238].

3.3 Practical considerations during the steps of mechanochemical synthesis & testing of the novel anti-hypoxic-antioxidants salts & co-crystals

The identification of the potent analgesic salts and co-crystals reported in the following manuscript is a result of a step by step progression from identification of potentially reactive drug-nutraceutical pairs, screening the performance of mechanochemical reactions, chemical validation of newly synthesized products, and behavioral testing to assess topical analgesic effects in CPIP rats. After screening based on chemical structure, ten of the twelve possible drug-nutraceutical pairs were identified from the three anti-hypoxic drugs pentoxifylline, clonidine and linsidomine and the four nutraceuticals antioxidants α-lipoic acid, caffeic acid, protocatechuic acid and gallic acid. Mechanochemical synthetic protocols were designed and reactions carried out between the ten pairs. This step revealed unprecedented results where some pairs failed to show the anticipated reactivity despite several modifications of their reaction conditions. A particular example was the

pairing between pentoxifylline and α-lipoic acid, where all LAG protocols failed to result in salt/co-crystal formation, but instead induced a loss of the substrates' crystallinity.

All the novel products that appeared to have successfully formed pairings were initially screened with PXRD, and those that exhibited efficient salt/co-crystal formation progressed into further chemical validation studies and subsequent screening for topical analgesic effects in CPIP rats. Though the amalgamated anti-hypoxic-antioxidant effects of the newly synthesized salts and co-crystals were anticipated to produce more effective analgesia in the CPIP rats, some of the novel products failed to meet the expectation. This was the case with the pentoxifylline-gallic acid co-crystal, where topical anti-allodynic effects produced in CPIP rats were lower and shorter-lasting as compared to the parent constituent, pentoxifylline.

These undertakings of analgesic salt and co-crystal synthesis demonstrated that theoretical predictions serve as only guides for the possible favorable outcomes of the synthesis and testing steps. It was only through experimental implementation of all required steps that salt and co-crystal pairings with enhanced pharmacological effects were able to be identified. The work presented in the following manuscript is a practical demonstration of the use of mechanochemical synthesis to prepare more effective analgesic salts and co-crystals by pairing agents of anti-hypoxic and antioxidant activity.

The generation of salts and co-crystals from two compounds of distinct therapeutic activity is a relatively scarce approach in drug development due to the intricacies of finding compatible drug molecules [334]. On the other hand, the addition of inert excipients to APIs to formulate salts and co-crystals of favorable physicochemical properties is a routine practice. [334]. The proposed synthesis of the multifunctional analgesic salt and co-crystals is aimed to achieve both of these purposes. The products are designed to simultaneously impact the complementary disease processes of tissue hypoxia and oxidative stress in CRPS and PNP in addition to achieving a new solid-state arrangement that yields enhanced pharmacokinetic attributes. The potential in this approach of pairing analgesic drugs of different mechanisms was demonstrated in studies on the synthesized tramadol-celecoxib co-crystal that has advanced successfully into clinical testing [335].

4. MANUSCRIPT 2: Drug-nutraceutical co-crystal and salts for making new and improved bi-functional analgesics

Oli A Fulas[1], Andre Laferriere[1], Ghada Ayoub[2], Gandrath Dayaker[2], Cristina Mottillo[2], Hatem M. Titi[2], Robin S. Stein[2], Tomislav Friščić[2], Terence J. Coderre[1]

Departments of Anesthesia[1] and Chemistry[2]

4.1 Contribution of authors

Prof. Terence J. Coderre conceived the idea for this project. The chemical synthesis and validation experiments were performed in Prof. Tomislav Friščić's laboratory, under his supervision and guidance. I performed all the chemical synthesis and most of the validation experiments reported in this work under the training and immediate supervision of Ghada Ayoub. Ghada Ayoub also performed the TGA validation experiments. Cristina Mottillo designed the synthesis steps of the salt between linsidomine and caffeic acid and oversaw my synthesis and validation of the product. Dayaker Gandrath designed the purification steps for clonidine hydrochloride and trained me on how to perform it. Hatem M Titi collected the DSC validation data on the products and solved the crystal structure of the co-crystal made from pentoxifylline and protocatechuic acid. Robin S. Stein collected and interpreted the ssNMR validation data on two of the products reported. I prepared the topical analgesic formulations. Andre Laferriere and I prepared the CPIP rats and performed the behavioral testing. I drafted the manuscript. Profs Coderre and Friščić, and Dr. Titi revised it.

4.2 Abstract

The discovery and development of effective analgesics are greatly lagging behind the steadily rising prevalence of chronic pain. Currently prescribed analgesics for chronic pain are lacking in efficacy mainly due to their narrowly targeted mechanism of action. Driving neuronal hyperexcitability that underlies symptoms of chronic pain are multiple non-neuronal processes, among which are tissue hypoxia and oxidative stress. Here we demonstrate the design, synthesis and activity of new multi-component bi-functional analgesic crystalline solids, co-crystals and salts, based on the pairing of vasodilatory anti-hypoxic drugs pentoxifylline, clonidine and linsidomine with antioxidant nutraceuticals protocatechuic acid, α-lipoic acid and caffeic acid. After validation, chemical and structural characterization of these novel salts and co-crystals,

topical formulations of the products were tested in a rat model of complex regional pain syndrome. Analgesic effects achieved with the salts and co-crystal exceeded the efficacy and/or potency of constituent compounds indicating that more effective, advanced analgesics can readily be developed by the careful pairing of compounds that simultaneously target multiple neural and non-neural processes driving chronic pain.

4.3 Introduction

Chronic pain is a highly prevalent and costly condition that causes physical disability and psychological debilitation to nearly 22% of the general population [1]. Its complex pathophysiology that involves interdependent neuronal, immune and vascular maladaptive processes has made it difficult to manage despite extensive research [2, 3]. Currently available drugs fail to provide optimal analgesia due to limited efficacy and dose-limiting side-effects [4]. One reason for the limited efficacy exhibited by most analgesics in clinical use is their narrowly directed mechanism of action that impacts a limited aspect of the disease process [5]. This can be circumvented by the introduction of broader-purposed multi-component analgesics.

The past decade has witnessed the rapid development of designs and uses of advanced multi-component pharmaceutical solids, based on the association of an active pharmaceutical ingredient (API) with additional counter molecules in order to provide a novel and unique crystalline material whose structure and physicochemical properties are distinct from those of pure component solid forms [6]. Two most significant types of such advanced pharmaceutical materials are salts, typically resulting from a proton transfer process between different components of the material, and co-crystals, in which the components of the material are assembled in a predictable fashion through principles of supramolecular chemistry and molecular recognition [7-9]. Whereas salt formation (salification) has been extensively used in the development of API solid forms and formulations, pharmaceutical co-crystals have only relatively recently emerged as a means to modify solid-state properties and bioavailability of drug molecules with no ionizable sites [7-11]. The design of pharmaceutical co-crystals is typically based on an API component, with one or more co-crystal formers (co-formers) that belong to the "Generally Regarded As Safe" (GRAS) list of compounds [10, 12, 13]. This modular design has enabled the design and synthesis of API forms with improved or modified physicochemical properties, such as melting point, tabletability, solubility, dissolution rate, bioavailability and stability [14-20]. A considerably less explored

design of pharmaceutical co-crystals involves the use of one or more API molecules as components, designed for the primary purpose of targeting multiple disease processes of a given pathology [21-24]. This application is even less explored in the field of analgesic development for chronic pain [25].

Here, we report the design and synthesis of advanced, bi-functional therapeutic co-crystal and salts made by combining vasodilatory anti-hypoxic drugs pentoxifylline (**pentx**), clonidine (**clon**) or linsidomine (**lin**), with antioxidant nutraceuticals protocatechuic acid (H**pca**), α-lipoic acid (H**ala**) and caffeic acid (H**cafa**), respectively. Our design is based on the observation that, in addition to their complementary mechanism of action that alleviates chronic pain symptoms, these two groups of compounds exhibit chemical structures that would be very suited for mutual co-crystallization and/or salt formation. Pentoxifylline is a methyl xanthine with an aromatic nitrogen in the imidazole ring and two carbonyl groups that should promote co-crystal formation with carboxylic acids through hydrogen bonding (Fig 10) [26]. In contrast, clonidine is an imidazoline derivative that contains a strongly basic guanidine group suitable for salt formation with carboxylic acids. Similarly, linsidomine is a morpholine derivative exhibiting a highly basic imine group that should allow for salt formation in the presence of a carboxylic acid (Fig 10). All the nutraceutical antioxidants, protocatechuic acid, α-lipoic acid and caffeic acid (3-(3,4-Dihydroxyphenyl)-2-propenoic acid), possess a carboxylic acid group capable of acting as a hydrogen bond donor for co-crystallization, or proton donors for salt formation (Fig 10).

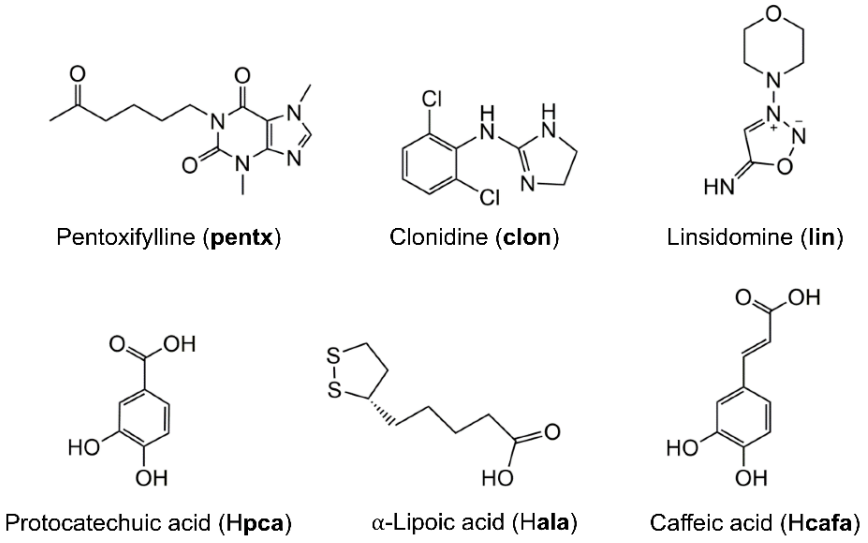

Figure 10. Molecular structures of pentoxifylline (**pentx**), clonidine (**clon**), linsidomine (**lin**), protocatechuic acid (H**pca**), α-lipoic acid (H**ala**) and caffeic acid (H**cafa**).

The synthesis of these salts and co-crystal was aimed at simultaneously relieving the pain-inducing hypoxia and oxidative stress seen in peripheral tissue affected by chronic pain conditions including complex regional pain syndrome (CRPS) and peripheral neuropathic pain [27, 28]. In a rat model of CRPS, we demonstrate the augmentation of analgesic efficacy and/or potency achieved by co-crystallization and salt formation between **pentx**, **clon** and **lin** and the nutraceutical antioxidants.

4.4 Methods

4.4.1 Materials

Pentoxifylline and caffeic acid were obtained from Sigma-Aldrich, St. Louis, MO. Linsidomine chloride, clonidine hydrochloride and protocatechuic acid were purchased from Cayman Chemicals, MI and α-lipoic acid from TCI chemicals, OR. Acetonitrile and diethyl ether were obtained from Fisher Chemical while ethanol was purchase from Commercial Alcohols, ON.

4.4.2 Synthesis of salts/co-crystals of drugs

Synthesis of **clon**, **lin** and **pentx** co-crystals/salts with the nutraceuticals H**ala**, H**cafa** and H**pca** was performed mechanochemically by liquid-assisted grinding (LAG), i.e. by milling in the presence of a small, near-stoichiometric amount of a liquid additive (measured by η, *i.e.* the ratio of added liquid volume to the weight of reactant mixture, in μL/mg) in order to improve the synthetic efficiency and provide a well-defined crystalline product [29, 30]. In particular, prior work has demonstrated that, whereas neat (dry) milling of molecular solids often leads to amorphization and poor reactivity, the addition of a liquid in the η-range below ca. 2 μL/mg leads to rapid and often quantitative co-crystal formation, independent of the solubility of each component. Ball milling was done for 30 minutes in a Retsch MM400 shaker mill (Hann, Germany) operating at 30 Hz, with samples placed in a 15 mL volume polytetrafluoroethylene (PTFE) jars, along with a small amount of ethanol (EtOH) and a single stainless steel ball of 10 mm diameter (4 g weight). For the reactions, **clon** and **pentx** were paired with equimolar quantities of H**ala** and H**pca**, respectively. For the pair **clon** and H**ala**, LAG was performed in the presence of 50 μL of EtOH (η = 0.47 μL/mg) as the liquid additive, while a 100 μL amount of EtOH (η = 0.46 μL/mg) was used for the pair **pentx** and H**pca**. The solid **clon** was first obtained from the commercially available hydrochloride salt (H**clon**$^{+}$Cl^{-}) form following a procedure described below (*vide infra*).

Due to the instability of the **lin** free base, the salt analgesic (H**lin**$^+$)(**cafa**$^-$) was synthesized by a mechanochemical anion exchange reaction. For this purpose, solid H**cafa** was first reacted with an equimolar quantity of sodium hydroxide (NaOH) by neat grinding for 30 minutes in a stainless-steel jar, using two stainless steel balls of 7 mm diameter (1.3 grams weight each) and a Retsch MM400 shaker mill operating at 30 Hz. The product, which was confirmed to be sodium caffeate (Na$^+$**cafa**$^-$), was then used for mechanochemical ion exchange with (H**lin**$^+$Cl$^-$), conducted by LAG in the presence of 10 μL EtOH, in a PTFE-based milling assembly. Milling for 45 minutes using a Retsch MM400 shaker mill operating at 30 Hz gave (H**lin**$^+$)(**cafa**$^-$) along with sodium chloride (NaCl).

4.4.2.1 Purification of clonidine hydrochloride

The API **clon** is commercially available as clonidine hydrochloride (H**clon**$^+$Cl$^-$) from which the free base was obtained by treatment with a concentrated solution of potassium hydroxide in deionized water. The reaction yielded water-soluble potassium chloride and the **clon** free base which readily precipitated out of solution. The resulting solid **clon** was separated and dried by vacuum filtration, and its composition confirmed *via* nuclear magnetic resonance (NMR) in $CDCl_3$ solution and Fourier-transform infrared attenuated total reflectance (FTIR-ATR) spectroscopy.

4.4.3 Validation and characterization of synthesized salts and co-crystals

4.4.3.1 FTIR-ATR spectroscopy

Measurements were performed on a Spectrum TWO FTIR with a single bounce diamond ATR from Perkin Elmer in the range of 4000-400 cm^{-1} and with a resolution of 1 cm^{-1}.

4.4.3.2 Powder X-ray diffraction (PXRD)

PXRD experiments on starting materials and reaction products were conducted on a Bruker D2 phaser equipped with CuKα X-ray source and a Nickel filter. Data was collected between 4-40 2θ with an increment of 0.05.

4.4.3.3 Single-crystal X-ray diffraction (SCXRD)

Single crystals used for single-crystal x-ray diffraction (SCXRD) were grown at room temperature by slow evaporation. The solvent used for the **(pentx)**(H**pca)** co-crystal was a mixture of EtOH and acetonitrile, and for the (H**clon**$^+$)(**ala**$^-$) salt it was diethyl ether.

Single crystal X-ray diffraction data for all compounds were collected on a Bruker D8 Advance diffractometer (Bruker-AXS, Madison, WI, USA) with a Photon 100 CMOS area

detector and an IμS microfocus X-ray source (Bruker AXS). X-ray diffraction experiments on single crystals of the (**pentx**)(H**pca)** co-crystal and the (H**clon**$^+$)(**ala**$^-$) salt were conducted using Cu*K*α radiation. Single crystals were often found to readily lose solvent upon exposure to air and were coated with Paratone oil (Hampton Research, Aliso Viejo, CA, USA) during X-ray single-crystal data collection.

Unit cell determination, data collection, data reduction, and correction for absorption were all conducted using the Apex3 software suite (Bruker AXS). The crystal structures were solved by an iterative dual space approach as implemented in SHELXT [29-31]. Non-hydrogen atoms were located from the difference map and refined anisotropically. Hydrogen atoms bonded to carbon atoms were placed in calculated positions. All hydrogen atoms coordination and thermal parameters were constrained to ride on the carrier atoms. Both co-crystal and salt were found to be twinned and the structure of (**pentx**)(H**pca)** was treated by CELL NOW (Version 2008-2, Bruker AXIS Inc., WI), in which three domains were found and separated.

4.4.3.4 Thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC)

TGA was performed using TGA Q500 (TA Instruments). Approximately 5 mg of the samples were heated over a temperature range of 25°C to 600°C while being purged with a flow of air and nitrogen gas throughout the experiment. The resulting thermograms were analyzed with TA Universal Analysis software. The DSC experiments were performed on a DSC2500 (TA instruments Ltd., Delaware, USA), under a stream (50 mL min^{-1}) of nitrogen gas. Samples (2 mg) were placed into hermetically sealed aluminum pans, which were first cooled to -20 °C and left in isotherm for 1 min before heating up the samples to 200 °C, in a heating rate of 5 °C s^{-1}.

4.4.3.5 Solid state nuclear magnetic resonance spectroscopy (ssNMR)

ssNMR was performed using a Varian 400 MHz VNMRS wide-bore spectrometer operating at a 1H frequency of 399.76 MHz, a ^{13}C frequency of 100.53 MHz. CPMAS ^{13}C spectra were acquired using a 4 mm double-resonance probe.

The ssNMR spectra of (Na$^+$**cafa**$^-$) and (H**lin**$^+$Cl$^-$) were acquired under spinning at 5 kHz, that of (H**lin**$^+$)(**cafa**$^-$) was acquired at 8 kHz, and that of H**cafa** was acquired at 13 kHz spinning. The spectrum of (H**lin**$^+$Cl$^-$) was compared with a CPTOSS spectrum (not shown) to identify spinning sidebands. A recycle delay of 4 s was used for H**cafa**, 5 s was used for (Na$^+$**cafa**$^-$) and (H**lin**$^+$Cl$^-$), and 6 s for (H**lin**$^+$)(**cafa**$^-$). A contact time of 3.5 ms was used for H**cafa** & (Na$^+$**cafa**$^-$),

1.5 ms was used for (H**lin**$^{+}$Cl^{-}), and 3.0 ms was used for (H**lin**$^{+}$)(**cafa**$^{-}$). 128 transients were acquired of (Na^{+}**cafa**$^{-}$), 1280 of (H**lin**$^{+}$Cl^{-}), 172 of (H**lin**$^{+}$)(**cafa**$^{-}$), and 8192 of H**cafa**. ^{13}C spectra were referenced to TMS using the carbonyl signal of glycine at 176.04 ppm.

4.4.4 Formulation of drugs into topical ointments

Topical drugs were formulated into ointment-type preparations using a composite, water-soluble polyethylene glycol (PEG) base system consisting of 40 % carbowax (PEG 3350) and 60 % PEG 400 (both from Sigma Aldrich). The required amounts of the active ingredients were first weighed and then added to the molten base and mixed. The vehicle treatment consisted of the same water-miscible base ointment without the addition of the active drugs.

4.4.5 Generation of the CPIP rat model of CRPS

A rat model of CRPS was performed by inducing prolonged hind paw ischemia and subsequent reperfusion previously described as chronic post-ischemia pain (CPIP) by Coderre et. al, 2004 [32]. In short, male Long Evans rats (300-400 g; Charles River, QC, Canada) were anesthetized over a 3-hour period with a bolus (55 mg/kg, intraperitoneally [i.p.]) followed by chronic i.p. infusion (0.15 mL/hour) of sodium pentobarbital (Ceva Sante Animale, Libourne, France) for 2 hours. Following induction of anesthesia, a Nitrile 70 Durometer O-ring (O-rings West, Seattle, WA) with an internal diameter of 5.5-mm was slipped around the rat's left hind limb proximal to the ankle joint to effect a complete blockage of arterial blood flow [33]. The ring was left in place for three hours, and the rats recovered from anesthesia 30 to 60 minutes following reperfusion.

4.4.6 Mechanical hypersensitivity testing

As a measure of mechanical hypersensitivity, paw withdrawal threshold (PWT), was tested on the plantar surface of the ipsilateral hind paw of the CPIP rats. The rats were first habituated for 30 minutes in the test chamber. Nylon monofilaments (von Frey hairs) were applied in either ascending (after negative response) or descending (after positive response) force as necessary to determine the filament closest to the threshold of response. Each filament was applied for 10 s or until a flexion reflex occurred. The minimum stimulus intensity was 0.25 g and the maximum was 15 g. Based on the response pattern, and the force of the final filament (5th stimulus after first direction change), the 50 % threshold (grams) was calculated as ($10^{[Xf+ k\delta]}$) /10000, where X_f = value (in log units) of the final von Frey hair used, k = value for the pattern of positive/negative

responses and δ=mean difference in log unit between stimuli (here, δ=0.224, for more detail see Chaplan et al., 1994) [34]. PWT was assessed before ischemia/reperfusion injury, and prior to and following topical treatments.

4.4.7 Statistics on animal behavioral data

Time-course measurements of PWTs after vehicle and drug administration were subjected to a repeated measure analysis of variance (ANOVA). Pairwise post-hoc comparisons were performed between PWTs obtained from drug and vehicle treatment groups measured at matching post-treatment times using Tukey's HSD test.

Cumulative anti-allodynic effect measured over 180-minute time course experiments was assessed by calculating the area under the curve (AUC) of PWT elevations plotted post-topical application. Comparisons of different drug doses versus vehicle were performed using repeated-measures ANOVA followed by Tukey's HSD test.

Dose-response curves for comparison of anti-allodynic potency were plotted on a semi-log scale with the amount of drug used per application on the X-axis and the AUC of the 180-minute PWTs measured or change in PWTs obtained post-drug application on the Y-axis. The linear regression of the dose-response curves was then calculated, and their difference analyzed using a 1-way ANOVA. Difference in anti-allodynic potency are stated in terms of the shift in the x-intercept of the regression line of the dose-response curve for each drug.

4.5 Results

4.5.1 Validation and characterization of synthesized co-crystal and salts

4.5.1.1 Co-crystal of pentoxifylline and protocatechuic acid

The co-crystal of pentoxifylline and protocatechuic acid (**pentx**)(H**pca**) was first obtained mechanochemically by LAG of the two solid components in the presence of EtOH. Co-crystal formation was evidenced by PXRD analysis of the solid mixture after milling, which revealed the complete disappearance of signals belonging to the starting materials and the appearance of new Bragg reflections at 2θ-values of 10.3°, 16.5° and 22.3°, that are not present in the PXRD patterns of starting materials (Fig 11A). Co-crystal formation also resulted in changes to the FTIR-ATR spectrum compared to the individual solid components and, notably, the C=O stretching band was found to shift slightly from 1274 cm^{-1} in the protocatechuic acid spectrum to 1283 cm^{-1} in **(pentx)(**H**pca)**, implying a change in the hydrogen bonding pattern that would be consistent with

co-crystallization (Fig 11B). Thermal analysis of (**pentx**)(H**pca**) was performed in the range from -20ºC to 200ºC, which revealed a sharp endothermic event at 148ºC, which is consistent with melting (see Appendix Fig S2.1). More importantly, the DSC thermograms of pure solids **pentx** and H**pca** exhibited melting point signals at 107ºC and 132ºC, which confirmed that (**pentx**)(H**pca**) is not a physical mixture of the starting materials, as well as that it exhibits higher thermal stability in the solid state (see Appendix Fig S2.4).

Single crystals of the (**pentx**)(H**pca)** co-crystal were obtained by slow evaporation from a solution of 1 mL in a mixture of EtOH and acetonitrile 50% (v/v). Structural analysis by single-crystal X-ray diffraction reveals that the asymmetric unit of the co-crystal consists of one **pentx** and one H**pca** molecule held together by a short O-H···N hydrogen bond of (O···N separation of 2.700(2) Å) involving the carboxylic acid group of protocatechuic acid and the imidazole nitrogen atom of pentoxifylline (Fig 11C).

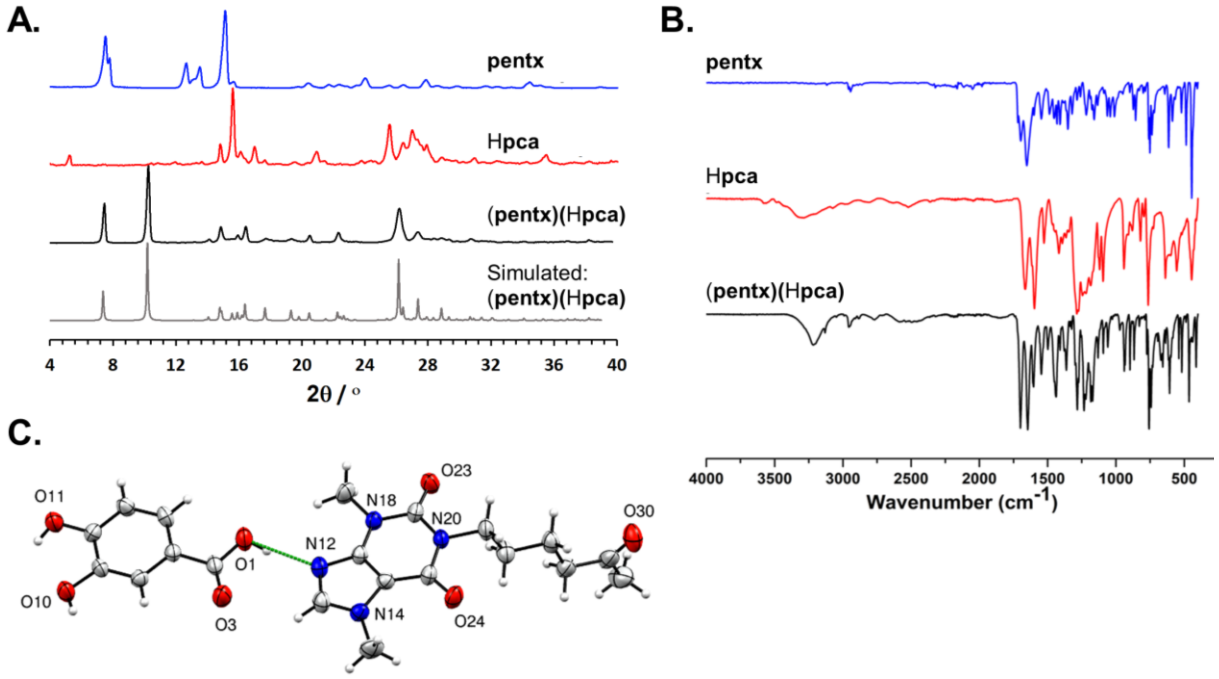

Figure 11. (**A**) PXRD, (**B**) FTIR **pentx**, H**pca** and (**pentx**)(H**pca)** co-crystal and (**C**) asymmetric unit in (**pentx**)(H**pca)**, in which the (**pentx**)(H**pca)** entities are held together through hydrogen bond depicted in dashed green line.

4.5.1.2 Salt of clonidine and α-lipoic acid (H**clon**$^+$)(**ala**$^-$)

After being separated from its hydrochloride salt using a neutralization reaction, solid **clon** was reacted with α-lipoic acid (H**ala**) using LAG in the presence of EtOH as the liquid additive. The PXRD pattern of the material after milling was distinct from those of the corresponding starting materials, with a prominent new Bragg reflection appearing at 2θ-value of 6.7°, indicating the formation of a new crystalline phase (Fig 12A). Analysis of the FTIR-ATR spectrum of the product revealed a significant shift of the C=O stretch of the H**ala** carboxylic acid moiety from 1694 cm^{-1} in the pure acid to 1666 cm^{-1} in the LAG product (see Appendix Fig S2.4). The shift is consistent with the deprotonation of the carboxylic acid moiety, indicating salt formation.

Analysis of the LAG product by TGA revealed a 5% loss in sample weight, taking place in one single step between ca. 50-75 ºC (see Appendix Fig S2.2). Such a well-defined step indicates that the material is a solvate containing one equivalent of EtOH per two units of (H**clon**$^+$)(**ala**$^-$). However, attempts to obtain the salt in the form of single crystals, using diethyl ether as the solvent, yielded the non-solvated salt of composition (H**clon**$^+$)(**ala**$^-$). Structural analysis by single-crystal X-ray diffraction revealed that the asymmetric unit of (H**clon**$^+$)(**ala**$^-$) consists of two unique sets of **ala**$^-$ anions and H**clon**$^+$ cations. In particular, the cations and anions are organized into pairs held together by $R_2^2(8)$ hydrogen bonding motifs involving two N-H groups of each H**clon**$^+$ cation and the carboxylate functionality of each **ala**$^-$ anion (Fig 12B) [35]. The resulting self-assembled ion pairs are further connected into one-dimensional (1-D) chains through N-H···O hydrogen bonds of lengths 2.601(8) Å - 2.762(8) Å.

Heating of the mechanochemically prepared (H**clon**$^+$)(**ala**$^-$) ethanol solvate 95ºC for one hour produced a material whose PXRD pattern shows resemblance to the one simulated for the crystal structure of non-solvated (H**clon**$^+$)(**ala**$^-$) (Fig 12A). Analysis of the ethanol solvated (H**clon**$^+$)(**ala**$^-$) by DSC was performed in the temperature range from -20ºC and 200ºC, which revealed a sharp endothermic event at 69ºC followed by a rapid exothermic process (see Appendix Fig S2.5). These observations are consistent with melting accompanied by decomposition.

Importantly, this behavior is different from either H**ala** or **clon**, which exhibit sharp melting point endothermic signals at 50ºC and 142ºC, respectively.

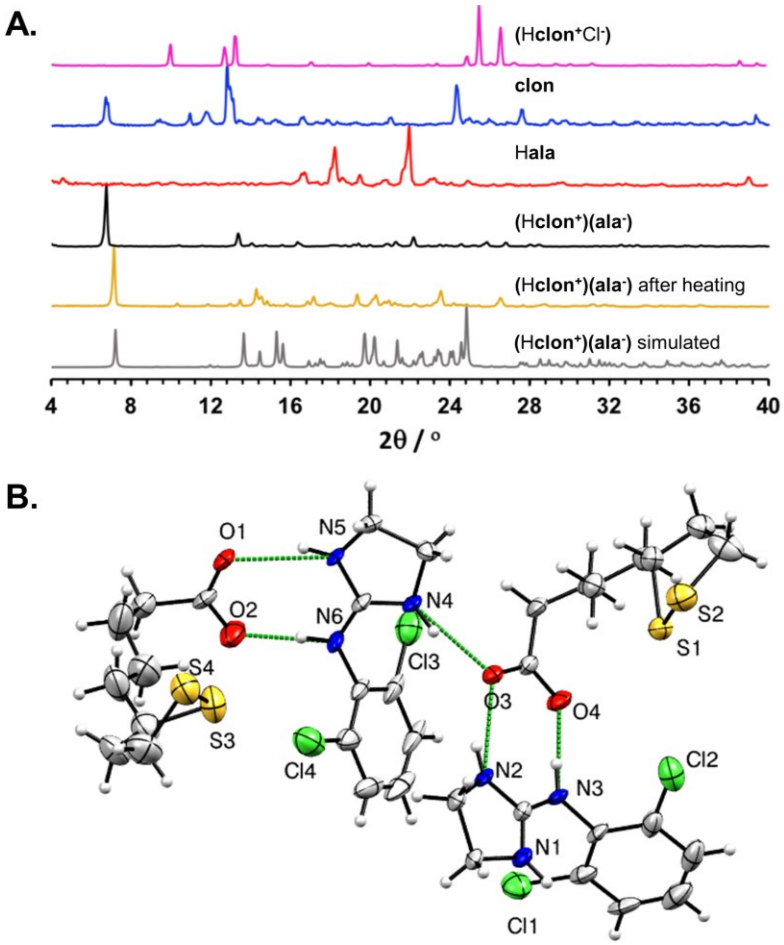

Figure 12. (**A**) Comparison of PXRD patterns for (top to bottom): (H**clon**$^{+}$Cl^{-}), solid **clon**, H**ala**, the LAG product of **clon** and H**ala** in presence of EtOH, the LAG product after heating at 95ºC for one hour and simulated for the herein reported crystal structure of (H**clon**$^{+}$)(**ala**$^{-}$); (**B**) View of the asymmetric unit of the (H**clon**$^{+}$)(**ala**$^{-}$) salt, illustrating the assembly of cations and anions via N-H···O hydrogen bonds. For clarity, only non-carbon and non-hydrogen atoms have been labeled.

4.5.1.3 Salt of linsidomine and caffeic acid (Hlin^{+})(cafa^{-})

As the **lin** free base is highly sensitive, with the API generally available in a stable solid form as the hydrochloride salt (H**lin**$^{+}$Cl^{-}), we devised a mechanochemical double ion metathesis route for the synthesis of linsidomine salt of caffeic acid (H**lin**$^{+}$)(**cafa**$^{-}$), by milling of (H**lin**$^{+}$Cl^{-}) with the pre-synthesized sodium salt of caffeic acid (Na^{+}**cafa**$^{-}$). The mechanochemical reaction

was expected to lead to the simultaneous formation of the new (H**lin**$^+$)(**cafa**$^-$) salt, accompanied by the byproduct NaCl (Fig 13).

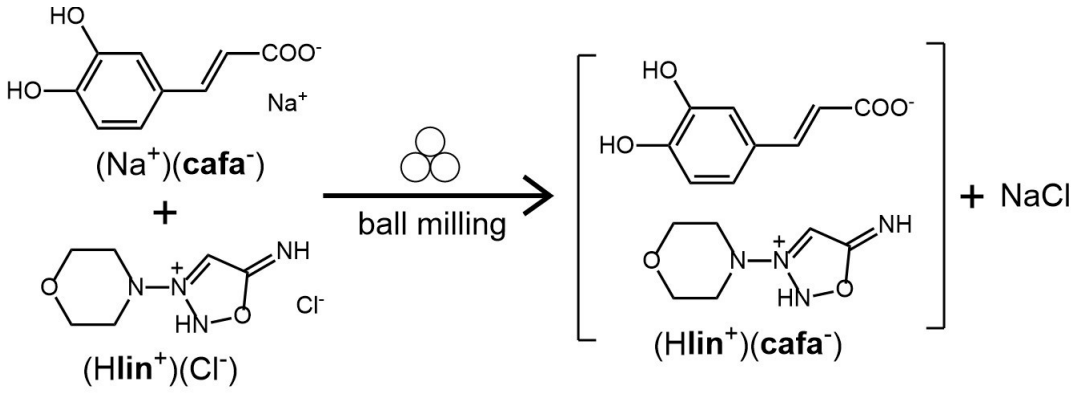

Figure 13. The mechanochemical double ion metathesis reaction used for the synthesis of (H**lin**$^+$)(**cafa**$^-$). The symbol for mechanochemical reaction conditions has been adopted from Rightmire & Hanusa [36].

Indeed, an inspection of the PXRD pattern of the product immediately after milling clearly revealed X-ray reflections of sodium chloride, with the particularly characteristic one at 2θ-value of 31.7° (Fig 14A), providing indirect evidence for the metathesis reaction to form (H**lin**$^+$)(**cafa**$^-$). Moreover, the FTIR-ATR spectrum of the milled reaction mixture clearly revealed a new absorption band at 1363 cm^{-1}, which is not present in the infrared spectra of either of the starting materials and is consistent with a C-O stretch of a carboxylate moiety (Fig 14B). Experiments to grow single crystals of (H**lin**$^+$)(**cafa**$^-$) suitable for structural characterization were unsuccessful, as the compound appeared to easily degrade upon dissolution in several different solvents. Consequently, (H**lin**$^+$)(**cafa**$^-$) was analyzed in more detail using DSC thermal analysis and ssNMR spectroscopy.

The DSC thermogram for (H**lin**$^+$)(**cafa**$^-$) exhibits a sharp endothermic signal at 70ºC that is consistent with melting and also confirms the absence of starting materials (see Appendix Fig S2.6). In particular, none of the starting material solids (H**lin**$^+$Cl$^-$), H**cafa** or (Na$^+$**cafa**$^-$) exhibits a sharp endothermic event that could be associated with melting: (H**lin**$^+$Cl$^-$) exhibits a weak endothermic event at 87ºC followed by exothermic decomposition at ca. 175ºC, the DSC thermogram for H**cafa** is featureless in the range -20ºC to 200ºC, and (Na$^+$**cafa**$^-$) undergoes a complex thermal transformation involving multiple endo- and exothermic events above ca. 150ºC.

All these observations are consistent with the formation of (H**lin**$^{+}$)(**cafa**$^{-}$) as a well-defined crystalline phase, different from any of the starting materials used.

The ^{13}C ssNMR CPMAS spectrum of (H**lin**$^{+}$)(**cafa**$^{-}$) generally resembles a combination of the solid-state spectra of the precursor salts (Na^{+}**cafa**$^{-}$) and (H**lin**$^{+}$Cl^{-}), which is consistent with the presence of both H**lin**$^{+}$ and **cafa**$^{-}$ in the final product (Fig 14C). However, the spectrum of (H**lin**$^{+}$)(**cafa**$^{-}$) also reveals minor changes in the chemical shifts of signals, which is consistent with a different crystalline environment compared to either of the starting material salts. The spectrum also reveals the appearance of additional resonances, which might suggest an increase in *Z'*, the number of moieties in the crystallographic asymmetric unit, compared to the precursor salts.

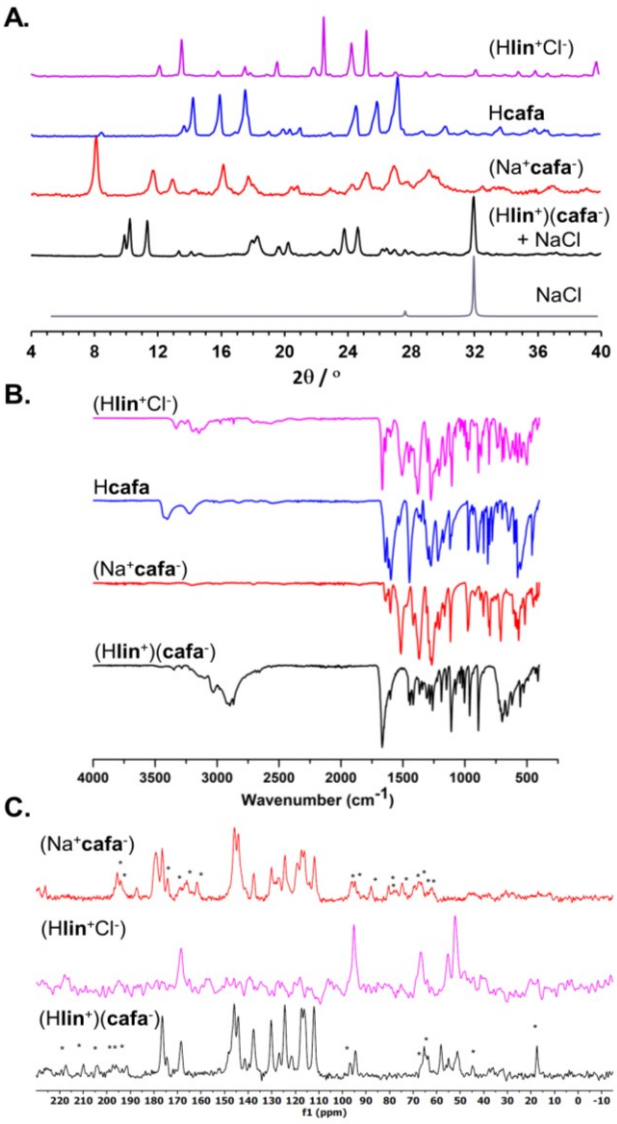

Figure 14. Comparison of relevant: (**A**) PXRD patterns; (**B**) FTIR-ATR spectra and (**C**) ^{13}C ssNMR spectra for the synthesis of (H**lin**$^{+}$)(**cafa**$^{-}$). Spinning side bands in c) are designated by '*'.

4.5.2 The enhanced analgesic effects of the nutraceutical co-crystal &salts of pentx, clon & lin

The synthesized co-crystals and salts were prepared in PEG as topical formulations and tested at different doses on the hind paw of CPIP rats. Occlusion of blood flow to the left hind paw with an O-ring tourniquet for three hours followed by reperfusion renders the hind paw edematous and hyperemic for 24-48 hours (Fig 15A). This subsides gradually gives way into hypersensitivity to thermal and mechanical stimuli (allodynia) for more than three weeks after the experimentally induced ischemia-reperfusion injury (IRI) [32]. The potency and efficacy of each co-crystal and salt in reducing mechanical allodynia was assessed by measuring the hind paw withdrawal threshold (PWT) of CPIP rats after topical application of the agents, as compared with that obtained with their corresponding constituent agents.

The topical application of the **(pentx**)(H**pca)** co-crystal produced significant anti-allodynic effects 30 to 120 minutes post-application at doses 2.5, 5 and 10% W/W (Fig 15B). A comparison of the log-dose response curve of **(pentx**)(H**pca)** with **pentx** showed a significant leftward shift by 0.59 log units which translates into a 3.9-fold increase in potency (p = 0.0113) (Fig 15C). Moreover, the cumulative three hours anti-allodynic effect of the lowest effective dose of **(pentx**)(H**pca)** (2.5%, constituted of 1.5% **pentx** and 1% H**pca** W/W) significantly exceeded the effect achieved by double the amount of its constituent drugs (3.5% **pentx** and 2% H**pca** W/W) (Fig 15D). Protocatechuic acid, topically applied at the different W/W doses did not produce significant anti-allodynic effects as compared to vehicle (Fig 15E).

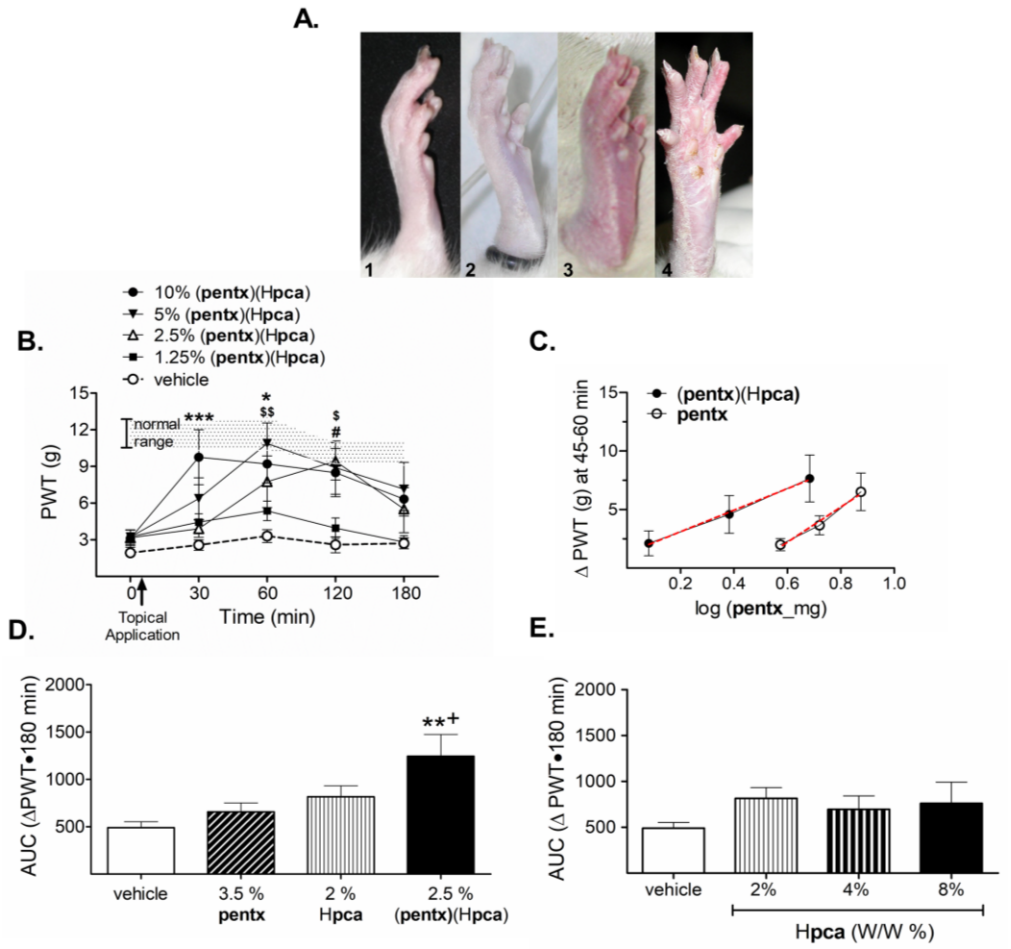

Figure 15. A comparison of the topical anti-allodynic effects of (pentx)(Hpca) to its constituent drugs on CPIP rats.

(**A**) The appearance of a rat hind paw before (1), during (2), 5 mins (3) and 24 hours (4) after application of O-ring tourniquet during the preparation of CPIP rats (**B**) time course of paw withdrawal threshold (PWT) response profile to topical application of different concentrations of (**pentx**)(H**pca)** (the shaded area represents PWT values from vehicle-treated normal animals). ($^{*}p < 0.05$, $^{***}p < 0.001$ pre- versus post-drug PWT of 10% W/W (**pentx**)(H**pca**) ; $^{\$}p < 0.05$, $^{\$\$}p < 0.01$ that of 5% W/W and $^{\#}p < 0.05$ that of 2.5% W/W) (n=8). (**C**) The anti-allodynic effect achieved by 2.5% (**pentx**)(H**pca)** exceeds the level obtained with 2 times the concentration of pentoxifylline and H**pca** contained in it ($^{**}p < .01$ compared to 3.5% **pentx**, $^{+}p < .05$ compared to 2% H**pca**) (**D**) The (**pentx**)(H**pca)** co-crystal has greater analgesic effect than pentoxifylline as evidenced by a leftward shift in its dose-response curve (**E**). The W/W amounts of H**pca** contained in the anti-allodynic concentrations of (**pentx**)(H**pca)** exhibit no significant anti-allodynic effect compared to vehicle. CPIP rat paw reprinted from Pain, Vol. 112, Coderre, T. J., et al., Chronic post-ischemia pain (CPIP): a novel animal model of CRPS-I produced by prolonged hind paw ischemia and reperfusion in the rat, 94-105 Copyright (2004), with permission from Elsevier [32].

With topical (H**clon**$^{+}$)(**ala**$^{-}$) (the ethanol solvate), a W/W concentration of 0.025% brought about a significant rise in PWTs at 30 minutes post-application and with a dose of 0.05% the PWTs stayed elevated for twice as long (Fig 16A). The change in PWTs observed over a three hours' time course of testing after topical application of (H**clon**$^{+}$)(**ala**$^{-}$), as compared to (H**clon**$^{+}$Cl^{-}), shows a small but significant improvement in its anti-allodynic potency. The x-intercept of the dose-response curve for (H**clon**$^{+}$)(**ala**$^{-}$), as compared to (H**clon**$^{+}$Cl^{-}), shifted significantly leftward by 0.265 log units (p =.04), which converts to a nearly two-fold increase in potency (Fig 16B).

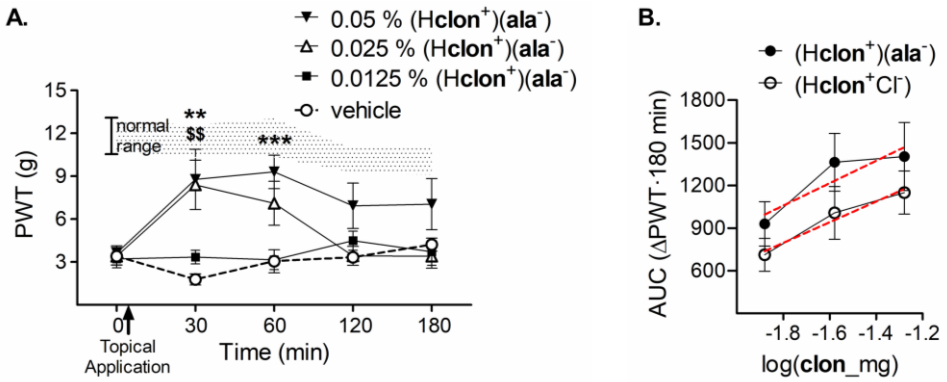

Figure 16. The effect of topical application of (Hclon^{+})(ala^{-}) and how it compares to its generically available counterpart (Hclon^{+}Cl^{-}).

(**A**) The time course of change in PWTs after topical application of different doses of (H**clon**$^{+}$)(**ala**$^{-}$). (the shaded area represents PWT values from vehicle-treated normal animals) ($^{**}p < .01$ and $^{***}p < .001$ pre- versus post-drug PWT of 0.05% W/W (H**clon**$^{+}$)(**ala**$^{-}$) and $^{\$\$}p < .01$ that of 0.025% W/W) (n=8). (**B**) The cumulative 3-hour change in PWTs achieved by topical application of (H**clon**$^{+}$)(**ala**$^{-}$) as compared to (H**clon**$^{+}$Cl^{-}) shows a leftward shift in its dose response curve implying a two-fold increase its potency.

The W/W concentrations of 1.6% of (H**lin**$^{+}$)(**cafa**$^{-}$) significantly elevated PWTs to non-allodynic values at 30 ($p < .0001$), 60 ($p < .0001$), 120 ($p < .001$) and 180 ($p < .01$) minutes post-application (Fig 17A). This anti-allodynic effect is dose-dependent with each doubling of dose producing a proportional rise in PWTs. A comparison of the dose-response curve of (H**lin**$^{+}$)(**cafa**$^{-}$) with (H**lin**$^{+}$)(**cafa**$^{-}$) showed the (H**lin**$^{+}$)(**cafa**$^{-}$) salt to have greater efficacy and potency. The maximum change in PWTs obtained after topical application of the HCl salt is (6.23 ± 0.99), but the caffeate salt brings about a greater rise in PWTs (9.6 ± 1.15) despite having half the **lin** content (Fig 17B). The x-intercept of the dose-response curve for (H**lin**$^{+}$)(**cafa**$^{-}$) is significantly shifted to the left by 0.3966 log units as compared to (H**lin**$^{+}$Cl^{-}) (p = .0026). This shift translates to a close

to 2.5-fold increase in potency (Fig 17B). Topical **cafa** produced no anti-allodynic effects on its own when administered at concentrations matching what is contained in (H**lin**$^{+}$)(**cafa**$^{-}$) (Fig 17C).

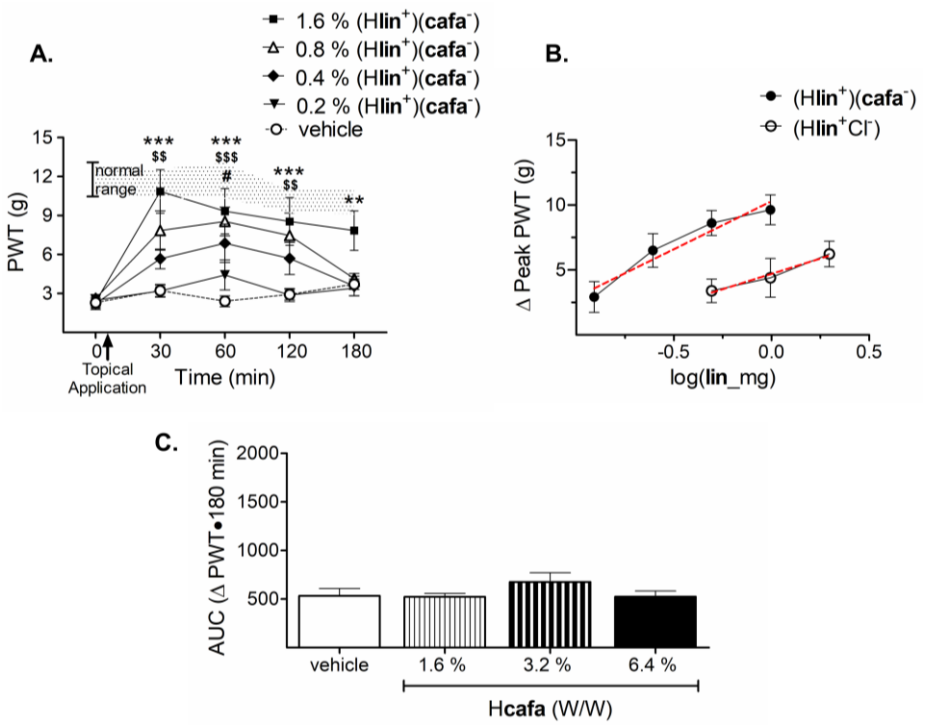

Figure 17. The anti-allodynic effects of topical (Hlin^{+})(cafa^{-}) and its dose-response comparison to (Hlin^{+}Cl^{-}).

(**A**) Topical (H**lin**$^{+}$)(**cafa**$^{-}$) significantly relieves CRPS allodynia at W/W doses of 1.6% and 0.8% for as long as 120 mins post-application (the shaded area represents PWT values from vehicle treated normal animals) ($^{**}p < .01$, $^{***}p < .001$ pre- versus post-drug PWT of 1.6% W/W (H**lin**$^{+}$)(**cafa**$^{-}$); $^{\$\$}p < .01$, $^{\$\$\$}p < .001$ that of 0.8% W/W and $^{\#}p < .05$ that of 0.4% W/W) (n=9). (**B**) The dose response curve of **lin-cafa** reaches a higher peak and is shifted leftward as compared to (H**lin**$^{+}$Cl^{-}), suggesting its greater efficacy and potency. (**C**) Topical administration of H**cafa** on its own exhibited no anti-allodynic effects.

4.6 Discussion

The co-crystal and salts made by pairing **pentx** with H**pca**, **clon** with H**ala** and **lin** with H**cafa** are, to the best of our knowledge, among the first demonstrations of effective analgesic drug-nutraceutical co-crystals and salts. The synthesis of these new solids was achieved through mechanochemical liquid-assisted grinding. In particular, the (**pentx**)(H**pca)** co-crystal was immediately obtained by liquid-assisted grinding in the presence of ethanol. The same approach yielded a solvated form of the salt (H**clon**$^{+}$)(**ala**$^{-}$), from which the non-solvated material was

obtained by thermal desolvation or re-crystallization from diethyl ether. Due to the instability of the free base **lin**, whose vasodilatory action is based in decomposition to release NO, the analgesic salt (H**lin**$^+$)(**cafa**$^-$) was prepared through a mechanochemical salt metathesis procedure involving liquid-assisted grinding of the commercially available stable solid hydrochloride form (H**lin**$^+$Cl$^-$) with sodium caffeate (Na$^+$**cafa**$^-$) [37].

The pairing of **pentx**, **clon** and **lin** with H**pca**, H**ala** and H**cafa** into salts and co-crystals was primarily aimed at targeting peripheral tissue hypoxia and oxidative stress seen in chronic post-ischemic pain [28, 32, 38]. Topical and systemic administration of agents that enhance tissue oxygenation and relieve oxidative damage has previously been shown to be analgesic in chronic pain due to IRI, peripheral neuropathy, diabetes and chemotherapeutic drug toxicity [32, 38-43]. However, targeting these mechanisms in unison has not been investigated. With the topical administration of (**pentx**)(H**pca**) co-crystal and (H**clon**$^+$)(**ala**$^-$) and (H**lin**$^+$)(**cafa**$^-$) salts to CPIP rats suffering from mechanical allodynia, we demonstrate for the first time that enhanced analgesia can be achieved with drugs that have simultaneous anti-hypoxic and antioxidant properties. We present the contrast between the anti-allodynic effect obtained with co-crystals and salts versus the individual constituent compounds. Our results indicate the enhanced analgesic effect obtained with the co-crystals and salts exceeds what could be achieved by the mere addition of their constituents. This is possibly a result of enhanced physicochemical properties acquired by the salts and co-crystals that yielded from the specific supramolecular interaction between their constituent drugs.

In conclusion, the synthesis of co-crystals and salts of compounds that impact the neural and non-neural processes involved in chronic pain is an approach that has yet to be explored to generate more effective analgesics. Through careful matching of drug compounds based on mechanism of action and chemical structure, the discovery of better analgesics for chronic pain can be enriched.

4.6 References

1. Tamburin S, Paolucci S, Smania N, Sandrini G. The burden of chronic pain and the role of neurorehabilitation: consensus matters where evidence is lacking. J Pain Res. 2017;10:101-3.
2. Woolf Clifford J, M.D., Ph.D., Max Mitchell B, M.D. Mechanism-based Pain Diagnosis: Issues for Analgesic Drug Development. Anesthesiology. 2001;95(1):241-9.
3. Ji R-R, Chamessian A, Zhang Y-Q. Pain regulation by non-neuronal cells and inflammation. Science. 2016;354(6312):572-7.
4. Mao J. Challenges of managing chronic pain. Br Med J. 2017;356:j741.

5. Woodcock J, Witter J, Dionne RA. Stimulating the development of mechanism-based, individualized pain therapies. Nat Rev Drug Discov. 2007;6:703.
6. Berry DJ, Steed JW. Pharmaceutical cocrystals, salts and multicomponent systems; intermolecular interactions and property based design. Adv Drug Deliv Rev. 2017;117:3-24.
7. Vishweshwar P, McMahon JA, Bis JA, Zaworotko MJ. Pharmaceutical co-crystals. J Pharm Sci. 2006;95(3):499-516.
8. Byrn SR, Zografi, G. , Chen, X. Pharmaceutical Salts. Solid State Properties of Pharmaceutical Materials. 1st ed. Hoboken (NJ): John Wiley & Sons, Inc.; c2017. p. 48-59.
9. Aitipamula S, Banerjee R, Bansal AK, Biradha K, Cheney ML, et al. Polymorphs, Salts, and Cocrystals: What's in a Name? Cryst Growth Des. 2012;12(5):2147-52.
10. Bolla G, Nangia A. Pharmaceutical cocrystals: walking the talk. Chem Commun. 2016;52(54):8342-60.
11. Cerreia Vioglio P, Chierotti MR, Gobetto R. Pharmaceutical aspects of salt and cocrystal forms of APIs and characterization challenges. Adv Drug Deliv Rev. 2017;117:86-110.
12. Shan N, Perry ML, Weyna DR, Zaworotko MJ. Impact of pharmaceutical cocrystals: the effects on drug pharmacokinetics. Expert Opin Drug Metab Toxicol. 2014;10(9):1255-71.
13. Gadade DD, Pekamwar SS. Pharmaceutical Cocrystals: Regulatory and Strategic Aspects, Design and Development. Adv Pharm Bull. 2016;6(4):479-94.
14. Žegarac M, Lekšić E, Šket P, Plavec J, et al. A sildenafil cocrystal based on acetylsalicylic acid exhibits an enhanced intrinsic dissolution rate. CrystEngComm. 2014;16(1):32-5.
15. Steed JW. The role of co-crystals in pharmaceutical design. Trends Pharmacol Sci. 2013;34(3):185-93.
16. McNamara DP, Childs SL, Giordano J, Iarriccio A, Cassidy J, Shet MS, et al. Use of a Glutaric Acid Cocrystal to Improve Oral Bioavailability of a Low Solubility API. Pharm Res. 2006;23(8):1888-97.
17. Karki S, Friščić T, Fábián L, Laity PR, Day GM, Jones W. Improving Mechanical Properties of Crystalline Solids by Cocrystal Formation: New Compressible Forms of Paracetamol. Adv Mater. 2009;21(38-39):3905-9.
18. Good DJ, Rodríguez-Hornedo N. Solubility Advantage of Pharmaceutical Cocrystals. Cryst Growth Des. 2009;9(5):2252-64.
19. Duggirala NK, Perry ML, Almarsson Ö, Zaworotko MJ. Pharmaceutical cocrystals: along the path to improved medicines. Chem Commun. 2016;52(4):640-55.
20. Aakeröy CB, Fasulo ME, Desper J. Cocrystal or Salt: Does It Really Matter? Mol Pharm. 2007;4(3):317-22.
21. Thipparaboina R, Kumar D, Chavan RB, Shastri NR. Multidrug co-crystals: towards the development of effective therapeutic hybrids. Drug Discov Today. 2016;21(3):481-90.
22. Thakuria R, Sarma B. Drug-Drug and Drug-Nutraceutical Cocrystal/Salt as Alternative Medicine for Combination Therapy: A Crystal Engineering Approach. Crystals. 2018;8(2).
23. Sekhon BS. Drug-drug co-crystals. DARU J Pharm Sci. 2012;20(1):45-45.
24. Grobelny P, Mukherjee A, Desiraju GR. Drug-drug co-crystals: Temperature-dependent proton mobility in the molecular complex of isoniazid with 4-aminosalicylic acid. CrystEngComm. 2011;13(13):4358-64.
25. Saleh A, Rizvi S. Utility of novel dual functionalized cocrystallized and ionic liquid based drugs for the pain management. Int J Pharm Res Bio Sci. 2016; 5: 97-109.
26. Stepanovs D, Jure Mr, Kuleshova LN, Hofmann DW, Mishnev AJCG, Design. Cocrystals of pentoxifylline: in silico and experimental screening. Cryst. Growth Des. 2015;15(8):3652-60.

27. Lim TK, Shi XQ, Johnson JM, Rone MB, Antel JP, David S, et al. Peripheral Nerve Injury Induces Persistent Vascular Dysfunction and Endoneurial Hypoxia, Contributing to the Genesis of Neuropathic Pain. J Neurosci. 2015;35(8):3346-59.
28. Coderre TJ. Complex regional pain syndrome: what's in a name? J Pain. 2011;12(1):2-12.
29. Friščić T, Mottillo C, Titi HM. Mechanochemistry for Synthesis. Angew Chem Int Ed Engl. 2020;59(3):1018-29.
30. Friščić T, Childs SL, Rizvi SA, Jones W. The role of solvent in mechanochemical and sonochemical cocrystal formation: a solubility-based approach for predicting cocrystallisation outcome. CrystEngComm. 2009;11(3):418-26.
31. Sheldrick GM. SHELXT–Integrated space-group and crystal-structure determination. Acta Crystallogr A Found Adv. 2015;71(1):3-8.
32. Coderre TJ, Xanthos DN, Francis L, Bennett GJ. Chronic post-ischemia pain (CPIP): a novel animal model of complex regional pain syndrome-Type I (CRPS-I; reflex sympathetic dystrophy) produced by prolonged hindpaw ischemia and reperfusion in the rat. Pain. 2004;112(1–2):94-105.
33. Laferrière A, Millecamps M, Xanthos DN, Xiao WH, Siau C, de Mos M, et al. Cutaneous tactile allodynia associated with microvascular dysfunction in muscle. Mol Pain. 2008;4:49..
34. Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL. Quantitative assessment of tactile allodynia in the rat paw. J Neurosci Methods. 1994;53(1):55-63.
35. Etter MC, MacDonald JC, Bernstein J. Graph-set analysis of hydrogen-bond patterns in organic crystals. Acta Crystallogr B. 1990;46 (Pt 2):256-62.
36. Rightmire NR, Hanusa TP. Advances in organometallic synthesis with mechanochemical methods. Dalton Trans. 2016;45(6):2352-62.
37. Rosenkranz B, Winkelmann BR, Parnham MJ. Clinical pharmacokinetics of molsidomine. Clin Pharmacokinet. 1996;30(5):372-84.
38. Coderre TJ, Bennett GJ. A hypothesis for the cause of complex regional pain syndrome-type I (reflex sympathetic dystrophy): pain due to deep-tissue microvascular pathology. Pain Medicine. 2010;11(8):1224-38.
39. Schratzberger P, Walter DH, Rittig K, Bahlmann FH, Pola R, et al. Reversal of experimental diabetic neuropathy by VEGF gene transfer. J Clin Invest. 2001;107(9):1083-92.
40. Ragavendran JV, Laferriere A, Xiao WH, Bennett GJ, Padi SS, Zhang J, et al. Topical combinations aimed at treating microvascular dysfunction reduce allodynia in rat models of CRPS-I and neuropathic pain. J Pain. 2013;14(1):66-78.
41. Kirchmair R, Walter DH, Ii M, Rittig K, Tietz AB, et al. Antiangiogenesis mediates cisplatin-induced peripheral neuropathy: attenuation or reversal by local vascular endothelial growth factor gene therapy without augmenting tumor growth. Circulation. 2005;111(20):2662-70.
42. Kirchmair R, Tietz AB, Panagiotou E, Walter DH, Silver M, et al. Therapeutic angiogenesis inhibits or rescues chemotherapy-induced peripheral neuropathy: taxol-and thalidomide-induced injury of vasa nervorum is ameliorated by VEGF. Mol Ther. 2007;15(1):69-75.
43. Fulas OA, Laferriere A, Stein RS, Bohle DS, Coderre TJ. Topical combination of meldonium and N-acetyl cysteine relieves allodynia in rat models of CRPS-1 and peripheral neuropathic pain by enhancing NO-mediated tissue oxygenation. J Neurochem. 2020;152(5):570-84.

5. Preamble to MANUSCRIPT 3:

Interdependent roles of tissue hypoxia & oxidative stress in PNP & CRPS

The enhanced potency of the analgesic salts and co-crystal reported in section 4, is indicative of the complementary analgesic effect produced by simultaneous alleviation of peripheral tissue hypoxia and oxidative stress in PNP and CRPS. The addition of the nutraceutical antioxidants to the vasodilators pentoxifylline, clonidine and linsidomine resulted in augmentation of their topical anti-allodynic effects in CPIP rats. Interestingly, the topical administration of the bare nutraceutical antioxidants produced no anti-allodynic effects in the rats. This implied the role played by the nutraceutical antioxidants in the novel salts and co-crystals was through the potentiation of the enhancement in tissue oxygenation produced by the vasodilatory anti-hypoxic drugs.

Tissue hypoxia and oxidative stress are observed both in the affected limbs of CRPS patients and the diseased peripheral nerves of patients with PNP [36, 50, 135, 336-338]. These two processes propagate one another in a vicious circle discussed earlier [50, 91]. On one hand, ROS and other oxidants induce microvascular injury that causes functional and structural changes impairing nutritive blood flow and tissue oxygenation [91, 141]. Some of that occurs through ROS, quenching and depleting the vasodilatory NO produced by the microvascular endothelium [104]. On the other hand, impaired tissue oxygenation impacts high energy phosphate metabolism in the mitochondria eventually resulting in dysfunction that results in excess production of ROS [91, 144]. Tissue hypoxia is also one of the drivers of inflammatory response causing the recruitment of immune cells that abundantly produce oxidant molecules [36]. This interdependent involvement of tissue hypoxia and oxidative stress in CRPS and PNP, allows the assessment of either processes to gain insight into the cumulative impact caused by both.

5.1 Measures of tissue oxygenation & downstream metabolic activity used as mechanism of action studies for the novel analgesic salts & co-crystals

Unlike tissue oxygenation, the direct measurement of ROS levels in live animals is difficult [339]. The indirect measures of change in tissue oxidative damage are unlikely to accurately reflect the acute impact of the topically administered treatments with the anti-hypoxic-antioxidant salts

and crystals. On the contrary, there are several non-invasive techniques that allow real-time *in vivo* measurement of tissue oxygenation [97, 340, 341]. These are mostly spectrophotometric techniques, including pulse oximetry, near-infrared spectroscopy (NIRS) and visible light spectroscopic oximetry, where light is shone onto the surface of a tissue and the unique light absorption characteristics of the oxygenated and deoxygenated states of hemoglobin are used to measure oxygenation. Other methods are non-spectroscopic techniques, including those that utilize phosphorescence or fluorescence, or functional magnetic resonance imaging (fMRI) with a 'BOLD' (Blood -oxygen-level-dependent) contrast imaging.

In pulse oximetry, cutaneous vascular beds like the digit or the ear lobe are sandwiched between a light-emitting probe and a detector [342]. The intensity of transmitted light originating from the variable-pulsatile arterial blood is filtered out and quantified by the detector. As the technique relies on the transmission of light through the tissue, skin pigmentation can limit adequate light penetration and affect signal detection [342]. NIRS oximetry and visible light spectroscopic oximetry, also utilize the principle of using light of suitable wavelength to measure tissue oxygenation [340, 343]. Both can utilize the intensity of the reflected or backscattered light to perform measurements from the same surface, and do not necessitate transillumination of a body part like pulse oximetry [340, 343]. The wavelength of light utilized by NIRS is long allowing deeper tissue penetration, while that of visible light oximetry is shorter with proportional limits to the depth of tissue probed [340, 343].

Methods that involve the use of phosphorescence and fluorescence to measure tissue oxygenation use a surface probe after the administration of an indicator material or use the implantation of a probe with the indicator bonded to it [344]. The need for the injection or implantation of exogenous indicator materials and probes limits the use of these techniques. BOLD fMRI is also another technique that measures tissue oxygenation by utilizing the signal generated by the paramagnetic deoxygenate hemoglobin. It is not an ideal technique for studies with the primary purpose of measuring tissue oxygenation as it has a relatively low sensitivity and gives an indirect indication of oxygenation by measuring levels of deoxyhemoglobin [344].

In the work described in the following manuscript, visible light spectroscopic oximetry was used to measure the change in tissue oxygenation induced by the novel analgesic co-crystal in CPIP rats. The technique provides a real-time, non-invasive measurement of local tissue

oxygenation with simple instrumentation. Although it has a shallower depth of measurement as compared with NIRS oximetry, it still allows the collection of valuable data on the oxygenation of the skin and the subdermal tissue [345]. Both visible light and NIRS oximetry have been used to assess local tissue oxygenation in CRPS patients. The data obtained from both techniques in these studies exhibited a similar outcome of compromised resting and post-ischemic tissue oxygenation in CRPS limbs [38, 341].

Local treatments that change microvascular flow can produce readily detectable changes in the level of tissue oxygenation. These observations have geared our mechanism of action studies on the novel salts and co-crystals towards measurements of tissue oxygenation and its downstream metabolic effects. The mitochondria are the primary site of oxygen consumption in the cell. Mitochondrial oxidative phosphorylation, which is the main pathway of ATP generation, is dependent on an adequate and continuous supply of oxygen. While oxygen is a substrate for the final step of the mitochondrial respiratory chain that involves cytochrome c oxidases, this final step is preceded by upstream reactions catalyzed by a series of dehydrogenase enzymes contained in the citric acid cycle [346]. Hypoxia disrupts mitochondrial activity including the activity of enzymes in the citric acid cycle [346]. Previous work in our laboratory has made use of a colorimetric assay that assessed the activity of mitochondrial dehydrogenases to demonstrate the impaired oxygen-dependent mitochondrial respiration exhibited by the CPIP hind paw muscle [141]. In the following manuscript, the same assay was utilized to assess for the alleviation of this impairment in CPIP rats after topical treatment with a novel analgesic co-crystal. In this biochemical colorimetric assay, freshly dissected tissue samples are incubated in a solution of one of the tetrazolium salts.

Tetrazolium salts are compounds known to serve as substrates for functional mitochondrial dehydrogenases in viable tissue and get reduced into a red-pigmented derivative called formazan that can be quantified spectrophotometrically [347]. A variant of this technique in the form of histochemical staining with tetrazolium salt derivatives has been routinely used in the past for the identification of ischemic and infarcted tissue in cardiovascular studies [348]. This tetrazolium-based assay is, therefore, likely to serve well in measuring the change in tissue perfusion and consequent oxygen-dependent metabolic activity produced by treatment of CPIP rat hind paws with the novel topical agent.

5.2 The direct role played by tissue oxygenation in alleviating mechanical hypersensitivity in CPIP rats

The theory that enhanced tissue oxygenation is the main mechanism through which the topical treatments produce their antiallodynic effect in the CPIP rats is supported by the direct effect oxygen therapy produces on CPIP allodynia. This observation was made from auxiliary experiments I performed in the laboratory, that investigated the effect of hyperbaric oxygen therapy on CPIP rats. In these set of experiments, CPIP rats were treated with 30-60 minutes of hyperbaric oxygen (at 3.5 atmospheres) for a period of 7 days at 1-day post-procedure, prior to the onset of mechanical hypersensitivity, and at 4 days after CPIP-induced mechanical hypersensitivity established. While the preventive administration of hyperbaric oxygen, at 1-day post-CPIP, delayed the occurrence of mechanical hypersensitivity in CPIP rats, its therapeutic use at 4 days post-CPIP reduced the level of hypersensitivity exhibited by the rats (Fig 18). These findings are in line with the analgesic effect of hyperbaric oxygen reported in CRPS patients and different rodent models of PNP [232, 349, 350].

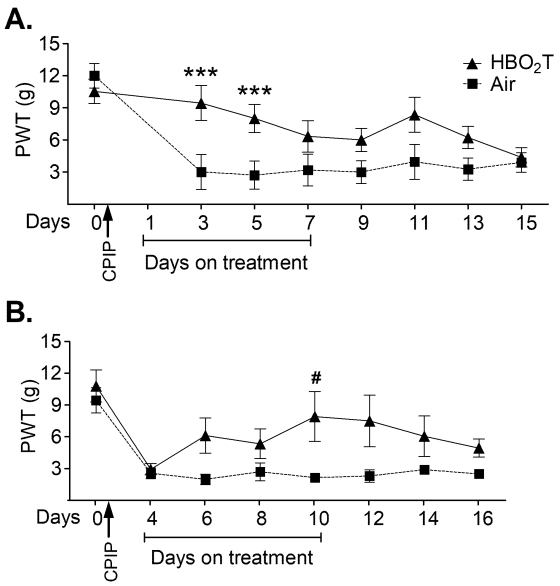

Figure 18. The effect of hyperbaric oxygen treatment (HBO_2T) on mechanical hypersensitivity in CPIP rats.

(**A**). The administration of hyperbaric oxygen treatment at 24 hours post-CPIP delays the onset of mechanical hypersensitivity. (*** $p < .001$, PWT of rats treated with HBO_2T versus those treated with air, n = 6-7 rats /group). (**B**) Mechanical hypersensitivity in CPIP rats, at 4-days post-procedure, is alleviated by HBO_2T. (# $p < .05$, PWT before (day 4) versus after HBO_2T (day 10), n = 6 rats /group).

5.3 Studies of mechanisms underlying the analgesic effects of a prototype of the novel anti-hypoxic-antioxidant salts & co-crystals

Mechanism of action studies on the novel analgesic salts and co-crystals were designed with consideration of the interactive roles of tissue hypoxia and oxidative stress in CRPS. The behavioral data indicate that, of the two constituents of the products, the anti-hypoxic agents played a more direct role in the topical anti-allodynic effects observed, while the nutraceutical antioxidants played a potentiating role. Given the anticipated dominant role of tissue oxygenation and its accessibility for direct measurement, follow up mechanism of action studies focused on assessing local changes in tissue oxygenation and its downstream impact on mitochondrial function.

The co-crystal of pentoxifylline and protocatechuic acid was chosen as a prototype of the newly synthesized anti-hypoxic-antioxidant salts and co-crystals. This was because a more complete chemical characterization was obtained for this product, and it produced potent topical anti-allodynic effects in the CPIP rats. As will be described in detail in the following manuscript, the mechanisms of action studies entail non-invasive percutaneous measurements of tissue oxygenation, along with a biochemical assay of oxygen-dependent mitochondrial enzyme function.

6. MANUSCRIPT 3: A novel co-crystal of pentoxifylline & protocatechuic acid relieves allodynia in a rat model of CRPS by alleviating local tissue hypoxia

Oli Abate Fulas[1], Andre Laferriere[1], Ghada Ayoub[2], Hatem M. Titi[2], Tomislav Friščić [2], Terence J. Coderre[1]

Departments of Anesthesia[1] and Chemistry[2]

6.1 Contribution of authors

I designed the experiments in this work with inputs from Prof Terence J Coderre and Andre Laferriere. I performed the chemical synthesis and validation experiments on the co-crystal of pentoxifylline and protocatechuic acid under the supervision of Ghada Ayoub and Prof Tomislav Friščić. I and Andre Laferriere generated the CPIP rats, performed the behavioural assays and mechanism of action studies. Hatem M Titi solved the single crystal structure of the co-crystal between pentoxifylline and protocatechuic acid. This manuscript was drafted by me and revised by Prof Coderre.

6.2 Abstract

Complex regional pain syndrome (CRPS) affected limbs exhibit extensive tissue ischemia and hypoxia, a pain-inducing peripheral process that can be locally targeted with topical treatments. We recently reported three novel salts and a co-crystal composed of vasoactive agents and antioxidant nutraceuticals, all of which produced potent topical anti-allodynic effects in the chronic post-ischemic pain (CPIP) rat model of CRPS. One of the products, pentx-pca, is a co-crystal synthesized from pentoxifylline and protocatechuic acid (pca). Pentx-pca exhibited potent topical anti-allodynic effects in CPIP rats exceeding effects produced by bare pentoxifylline and pca. We hypothesized that the anti-allodynic effects of pentx-pca in CPIP rats were due to its impact on local tissue oxygenation and subsequent oxygen dependent mitochondrial respiration. Percutaneous tissue oxygen saturation (SaO_2) measurements taken from the hind paw of the CPIP rats revealed that anti-allodynic doses of topical pentx-pca increased local tissue SaO_2. Moreover, assessment of oxygen dependent mitochondrial function using a triphenyl tetrazolium chloride (TTC) assay revealed CPIP-induced mitochondrial dysfunction significantly declined in plantar muscle collected from CPIP rats topically treated with anti-allodynic doses of pentx-pca.

Furthermore, time-dependent resolution of plantar muscle mitochondrial dysfunction, that occurred in the CPIP rats at 6-weeks post procedure, paralleled the loss of the anti-allodynic response to topical treatment with pentx-pca. Our results indicated that pentx-pca produced potent anti-allodynic effects in the CPIP rat model of CRPS by alleviating peripheral tissue ischemia/hypoxia and downstream hypoxia-driven mitochondrial dysfunction.

6.3 Introduction

Impaired nutritive blood flow and inadequate tissue oxygenation contribute to the generation and maintenance of pain and hypersensitivity in complex regional pain syndrome (CRPS) where inciting injury-induced disruption of microvascular function results in ischemia and hypoxia of skin, muscle, nerve and bone in the affected limb [1-3]. Fluctuating microvascular perfusion and tissue oxygen supply generate reactive oxygen species that further propagate the dysfunction of the microvasculature [2, 4]. The resulting hypoxic, acidic and inflammatory microenvironment has been implicated in the sensitization of peripheral nerve fibers and consequent pain symptoms of CRPS [5-7]. The rodent CPIP model of CRPS, generated by inducing 3-hr long limb ischemia followed by reperfusion, clearly reveals the microvascular dysfunction observed in CRPS along with the phenotypic appearance of the diseased limb and painful response to various somatosensory stimuli [4, 8].

Ischemic hypoxia of limb tissue observed in CRPS and CPIP are pain inducing peripheral processes that can be targeted locally by the administration of topical agents that enhance blood flow, microvascular perfusion and oxygen supply [4, 9-11]. Studies have reported analgesia produced by vasoactive agents, administered systemically and topically, alone or in combination, in CPIP rats [10-12]. There are also human studies in which topical treatments containing vasodilators have yielded pain relief in CRPS patients [9, 13, 14].

We recently reported a group of analgesic salts and co-crystals made from vasodilator drugs and antioxidant nutraceuticals. Salts and co-crystals are distinguished as patentable new solid forms, presenting new compounds for quick development and clinical translation [15, 16]. The addition of nutraceutical antioxidants to vasodilator agents boosted anti-allodynic efficacy and potency in the CPIP rat model of CRPS. In here, we present one of the new products, a co-crystal synthesized from the vasoactive agent pentoxifylline and a nutraceutical antioxidant protocatechuic acid, also detailing its mechanism of action.

Pentoxifylline is a hemorheologic vasodilator used to enhance regional microcirculation in the treatment of peripheral vascular disease [17]. In addition to decreasing vascular tone, it increases red blood cell malleability, prevents platelet aggregation and white blood cell adhesion to the endothelium, all promoting microvascular perfusion [17-21]. Treatment with pentoxifylline has resulted in enhanced microelectrode-measured oxygen tension in the muscle of patients with peripheral arterial disease at baseline and post-exercise [17, 22, 23]. Similarly, enhanced oxygenation post-treatment with pentoxifylline has been reported in the CSF of patients with cerebral vascular disease and the retinal tissue of patients with retinopathy [17, 24, 25]. The vascular effects of pentoxifylline produce improved neurologic function and pain relief when administered to patients with chronic pain pathologies involving microvascular compromise, including diabetic neuropathy and CRPS [9, 26]. Enhanced vascular function and pain relief are also achieved after both systemic and topical treatment with pentoxifylline in CPIP rats [10, 11, 19].

The nutraceutical protocatechuic acid is plant derived phenolic compound that has potent antioxidant and reactive oxygen species (ROS) scavenging activities [27]. It is reported to alleviate oxidative stress and prevent tissue injury after cerebral, retinal or myocardial ischemia [28-31]. Its therapeutic effects are also reported in conditions like neurodegeneration, depression and aging, where oxidative stress plays a pathophysiologic role [32-34].

Pentoxifylline, as a methyl xanthine, has a basic aromatic nitrogen in its imidazoline ring that is complementary to the hydroxyl group of protocatechuic acid, greatly increasing the potential for the bonding of the two compounds into a compact co-crystal of defined stochiometric ratio [35, 36]. Such a co-crystallization yields a novel solid form with expected modifications of physical and chemical properties like solubility, bioavailability and stability that may translate into enhanced therapeutic efficacy and potency [37, 38].

In addition to having the chemical congruence that suits co-crystallization, the vasoactive pentoxifylline and antioxidant protocatechuic acid have complementary biological actions that should alleviate microvascular dysfunction and tissue hypoxia [39]. Oxidative stress due to excess production of ROS and other oxidants results in injury of the endothelium, disrupting its normal vasodilatory, anticoagulant and antiadhesive properties with consequent microvascular compromise [39, 40]. The addition of the antioxidant protocatechuic acid to the vasodilator

pentoxifylline should reduce both oxidative stress and microvascular dysfunction yielding a compound that efficiently restores microvascular flow and tissue oxygenation.

In this study, we report the effects of topical treatment with the novel co-crystal pentoxifylline-protocatechuic acid (pentx-pca) on mechanical allodynia in CPIP rat models of CRPS as well as its impact on local tissue oxygen saturation and consequent oxygen-dependent mitochondrial respiration.

6.4 Methods

6.4.1 Drugs

Pentoxifylline was obtained from Sigma-Aldrich, St. Louis, MO. Protocatechuic acid was purchased from Cayman Chemicals, MI. Acetonitrile was obtained from Fisher Chemical, while ethanol was purchase from Commercial Alcohols, ON.

6.4.2 Synthesis of the pentx-pca co-crystal

Synthesis of the pentx-pca co-crystal was performed by liquid-assisted grinding (LAG) using a Retsch MM400 shaker mill (Hann, Germany). The milling was conducted at 30 Hz for 30 min using one stainless steel ball (10 mm diameter, 4 g weight) in a 14-ml polytetrafluoroethylene (PTFE) jar. Equimolar quantities of pentoxifylline and protocatechuic acid were milled using 100 µl of ethanol as a LAG solvent.

6.4.3 Powder X-ray diffraction (PXRD)

PXRD experiments on pentoxifylline, protocatechuic acid and pentx-pca were conducted on a Bruker D2 phaser equipped with Cu*K*α X-ray source and a Nickel filter. Data was collected between 4-40° with increment of 0.05°.

6.4.4 Single crystal X-ray diffraction (SCXRD)

Single crystals used for single crystal x-ray diffraction (SCXRD) were grown at room temperature by slow evaporation in a mixture of ethanol and acetonitrile. Single crystal X-ray diffraction data were collected on a Bruker D8 Advance diffractometer (Bruker-AXS, Madison, WI, USA) with a Photon 100 CMOS area detector and an IµS microfocus X-ray source (Bruker AXS). X-ray diffraction experiments on single crystals of the pentx-pca co-crystal were conducted using Cu*K*α radiation. Single crystals were often found to readily lose solvent upon exposure to air, and were coated with Paratone oil (Hampton Research, Aliso Viejo, CA, USA) during X-ray single crystal data collection.

Unit cell determination, data collection, data reduction, and correction for absorption were all conducted using the Apex3 software suite (Bruker AXS). Crystals were solved by an iterative dual space approach as implemented in SHELXT. Non-hydrogen atoms were located from the difference map and refined anisotropically. Hydrogen atoms bonded to carbon atoms were placed in calculated positions. All hydrogen atoms coordination and thermal parameters were constrained to ride on the carrier atoms. Pentx-pca was found to be twinned and its structure was treated by CELL NOW (Version 2008-2, Bruker AXIS Inc., WI), in which three domains were found and separated.

6.4.5 Formulation of drugs into topical ointments

Topical drugs were formulated into ointment-type preparations using a composite, water-soluble polyethylene glycol base system consisting of 40 % carbowax (PEG 3350) and 60 % PEG 400 (both from Sigma Aldrich). The required amounts of the active ingredients were first weighted out and then added to the already molten base and mixed. The vehicle treatment consisted of the same water-miscible base ointment without the addition of the active drugs.

6.4.6 Generation of the chronic post-ischemic pain (CPIP) rat model of CRPS

The CPIP rat model of CRPS was generated by inducing prolonged hind paw ischemia and subsequent reperfusion as previously described by Coderre et. al, 2004 [8]. In short, male Long Evans rats (300-400 g; Charles River, QC, Canada) were anesthetized over a 3-hour period with a bolus (55 mg/kg, intraperitoneally (i.p.)) followed by chronic i.p. infusion (0.15 mL/hour) of sodium pentobarbital (Ceva Sante Animale, Libourne, France) for 2 hours. Following induction of anesthesia, a Nitrile 70 Durometer O-ring (O-rings West, Seattle, WA) with an internal diameter of 5.5 mm was slipped around the rat's left hind limb proximal to the ankle joint to effect a complete blockade of arterial blood flow [4]. The ring was left in place for three hours, and the rats recovered from anesthesia 30 to 60 minutes following O-ring removal and reperfusion.

6.4.7 Mechanical sensitivity testing

As a measure of mechanical sensitivity, paw withdrawal thresholds (PWTs), were tested on plantar surface of the ipsilateral injured hind paw of the CPIP rats. The rats were first habituated for 20 minutes in the test chamber. Nylon monofilaments (von Frey hairs) were applied in either ascending (after negative response) or descending (after positive response) force as necessary to determine the filament closest to the threshold of response. Each filament was applied for 10 s or until a flexion reflex occurred. The minimum stimulus intensity was 0.25 g and the maximum was

15 g. Based on the response pattern, and the force of the final filament (5^{th} stimulus after first direction change), the 50% threshold (grams) was calculated as (10[Xf+ kδ]) /10000 where Xf=value (in log units) of the final von Frey filament used, k=value for the pattern of positive/negative responses and δ=mean difference in log unit between stimuli (here, δ=0.224, for more detail see Chaplan et al., 1994) [41]. PWTs were assessed before the ischemia/reperfusion injury of the CPIP procedure, and before and after topical treatments.

6.4.8 Measurement of plantar tissue oxygen saturation

Using a visible light based spectroscopic vascular monitoring system (moorVMS-OXY, Moor Instrument Ltd, Devon UK), percent oxygen saturation of hemoglobin (SaO_2) was measured from the plantar side of the rat's hind paw before the O-ring occlusion of the CPIP procedure, 15 minutes after reperfusion and 5-7 days after the CPIP procedure following topical hind paw treatment with vehicle or 5% (W/W) pentx-pca. To perform the hind paw SaO_2 measurements, a light emitting diode (LED) probe (7 mm) that transmits low-power white light and collects reflectance light from blood cells in the tissue was apposed to the hind paw of the rats using a loose-fitting elastic bandage (3 cm inner diameter) after light anesthesia with IP pentobarbital (40 mg/kg). Continuous measurement of total hemoglobin, oxygenated hemoglobin, deoxygenated hemoglobin and SaO_2, were recorded at 2 HZ for 30 seconds. The rats underwent SaO_2 measurement sessions 60 minutes following topical treatment with vehicle or 5% (W/W) pentx-pca on two alternate days. A mean of each rat's 30 second recording was calculated for comparison between sessions.

6.4.9 Colorimetric assay of oxygen-dependent mitochondrial respiration in the CPIP rat plantar muscle

Oxygen dependent mitochondrial respiration in plantar muscle samples collected from vehicle-treated and pentx-pca treated CPIP rats was estimated from the reduction of triphenyl tetrazolium chloride (TTC) according to a protocol described by Laferriere et al, 2008 with some modifications [4] .

Plantar muscle samples were dissected and collected in chilled lactated ringer on ice. The samples were then weighed and grossly chopped with a surgical blade then incubated in 2% triphenyl tetrazolium chloride (TTC; Sigma, St Louis, MO) in 0.05 M PBS/0.25 M sucrose for 60 minutes at 37°C. Formazan (1,3,5-triphenylformazan), a red pigmented product of oxygen-dependent mitochondrial enzyme processing of the TTC substrate by the muscle samples, was

then extracted by incubation in acetone (60 min, 37ºC) and centrifugation (3 min at 5000 rpm). The quantity of formazan in each sample was then estimated by sample absorbance (485 nm) of 200 µl of supernatant per weight of tissue in mg.

6.4.10 Statistics

Time course measurements of PWTs after vehicle and drug administration were subjected to a repeated measure analysis of variance (ANOVA). Pairwise post-hoc comparisons were performed between mean PWTs obtained from drug and vehicle treatment groups measured at matching post-treatment times using Tukey's HSD test.

Cumulative anti-allodynic effects measured over 180-minute time course experiments were assessed by calculating the area under the curve (AUC) of plotted PWT elevations observed post-topical application. Comparisons of different drug doses versus vehicle were performed using repeated measures ANOVA followed by Tukey's HSD test.

Dose response curves for comparison of anti-allodynic potency were plotted on a semi-log scale with the amount of drug used per application on the X-axis and the AUC of the 180-minute PWTs measured post-drug application, on the Y-axis. A linear regression of each dose-response curve was then calculated, and their differences analyzed using a 1-way ANOVA. Difference in anti-allodynic potency are stated in terms of the shift in the x-intercept of the regression line of the dose-response curve for each drug.

The SaO_2 data collected were analyzed using a repeated measures design; comparisons were made between each rat's SaO_2 value collected pre-occlusion, after reperfusion and post-topical treatment with vehicle and pentx-pca. The repeated measure design minimized SaO_2 variabilities introduced due to differences between individual rats in skin thickness, pigmentation and cardiovascular response to sedation with pentobarbital. Differences between mean SaO_2 values were calculated with a 1-way repeated measure ANOVA followed by a post-hoc pairwise comparison using Tukey's HSD test.

For data from the TTC assay of plantar muscle samples, statistical differences in the quantity of formazan produced in normal, vehicle-treated and drug-treated samples was calculated using a one-way ANOVA followed by a post-hoc pairwise orthogonal contrast.

6.5 Results

6.5.1 Co-crystallization of pentoxifylline and protocatechuic acid after liquid assisted grinding

The LAG of equimolar quantities of pentoxifylline (pentx) and protocatechuic acid (pca) resulted in the formation of a novel co-crystal: pentx-pca (Fig 19). This was verified after obtaining the structure of the product's single crystal grown in a mixture of ethanol and acetonitrile. The structure revealed the carboxylic acid group of one protocatechuic acid molecule hydrogen bonded with the N12-atom of one pentoxifylline molecule at an angle of O1-H···N12, 2.700(2) Å (Fig 19B). Data obtained from characterization studies with PXRD and other spectroscopic techniques also aligned with the single crystal structure of the product (Fig. 19C, see Appendix).

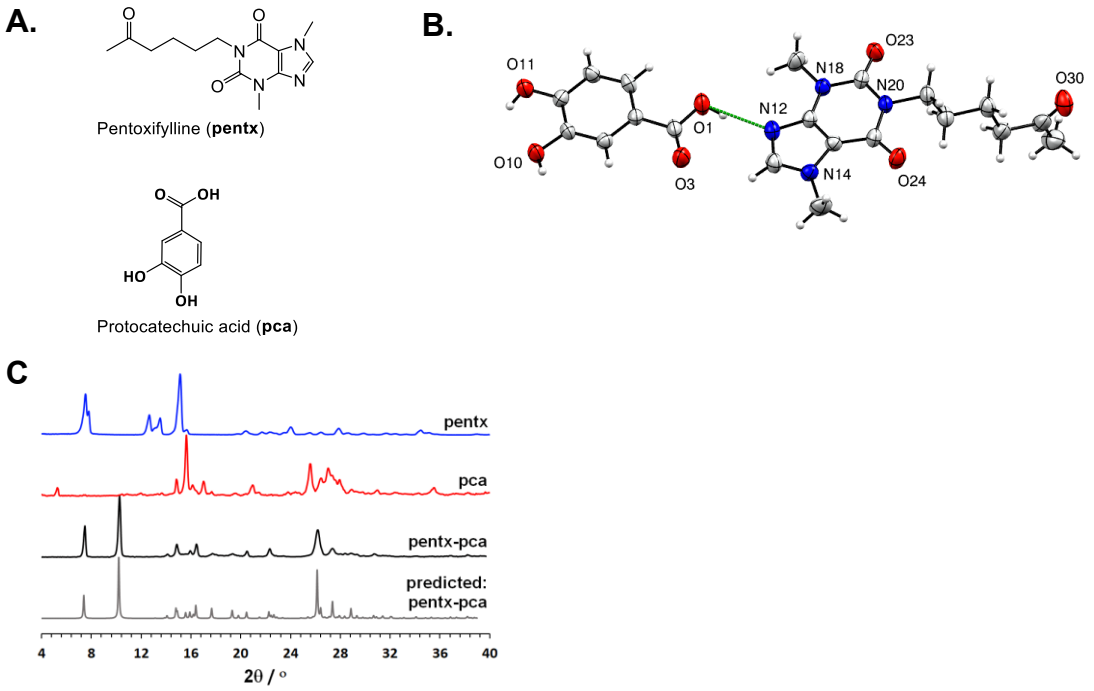

Figure 19. Chemical characterization of the co-crystal of pentoxifylline & protocatechuic acid.
(**A**) molecular structures of pentoxifylline (**pentx**) and protocatechuic acid (**pca**) (**B**) single crystal structure of the pentx-pca co-crystal (**C**) PXRD of pentx, pca & pentx-pca.

6.5.2 Anti-allodynic effects of topical pentx-pca in CPIP rats

The anti-allodynic effect of pentx-pca was investigated by measuring withdrawal thresholds of the injured hind paw of CPIP rats after topical treatment with 1.25, 2.5 and 5% W/W ointment formulations. The PWT data collected over 180 mins post-topical application revealed

significant main effects of dose ($F_{3,18} = 8.59$, $p < .001$) and time ($F_{4,24} = 7.13$, $p < .001$), as well as a significant dose x time interaction ($F_{12,72} = 14.99$, $p < .05$). Topical pentx-pca, at W/W dose of 5% elevated PWT to near normal values at 60 ($p < .001$) and 120 ($p < .01$) minutes post-application as compared to vehicle, while the 2.5% W/W dose produced significant effects only after 120 minutes ($p < .01$) (Fig 20A). A cumulative assessment of the change in PWT produced over 180 minutes post-topical treatment with pentx-pca also revealed the formulation, at W/W doses of 2.5% ($p < .001$) and 5% ($p < .0001$) produced significant elevations in PWTs as compared to vehicle (Fig 20B).

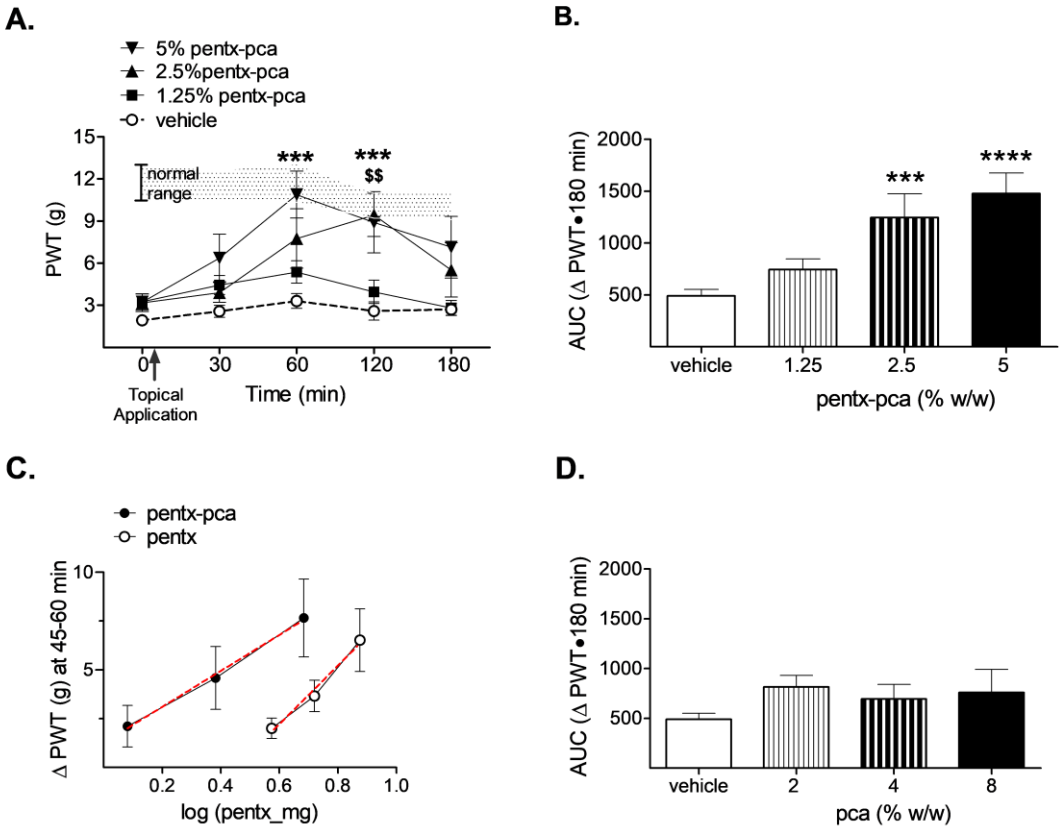

Figure 20. The topical anti-allodynic effect of pentx-pca and its constituents pentoxifylline and protocatechuic acid (pca) on CPIP rats.

(**A**) Time course of paw withdrawal threshold (PWT) response of CPIP rats to topical treatment with different W/W doses of pentx-pca. (*** $p < .001$ for 5% W/W, $^{\$\$}$ $p < .01$ for 2.5% W/W pentx-pca versus vehicle) (**B**) Three-hour cumulative change in PWT produced by topical pentx-pca (*** $p < .001$, **** $p < .0001$ versus vehicle) (**C**) Dose-response curve of topical pentx-pca in comparison to pentoxifylline (pentx) (**D**) PWT response of CPIP rats to different W/W doses of pca (n = 7 rats).

Topical treatment with 2, 4, and 8% W/W doses of pca produced no anti-allodynic effect in CPIP rats (Fig 20D). On the other hand, topical pentoxifylline has been reported to exhibit anti-allodynic effects in CPIP rats. To gain insight into how the topical anti-allodynic effects of pentoxifylline and pentx-pca compare, a log-dose response curve of the two formulations was generated. The curve of pentx-pca is shifted leftwards by 0.59 log units as compared to pentoxifylline, which translated into a 3.9-fold increase in potency ($p = .01$) (Fig 20C).

6.5.3 Local oxygen saturation changes produced by topical pentx-pca in CPIP rats

To investigate the mechanism of anti-allodynic effects produced by topical pentx-pca, we percutaneously measured the local oxygen saturation (SaO_2) in the plantar tissue of the hind paw of CPIP rats using a LED-based spectroscopic device. SaO_2 measurements were obtained at four time points from each rat: at the start of the CPIP procedure before placement of the O-ring tourniquet, 30-mins post-reperfusion at the end of the CPIP procedure, and 5-7 days post-CPIP in the allodynic rats, 60 minutes after treatment with vehicle or 5% W/W topical pentx-pca.

Local SaO_2 in the plantar tissue of the rats prior to the CPIP procedure amounted to 54.18 ± 1.4 % and after 5-7 days post-procedure, there was a significant decline in SaO_2 to 45.86 ± 1.8% ($p < .01$) (Fig. 3A). Topical treatment with 5% W/W pentx-pca significantly elevated the CPIP-induced decline in local plantar tissue SaO_2 to 66.49 ± 1.2 % ($p < .001$) (Fig 21A). This value compared closely to the 30-min post-reperfusion SaO_2 measures of the rats obtained during the reactive hyperemic period of the CPIP procedure (Fig 21A) when SaO_2 is normally elevated.

6.5.4 Effect of topical pentx-pca on oxygen-dependent mitochondrial respiration in the CPIP rat plantar muscle

Subsequent experiments were aimed at measuring the effect of topical pentx-pca on oxygen-dependent mitochondrial respiration in the plantar muscle of CPIP rats, as previous studies have directly correlated the allodynia exhibited by CPIP rats with plantar muscle mitochondrial dysfunction and lactate production [4]. The TTC assay, a substrate-based colorimetric assay that measures the activity of oxygen dependent mitochondrial enzymes, was used to assay the hind paw plantar muscle of naïve and topical pentx-pca or vehicle treated CPIP rats. This was estimated from the quantity of spectrophotometrically measured formazan, the red pigmented product made from the TTC substrate by active mitochondrial dehydrogenases in the plantar muscle samples. The highest concentration of formazan was measured form the plantar muscle of uninjured naïve

rats (Fig 21B). Plantar muscle obtained from vehicle treated CPIP rats exhibited significantly lower levels of formazan as compared to that in naïve rat samples ($p < .001$) (Fig. 3B). After 60 minutes of topical treatment with pentx-pca, the plantar muscle samples collected from CPIP rats yielded significantly greater levels of formazan as compared to the vehicle-treated group ($p = .03$) indicating recovery of oxygen dependent mitochondrial activity (Fig 21B).

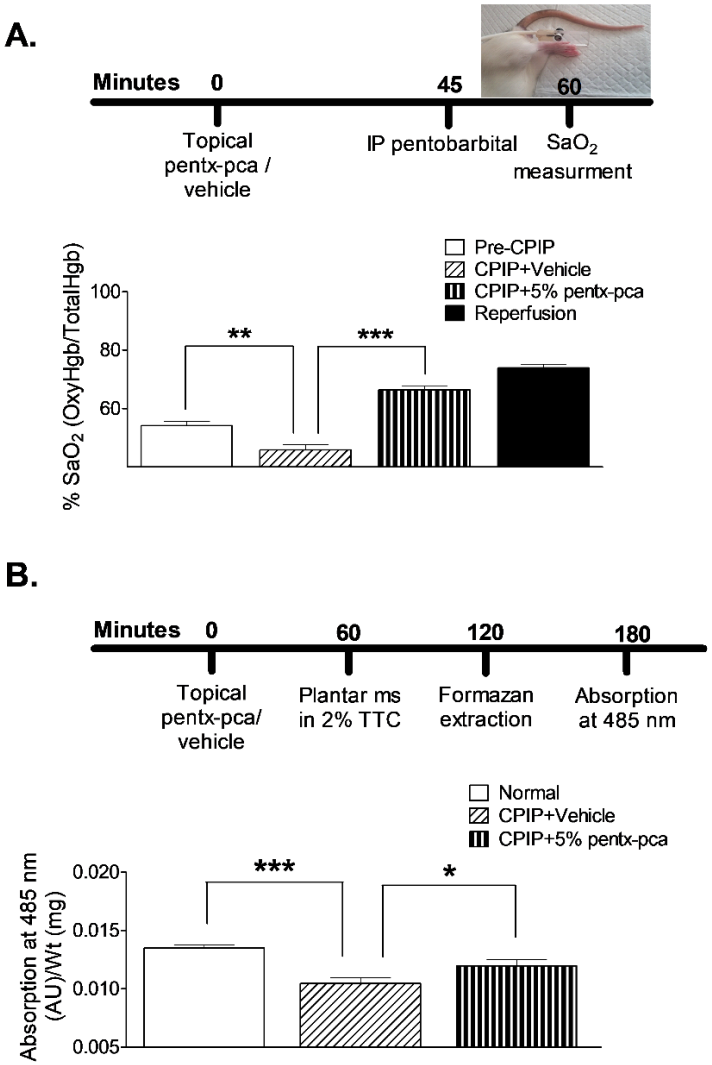

Figure 21. Topical treatment with pentx-pca alleviates local tissue hypoxia and plantar muscle mitochondrial dysfunction in CPIP rats.

(**A**) CPIP induced-decline in local tissue oxygen saturation (SaO_2) is reversed by topical treatment with an anti-allodynic (5% W/W) dose of pentx-pca (n = 10 rats). (**B**) Plantar muscle mitochondrial dysfunction exhibited by CPIP rats, as measured by a substrate based colometeric assay, is relieved after topical treatment with pentx-pca. (* $p < .05$, ** $p < .01$ and *** $p < .001$) (n = 6 rats).

CPIP rats exhibit mechanical allodynia for at least 4-weeks post-procedure and the mechanisms implicated to underly CPIP allodynia evolve with increasing time post-injury [4]. Accordingly, the anti-allodynic response of CPIP rats to topical pentx-pca was assessed at 1 week and 6 weeks post-CPIP. At 1-week post-CPIP, 5% W/W topical pentx-pca produced significant elevations ($p < .001$) in PWTs as compared to vehicle, while topical treatment with the formulation had no effects on PWTs in 6-week post-CPIP rats (Fig. 22A). PWT responses of CPIP rats to topical pentx-pca were significantly lower at 6 weeks, as compared to 1 week, post-injury ($p < .001$) (Fig 22A).

In a parallel experiment, the TTC assay was performed on plantar muscle samples from CPIP rats at 1 week and 6 weeks post-procedure (Fig 22B). The quantity of formazan produced by uninjured contralateral paw of the rats was used as a reference to estimate the CPIP-induced change in mitochondrial respiration. Plantar muscle samples obtained at 1-week post-CPIP exhibited a significant reduction in the quantity of formazan produced by the CPIP verses the contralateral hind paw, and this reduction declined significantly in plantar muscles samples collected at 6 weeks post-CPIP ($P < .01$) (Fig 22B). These results demonstrated that CPIP-induced impairment in plantar muscle mitochondrial respiration resolved over a period of 6 weeks post-procedure, a time point at which a significant decline in the anti-allodynic effect of pentx-pca also occurred (Fig. 22).

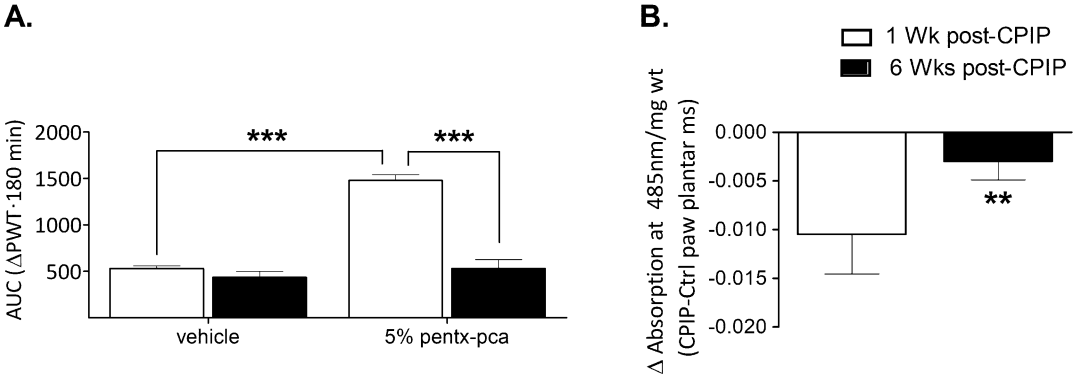

Figure 22. A parallel between the progression of anti-allodynic response to topical pentx-pca and plantar muscle mitochondrial dysfunction at 1-week and 6-weeks post-procedure in CPIP rats.

(**A**) Mechanical allodynia in CPIP rats is alleviated by topical treatment with pentx-pca at 1-week (n = 8 rats), but not 6-weeks (n = 6 rats) post-procedure. (**B**) The sizable difference in the oxygen dependent respiration of plantar muscle samples between the injured (CPIP) and contralateral (Ctrl) hind-paws of rats 1-week post-CPIP (n = 5 rats) significantly declines at 6-weeks after CPIP (n = 6 rats). (** $p < .01$ and *** $p < .001$).

6.6 Discussion

Tissue hypoxia and oxidative stress are prominent pathologic processes observed in the peripheral tissue of CRPS patients and can be locally targeted with topical vasodilatory and antioxidant agents to alleviate pain symptoms [1, 3, 9, 10, 42]. Although previous studies report the systemic and topical effects of vasoactive and antioxidants agents individually, the anti-allodynic effects of the two drug groups in combination have not been well explored [9-11]. In this study, we report the synthesis, anti-allodynic effects and mechanism of action of a novel co-crystal, pentx-pca, composed of the vascular agent, pentoxifylline, and the nutraceutical, protocatechuic acid. It is one of the three novel analgesic drug-nutraceutical salts and co-crystals synthesized and patented by our group [43]. The pentx-pca co-crystals exhibited anti-allodynic effects in CPIP rats that exceeded the potency of its individual constituents pentoxifylline and protocatechuic acid. Topically non-analgesic doses of protocatechuic acid added to pentoxifylline via co-crystallization, boosted the anti-allodynic effects of the co-crystal pentx-pca as compared to pentoxifylline on its own. Co-crystals, in contrast to plain drug mixtures, are held together by intermolecular interactions with resulting altered physiochemical properties that modify absorption, distribution and half-life, impacting drug efficacy and potency [15, 37, 44].

Our studies of the anti-allodynic mechanism of action of topical pentx-pca focused on assessing alterations in tissue oxygenation in the hind paw of drug-treated CPIP rats, and downstream changes in oxygen dependent mitochondrial function in the plantar muscle. Antioxidants, by preventing ROS formation, conserve available oxygen for proper tissue utilization and reduce the quenching of vasodilatory NO in the microvasculature [3,45]. Thus, our mechanism of action studies indirectly reflect the anti-oxidant effects of protocatechuic acid

Measures of local tissue oxygen saturation (SaO_2), which is a percentage of oxygenated hemoglobin in the vascular bed of a tissue, are reflections of regional microvascular caliber and perfusion with oxygen laden red blood cells [46-48]. Our results reveal a significant decline the SaO_2 of plantar hind paw tissue when values 5-7 days post-CPIP are compared to those pre-CPIP. This was expected given prior studies reporting evidence of microvascular dysfunction and plantar muscle ischemia in CPIP rats [4]. Patients with CRPS also exhibit impaired microcirculation, hypoxia and acidosis in their affected limb [49, 50]. The significant increase in plantar hind paw SaO_2, observed in the pentx-pca treated CPIP rats, is an indication of the effect of the drug in

improving microvascular perfusion and blood rheology. The elevated SaO_2 values occurred at a time point that coincided with maximal PWT responses to topical treatment with pentx-pca, implying local increase in tissue oxygenation contributed to the anti-allodynic effects of the drug. In previous studies, intraperitoneally administered pentoxifylline in doses that relieved allodynia in CPIP rats 1-week post-procedure, also improved laser Doppler flux measures of post-occlusive hyperemic response - an indicator of microvascular function and reserve [19]. The pentx-pca treatment-induced change in plantar hind-paw SaO_2 exceeded the pre-CPIP injury SaO_2 values of the rats. The degree of SaO_2 increment, however, remains physiologically plausible as it still falls below the reperfusion SaO_2 values of the rats measured during the reactive-hyperemic phase of the CPIP procedure following the removal of the O-ring tourniquet [8, 51].

Tissue oximetry using visible light spectroscopy provides a continuous, non-invasive and localized measurement of SaO_2 [52]. The depth of penetration of tissue by the light probe used is determined by the wavelength of light produced, with a wavelength range of 600-1600 nm penetrating up to a depth of 8-10 mm [53]. The SaO_2 vascular monitoring system used in our experiments had a laser probe that emitted wavelengths in the range of 400-700 nm, making possible collection of SaO_2 data in the plantar skin and possibly, the deeper lying plantar muscle. Regardless, microvascular dysfunction in the skin provides a surrogate measure of oxygenation status in deeper and less-accessible tissue [54]. Studies that reported skin hypoxia in CRPS patients were assumed to also imply hypoxia in the deeper lying tissue [3, 6].

In following experiments, plantar muscle samples of CPIP rats treated with topical pentx-pca were collected to determine oxygen-dependent mitochondrial respiration using the triphenyl tetrazolium chloride (TTC) assay. In this assay, the colourless solution of the tetrazolium compound, when incubated with freshly dissected muscle samples, converts into a red formazan dye indicating the reducing activity of the tissue's mitochondrial dehydrogenases that are responsible for cellular oxidative respiration [55]. Tissue hypoxia impairs mitochondrial integrity and the activity of its enzymes including dehydrogenases [56]. Moreover, reduced formazan production by ischemic muscle has been linked with morphologic evidences of mitochondrial injury on microscopy [57]. In previous studies, plantar muscle of CPIP rats collected between 2 and 7-days post-procedure exhibited reduced formazan production and increased lactate content, and this correlated to the severity of mechanical allodynia as measured by PWTs [4]. While plantar

muscle formazan production was also reduced in our vehicle-treated CPIP rats 7-days post procedure, topical treatment with anti-allodynic doses of pentx-pca significantly alleviated the reduction. This indicates that the increase in plantar tissue SaO_2 produced by pentx-pca positively impacts the function of oxygen-driven mitochondrial dehydrogenases. In addition to confirming increased oxygen availability on a cellular basis, such a recovery of mitochondrial enzyme function is also reported to be a signal for reduction in oxidative stress [58]. Excess ROS and oxidative stress are one of the pain-inducing mechanisms in CPRS patients and CPIP rats [4, 59]. Being partly composed of an antioxidant, the anti-allodynic effects seen in pentx-pca are likely to be a result of its combined anti-hypoxic and antioxidant effects.

The underlying pathophysiology of chronic pain in CRPS has been reported to evolve with time [60, 61]. While peripheral tissue hypoxia, inflammation and oxidative stress play a significant role in the early stages, these changes give way to central neuronal plasticity and reorganization causing bilateral and widespread hyperalgesia, later in the course of the disease [61]. This is also an observation in CPIP and other rat models of CRPS, where early post-injury mechanisms of pain genesis involve prominent microvascular dysfunction with capillary slow-flow/no-reflow, poor tissue perfusion, lactate and ROS accumulation, and consequent florid inflammatory response [4, 19, 62, 63]. At later CPIP time points, peripheral processes subside leaving behind the pain phenotype, mostly explained by central changes in sensory processing [64]. In line with this evidence, the TTC assay we performed on the plantar muscle of CPIP rats at 1 week and 6 weeks post-procedure showed the occurrence of a time-dependent resolution of mitochondrial enzyme dysfunction. Anti-allodynic response to topical pentx-pca showed a similar pattern with robust anti-allodynic effects seen at 1 week post-CPIP when microvascular compromise and tissue hypoxia also existed. Anti-allodynic effects disappeared 6 weeks post-procedure, coinciding with the resolution of the peripheral vascular processes. Previous studies with systemic administration of pentoxifylline have revealed a similarly time dependent change on its effects on mechanical allodynia and post-occlusive hyperemic response [19]. While IP pentoxifylline restored the impaired microvascular function in early CPIP rats, late CPIP rats failed to show anti-allodynic response and no longer exhibited impairment in post-occlusive hyperemic response. These evidences identify ischemic tissue hypoxia and the resulting compromise of mitochondria respiration in the plantar muscle to be the processes impacted by topical pentx-pca to alleviate mechanical allodynia in CPIP rats.

In conclusion, this study presents anti-allodynic and tissue restorative effects of pentx-pca, a novel analgesic compound of enhanced potency, synthesized via co-crystallization of the vasoactive agent pentoxifylline and the natural antioxidant protocatechuic acid. This analgesic and mechanism of action data for the co-crystal provide strong evidence for the potential of targeting peripheral processes like tissue hypoxia to alleviate the pain symptoms of CRPS. Moreover, the study demonstrates tissue hypoxia in CRPS can be sufficiently impacted with local treatments via topical application to produce effective analgesia greatly minimizing side effects that could result from systemic administration.

6.7 References

1. Coderre TJ, Bennett GJ. A hypothesis for the cause of complex regional pain syndrome-type I (reflex sympathetic dystrophy): pain due to deep-tissue microvascular pathology. Pain Medicine. 2010;11(8):1224-38.
2. Coderre TJ. Complex regional pain syndrome: what's in a name? J Pain. 2011;12(1):2-12.
3. Koban M, Leis S, Schultze-Mosgau S, Birklein F. Tissue hypoxia in complex regional pain syndrome. Pain. 2003;104(1-2):149-57.
4. Laferrière A, Millecamps M, Xanthos DN, Xiao WH, Siau C, de Mos M, et al. Cutaneous tactile allodynia associated with microvascular dysfunction in muscle. Mol Pain. 2008;4:49.
5. Heerschap A, Hollander JAD, Reynen H, Goris RJA. Metabolic changes in reflex sympathetic dystrophy: A 31P NMR spectroscopy study. Muscle Nerve. 1993;16(4):367-73.
6. Birklein F, Weber M, Neundörfer B. Increased skin lactate in complex regional pain syndrome: Evidence for tissue hypoxia? Neurology. 2000;55(8):1213-5.
7. Birklein F, Weber M, Ernst M, Riedl B, Neundorfer B, Handwerker HO. Experimental tissue acidosis leads to increased pain in complex regional pain syndrome (CRPS). Pain. 2000;87(2):227-34.
8. Coderre TJ, Xanthos DN, Francis L, Bennett GJ. Chronic post-ischemia pain (CPIP): a novel animal model of complex regional pain syndrome-Type I (CRPS-I; reflex sympathetic dystrophy) produced by prolonged hindpaw ischemia and reperfusion in the rat. Pain. 2004;112(1–2):94-105.
9. Russo MA, Santarelli DM. A Novel Compound Analgesic Cream (Ketamine, Pentoxifylline, Clonidine, DMSO) for Complex Regional Pain Syndrome Patients. Pain Pract. 2016;16(1):E14-E20.
10. Ragavendran JV, Laferriere A, Xiao WH, Bennett GJ, Padi SS, Zhang J, et al. Topical combinations aimed at treating microvascular dysfunction reduce allodynia in rat models of CRPS-I and neuropathic pain. J Pain. 2013;14(1):66-78.
11. Laferriere A, Abaji R, Tsai CY, Ragavendran JV, Coderre TJ. Topical combinations to treat microvascular dysfunction of chronic postischemia pain. Anesth Analg. 2014;118(4):830-40.
12. Xanthos DN, Coderre TJ. Sympathetic vasoconstrictor antagonism and vasodilatation relieve mechanical allodynia in rats with chronic postischemia pain. The Journal of Pain. 2008;9(5):423-33.
13. Davis K, Treede R, Raja SN, Meyer R, Campbell JN. Topical application of clonidine relieves hyperalgesia in patients with sympathetically maintained pain. Pain. 1991;47(3):309-17.

14. Groeneweg G, Niehof S, Wesseldijk F, Huygen F, Zijlstra FJ. Vasodilative effect of lsosorbide dinitrate ointment in complex regional pain syndrome type 1. Clin J Pain. 2008;24(1):89-92.
15. Vishweshwar P, McMahon JA, Bis JA, Zaworotko MJ. Pharmaceutical co-crystals. J Pharm Sci. 2006;95(3):499-516.
16. Aitipamula S, Banerjee R, Bansal AK, Biradha K, Cheney ML, et al. Polymorphs, Salts, and Cocrystals: What's in a Name? Cryst Growth Des. 2012;12(5):2147-52.
17. Ward A, Clissold SP. Pentoxifylline. A review of its pharmacodynamic and pharmacokinetic properties, and its therapeutic efficacy. Drugs. 1987;34(1):50-97.
18. Magnusson M, Bergstrand IC, Bjorkman S, Heijl A, Roth B, Hoglund P. A placebo-controlled study of retinal blood flow changes by pentoxifylline and metabolites in humans. Br J Clin Pharmacol. 2006;61(2):138-47.
19. Ragavendran JV, Laferriere A, Khorashadi M, Coderre TJ. Pentoxifylline reduces chronic post-ischaemia pain by alleviating microvascular dysfunction. Eur J Pain. 2014;18(3):406-14.
20. Bowton DL, Stump DA, Prough DS, Toole JF, Lefkowitz DS, Coker L. Pentoxifylline increases cerebral blood flow in patients with cerebrovascular disease. Stroke. 1989;20(12):1662-6.
21. Samlaska CP, Winfield EA. Pentoxifylline. J Am Acad Dermatol. 1994;30(4):603-21.
22. Ehrly AM, Saegerlorenz K. Effect of pentoxifylline on the muscle-tissue oxygen-pressure of claudicants after pedoergometric exercise. Vol. 37(5), Angiology. NY:Westminster Publ inc; c1986. p. 398-398
23. Salhiyyah K, Forster R, Senanayake E, Abdel-Hadi M, Booth A, Michaels JA. Pentoxifylline for intermittent claudication. Cochrane Database Syst Rev. 2015; 9(9):Cd005262.
24. Iijima, M. Changes in gas content of blood and CSF in CVD patients, especially those at the chronic stage of CVD. Proceeding of the 3rd General Meeting of the Japanese Society of Stroke Report, 1975.
25. Kaznelson L, et al, Experience with pentoxifylline in various vascular disorders of fundus oculi. Pharmatherapeutica. 1978: 2:146-149.
26. Rendell M, Bamisedun O. Skin blood flow and current perception in pentoxifylline-treated diabetic neuropathy. Angiology. 1992;43(10):843-51.
27. Semaming Y, Pannengpetch P, Chattipakorn SC, Chattipakorn N. Pharmacological properties of protocatechuic Acid and its potential roles as complementary medicine. Evid Based Complement Alternat Med. 2015;2015:593902.
28. Yüksel M, Yıldar M, Başbuğ M, Çavdar F, Çıkman Ö, Akşit H, et al. Does protocatechuic acid, a natural antioxidant, reduce renal ischemia reperfusion injury in rats? Ulus Travma Acil Cerrahi Derg. 2017;23(1):1-6.
29. Jiang L, Zeng H, Ni L, Qi L, Xu Y, et al. HIF-1α Preconditioning Potentiates Antioxidant Activity in Ischemic Injury: The Role of Sequential Administration of Dihydrotanshinone I and Protocatechuic Aldehyde in Cardioprotection. Antioxid Redox Signal. 2019;31(3):227-42.
30. Tang XL, Liu JX, Dong W, Li P, Li L, et al. Cardioprotective effect of protocatechuic acid on myocardial ischemia/reperfusion injury. J Pharmacol Sci. 2014;125(2):176-83.
31. Yin X, Zhang X, Lv C, Li C, Yu Y, Wang X, et al. Protocatechuic acid ameliorates neurocognitive functions impairment induced by chronic intermittent hypoxia. Sci Rep. 2015;5:14507.
32. Krzysztoforska K, Mirowska-Guzel D, Widy-Tyszkiewicz E. Pharmacological effects of protocatechuic acid and its therapeutic potential in neurodegenerative diseases: Review on the basis of in vitro and in vivo studies in rodents and humans. Nutr Neurosci. 2019;22(2):72-82.

33. Thakare VN, Dhakane VD, Patel BM. Attenuation of acute restraint stress-induced depressive like behavior and hippocampal alterations with protocatechuic acid treatment in mice. Metab Brain Dis. 2017;32(2):401-13.
34. Shin S, Cho SH, Park D, Jung E. Anti-skin aging properties of protocatechuic acid in vitro and in vivo. J Cosmet Dermatol. 2019.
35. Stepanovs D, Jure Mr, Kuleshova LN, Hofmann DW, Mishnev AJCG, Design. Cocrystals of pentoxifylline: in silico and experimental screening. Cryst. Growth Des. 2015;15(8):3652-60.
36. Sinha AS, Maguire AR, Lawrence SE. Cocrystallization of nutraceuticals. Crystal Growth & Design. 2015;15(2):984-1009.
37. Steed JW. The role of co-crystals in pharmaceutical design. Trends Pharmacol Sci. 2013;34(3):185-93.
38. Duggirala NK, Perry ML, Almarsson Ö, Zaworotko MJ. Pharmaceutical cocrystals: along the path to improved medicines. Chem Commun. 2016;52(4):640-55.
39. Crimi E, Ignarro LJ, Napoli C. Microcirculation and oxidative stress. Free Radic Res. 2007;41(12):1364-75.
40. Cooper D, Stokes KY, Tailor A, Granger DN. Oxidative stress promotes blood cell-endothelial cell interactions in the microcirculation. Cardiovasc Toxicol. 2002;2(3):165-80.
41. Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL. Quantitative assessment of tactile allodynia in the rat paw. J Neurosci Methods. 1994;53(1):55-63.
42. Kwak KH, Han CG, Lee SH, Jeon Y, Park SS, Kim SO, et al. Reactive oxygen species in rats with chronic post-ischemia pain. Acta Anaesthesiol Scand. 2009;53(5):648-56.
43. Coderre TJ, Fulas OA, Laferriere A, Ayoub G, Friscic T, Gandrath D, Mottillo C. Pharmaceutical salts/co-Crystals of pentoxifylline, clonidine & linsidomine with caffeic, protocatechuic or α-lipoic acid & use thereof for treatment of pain. 2019; PCT/CA2019/050719.
44. Miroshnyk I, Mirza S, Sandler N. Pharmaceutical co-crystals-an opportunity for drug product enhancement. Expert Opin Drug Deliv. 2009;6(4):333-41.
45. Hermes-Lima M, Storey JM, Storey KB. Antioxidant defenses and animal adaptation to oxygen availability during environmental stress. In: Storey KB, Storey JM, eds. Vol. 2. Cell and Molecular Responses to Stress. Amesterdam (BV): Elsevier Science; 2001;2:263-87.
46. Intaglietta M, Johnson PC, Winslow RM. Microvascular and tissue oxygen distribution. Cardiovasc Res. 1996;32(4):632-43.
47. Pittman RN. Oxygen transport in the microcirculation and its regulation. Microcirculation. 2013;20(2):117-37.
48. Lipowsky HH. Microvascular rheology and hemodynamics. Microcirculation. 2005;12(1):5-15.
49. Groeneweg G, Huygen FJ, Coderre TJ, Zijlstra FJ. Regulation of peripheral blood flow in complex regional pain syndrome: clinical implication for symptomatic relief and pain management. BMC Musculoskeletal Disorders. 2009;10:116.
50. Bellingham GA, Smith RS, Morley-Forster P, Murkin JM. Use of near infrared spectroscopy to detect impaired tissue oxygen saturation in patients with complex regional pain syndrome type 1. Can J Anaesth. 2014;61(6):563-70.
51. Tomiyama H, Yamashina A. Non-invasive vascular function tests: their pathophysiological background and clinical application. Circ J. 2010;74(1):24-33.

52. Benaron DA, Parachikov IH, Friedland S, Soetikno R, Brock-Utne J, van der Starre PJ, et al. Continuous, noninvasive, and localized microvascular tissue oximetry using visible light spectroscopy. Anesthesiology. 2004;100(6):1469-75.
53. Douplik A, Saiko G, Schelkanova I, Tuchin VV. The response of tissue to laser light. In: Jelínková H, ed. Lasers for Medical Applications. Philadelphia: Woodhead Publishing; c2013. p. 47-109.
54. Kuliga KZ, McDonald EF, Gush R, Michel C, Chipperfield AJ, Clough GF. Dynamics of microvascular blood flow and oxygenation measured simultaneously in human skin. Microcirculation. 2014;21(6):562-73.
55. Belkin M, Brown RD, Wright JG, LaMorte WW, Hobson RW. A new quantitative spectrophotometric assay of ischemia-reperfusion injury in skeletal muscle. Am J Surg. 1988;156(2):83-6.
56. Chandel NS, Budinger GR, Schumacker PT. Molecular oxygen modulates cytochrome c oxidase function. J Biol Chem. 1996;271(31):18672-7.
57. Blebea J, Kerr JC, Shumko JZ, Feinberg RN, Hobson RW. Quantitative histochemical evaluation of skeletal muscle ischemia and reperfusion injury. J Surg Res. 1987;43(4):311-21.
58. Stepien KM, Heaton R, Rankin S, Murphy A, Bentley J, Sexton D, et al. Evidence of Oxidative Stress and Secondary Mitochondrial Dysfunction in Metabolic and Non-Metabolic Disorders. J Clin Med. 2017;6(7).
59. Tan EC, Janssen AJ, Roestenberg P, van den Heuvel LP, Goris RJ, Rodenburg RJ. Mitochondrial dysfunction in muscle tissue of complex regional pain syndrome type I patients. Eur J Pain. 2011;15(7):708-15.
60. Bruehl S. Complex regional pain syndrome. Br Med J. 2015;351:h2730.
61. Birklein F, Schlereth T. Complex regional pain syndrome-significant progress in understanding. Pain. 2015;156 Suppl 1:S94-103.
62. Wei T, Guo T-Z, Li W-W, Kingery WS, Clark JD. Acute versus chronic phase mechanisms in a rat model of CRPS. J Neuroinflammation. 2016;13:14.
63. Guo TZ, Wei T, Huang TT, Kingery WS, Clark JD. Oxidative stress contributes to fracture/cast-induced inflammation and pain in a rat model of complex regional pain syndrome. J Pain. 2018;19(10):1147-56.
64. Laferriere A, Pitcher MH, Haldane A, Huang Y, Cornea V, Kumar N, et al. PKMzeta is essential for spinal plasticity underlying the maintenance of persistent pain. Mol Pain. 2011;7:99.

7. Preamble to MANUSCRPT 4:

Topical anti-hypoxic agents as potential analgesics for the treatment of patients with PNP

Extensive evidence has so far accumulated for the role of microvascular dysfunction and tissue hypoxia in the genesis and maintenance of peripheral hypersensitivity and pain in PNP [37, 101, 351, 352]. However, the targeting of these pathologic processes to develop viable therapeutic interventions for PNP has not been thoroughly implemented. Only a handful of local vasodilatory treatments have achieved the stages of clinical testing for painful diabetic neuropathy [170, 220, 221, 353]. With the existing need for more effective and tolerable analgesics for PNP, this avenue of therapy warrants further exploration.

Effective alleviation of peripheral nerve hypoxia in PNP requires the use of agents that can compensate for the multifactorial structural and functional pathologic changes that occur in this condition. This can be achieved with the use of a combination of agents that increase thermoregulatory blood flow through actions on the vascular smooth muscle and endothelium. This ultimately enhances nutritive capillary blood flow which increases tissue perfusion with oxygen-delivering RBC. The work described in the preceding manuscripts, included in this thesis, and previous publications from our laboratory provide the preclinical evidence for the viability of this approach in developing effective analgesics for PNP [215].

One of the topical combinations of anti-hypoxic agents, pentoxifylline and clonidine, had progressed into the stage of testing on healthy volunteers after it demonstrated robust analgesic effects in animal models of PNP [215, 228]. The healthy volunteer study, that involved the use of an experimental surrogate for neuropathic pain, revealed the analgesic efficacy of this topical combination [228]. In this study, intraepidermal capsaicin was administered on the forearm of healthy subjects and this was followed by the application of a tourniquet block proximal to the injection site. The resulting post-capsaicin tourniquet-induced pain resembled neuropathic pain and had both a superficial burning and a deep cramping quality. The experimental treatment also produced punctate and dynamic mechanical allodynia. The study involved subject groups for the investigation of the topical analgesic effect of single-drug treatments with clonidine and pentoxifylline, and both low-dose and high-dose combinations of the two drugs. The outcome

measures used were primarily VAS rating of pain intensity and dynamic mechanical allodynia, and secondarily area of punctate mechanical allodynia. While the lower dose combination and the single agent treatments produced some changes in the outcome measures, the high dose combination of pentoxifylline and clonidine exhibited superior analgesic effects in comparison. Singular administration of topical clonidine, in the form of a gel, has been previously demonstrated to reduce the level of foot pain in patients with painful diabetic neuropathy [170]. This current evidence from the healthy volunteer study indicated a potential for greater analgesic effects by combining pentoxifylline and clonidine in a topical formulation. The next steps have been taken to advance the high-dose topical combination of clonidine and pentoxifylline for a clinical study on a cohort of post-traumatic neuropathic pain patients. The following manuscript details the protocol being used for this follow up phase-II clinical trial. A manuscript describing the protocol for this investigation has been included in this thesis, in lieu of the final report, since the recruitment of patient for the completion of this trail has been indeterminately stalled due to the COVID-19 pandemic.

8. MANUSCRIPT 4: The effect of a topical combination of clonidine & pentoxifylline on post-traumatic neuropathic pain patients:

Study protocol for a randomized, double-blind placebo-controlled trial

Oli Abate Fulas [1], André Laferriere [1], D. Mark A Ware [2], Yoram Shir [2], Terence J. Coderre [1*]

Department of Anesthesia[1]; Alan Edwards Pain Management Unit, McGill University Health Centre[2]

8.1 Contribution of authors

Prof Terence J Coderre and Dr. Mark A Ware designed the study. I prepared the first draft of the manuscript and performed the recommended revisions. Andre Laferriere, Dr. Mark A Ware, Prof Terence J Coderre and Dr. Yoram Shir revised the manuscript.

In the proceedings of the trial, I and Andre Laferriere are the study co-ordinators. Dr. Yoram Shir is the study physician and Prof Terence J Coderre the study sponsor-investigator.

8.2 Abstract

8.2.1 Background

First-line pharmacotherapy for neuropathic pain entails the use of systemic antidepressants and anticonvulsants. These drugs are not optimally effective, and poorly tolerable, especially for older patients with comorbid conditions. Given the high number of such patients, there is a need for a greater repertoire of safer and more effective analgesics. Clonidine and pentoxifylline are vasodilator agents that work synergistically to enhance tissue perfusion and oxygenation. The topical administration of these drugs, individually and in combination, has shown anti-nociceptive properties in rodent models of neuropathic pain. A topically-administered combination of clonidine and pentoxifylline also effectively reduced the intensity of both spontaneous and evoked pain in healthy volunteers with experimentally-induced neuropathic pain. The next step in advancing this formulation to clinical use is the undertaking of a phase-II clinical study to assess its efficacy and safety in neuropathic pain patients.

8.2.2 Methods/Design

This is a study protocol for a randomized, double-blind, placebo-controlled, phase II clinical trial with a cross-over design. It is a single-centered, 5-week study that will enroll a total of 32 patients with post-traumatic peripheral neuropathic pain. Patients will be treated topically with either a combination of clonidine and pentoxifylline or placebo for a period of two weeks each, in randomly assigned order across patients, with an intervening washout period of one week. The primary outcome measures of the study are the intensity of spontaneous pain recorded daily in a pain diary with a visual analog scale, and the degree of mechanical allodynia evoked by a brush stimulus. The secondary outcome measures of the study include scores of pain relief and change in the area of punctate hyperalgesia. This trial has been prospectively registered with ClinicalTrials.gov on November 01, 2017. ClinicalTrials.gov Identifier: NCT03342950.

8.2.3 Discussion

The analgesic use of topical treatment with clonidine and pentoxifylline in combination has not been investigated in post-traumatic neuropathic pain. This study could generate the first evidence for the efficacy and safety of the formulation in alleviating pain in patients with neuropathic pain. Furthermore, this trial will provide objective grounds for the investigation of other agents that enhance tissue oxygenation in the topical treatment of peripheral neuropathic pain.

8.2.4 Trial registration

This trial has been registered with ClinicalTrials.gov owned by NIH's U.S. National Library of Medicine.

ClinicalTrials.gov Identifier: NCT03342950

Date of registration: November 01, 2017 (The trial was prospectively registered.)

8.2.5 Protocol version and identifiers

This is protocol version 5, dated June 2018. McGill University Health Center (MUHC) Research Ethics Board (REB) identification number: TTNP 2018-3906.

8.3 Background

Peripheral neuropathic pain is a chronic pain condition that is a direct outcome of damage to the peripheral nerves [1]. The damage could result from a myriad of etiologies like physical trauma, metabolic dysfunctions like diabetes mellitus or toxic effects of chemotherapeutic agents [2]. The patients suffer from debilitating pain of burning, tingling or electric shock-like quality, along with hypersensitivity to touch, pressure and changes in temperature [2]. Although the pathophysiology of peripheral neuropathic pain involves an interplay between neuronal, immune, neuroimmune, vascular and metabolic factors, first-line pharmacological analgesics in clinical use are mostly antidepressants and anticonvulsants that solely target modulatory processes in the central nervous system [3]. In addition to being sub-optimally therapeutic, these agents have significant side effects often making them intolerable especially to patients of older age and co-morbid conditions [4, 5]. There is an indisputable need for effective and safe analgesic options for patients with peripheral neuropathic pain.

Peripheral neuropathic pain of varying etiologies involves microvascular changes that result in ischemic hypoxia of the affected nerves. Functionally, deficits have been reported in the endothelial production of vasodilatory nitric oxide (NO), which normally acts to counteract the basal vasoconstrictive sympathetic tone [6]. Structural changes to the endoneurial vessels have also been observed, including endothelial cell layer swelling and capillary basement membrane thickening [7, 8]. These microvascular changes impair endoneurial nutritive perfusion and oxygenation, which can directly impact the excitability of the peripheral nerves [9]. The tactile allodynia and thermal hyperalgesia exhibited by neuropathic rats are significantly correlated to the extent of endoneurial hypoxia in the affected peripheral nerves [10, 11]. Therefore, treatments aimed at enhancing tissue oxygenation by increasing arterial and capillary flow may effectively relieve symptoms in patients with neuropathic pain.

Microvascular perfusion is under the regulation of vasodilatory molecules produced by the endothelium like, NO, and the vasoconstrictive transmitter norepinephrine, which is released from sympathetic post-ganglionic neurons [12, 13]. NO's vasodilator effect occurs by the augmentation of vascular smooth muscle cyclic mononucleotide level that in turn inhibits calcium influx and smooth muscle contraction, a mechanism that is counterbalanced by the activity of phosphodiesterase enzymes that degrade cyclic mononucleotides [14]. Drugs with phosphodiesterase inhibitor activity, including pentoxifylline, produce vasodilation and enhance

blood flow [15],[16]. Primarily indicated for peripheral arterial disease, pentoxifylline inhibits a broad isoform of phosphodiesterase enzymes resulting in enhanced blood flow, reduced platelet aggregation, decreased blood viscosity and increased red blood cell flexibility, all of which aid in alleviating poor microvascular perfusion [16-18]. On the other hand, microvascular perfusion is further degraded by the vasoconstrictive effect of sympathetic activity when norepinephrine binds to α-adrenergic receptors on vascular smooth muscle cells [19]. Clonidine is a drug that counteracts sympathetic vasoconstriction by activating presynaptic α_2-adrenergic receptors and reducing the release of norepinephrine both centrally and at peripheral sympathetic nerve endings [20]. It has also been reported to activate endothelial α_2-receptors resulting in increased release of NO and subsequent vasodilation [21]. Therefore, clonidine and pentoxifylline act via complementary mechanisms to increase microvascular caliber, blood flow and tissue perfusion.

In neuropathic pain, the effects of microvascular dysfunction are most adverse on the peripheral nerve's distal endings in the skin, also called intraepidermal nerve fibers [22, 23]. Thus, topical microvascular-targeted local and cutaneous interventions that are effective in combating poor nutritive perfusion of the peripheral nerve endings are beneficial. The topical route allows the delivery of effective drugs in high concentrations at the local target site while avoiding undesired systemic and adverse effects [24]. Prior studies by our group have demonstrated that topical combination of α-adrenergic receptor agonists and phosphodiesterase inhibitors effectively alleviate tactile allodynia and microvascular dysfunction in rodent models of post-traumatic, diabetic and chemotherapy-induced neuropathic pain [25]. α_2-Adrenergic receptor agonists have also been used topically to alleviate chronic pain in humans [26, 27]. Moreover, as an initial step in determining the translatability of topical combination of clonidine and pentoxifylline as analgesics, we have tested the effect of the formulation in healthy volunteers in an experimentally-induced surrogate for neuropathic pain using a double-blind randomized and controlled design [28]. In the study, an experimental model of neuropathic pain was induced by tourniquet application following intradermal capsaicin injection. Subsequent topical treatment with the combination of clonidine and pentoxifylline significantly reduced the superficial and deep "neuropathic-like" pain reported by the volunteers. Moreover, areas of dynamic allodynia and mechanical hyperalgesia were significantly reduced to an extent that exceeded the published effectiveness of most analgesics, including opioids and gabapentinoids [29, 30]. The current protocol outlines the second translational investigation to determine the effectiveness and safety

of the same topical combination of clonidine and pentoxifylline in relieving pain in patients with post-traumatic neuropathic pain.

8.4 Objectives

This protocol aims to examine the analgesic effects of a topical combination of clonidine and pentoxifylline in patients with post-traumatic neuropathic pain. Using a phase-II proof-of-concept clinical trial design, the focus is on providing preliminary evidence for the analgesic efficacy and safety of the treatment using a small and well-defined patient population. More specifically, the tests employed are designed to measure the magnitude of relief of spontaneous pain using daily recordings of pain level with visual analog scale (VAS)-based pain diaries. To estimate the effect of the treatment on evoked pain, measures for the severity of dynamic mechanical hyperalgesia and area of punctate hyperalgesia will also be used.

8.5 Methods

8.5.1 Trial design

This trial uses a randomized, double-blind, placebo-controlled cross-over study design. In this counterbalanced cross-over study, enrolled patients will be treated with active drug and placebo for periods of 2 weeks each, with an intervening 1-week wash-out period (Figure 23).

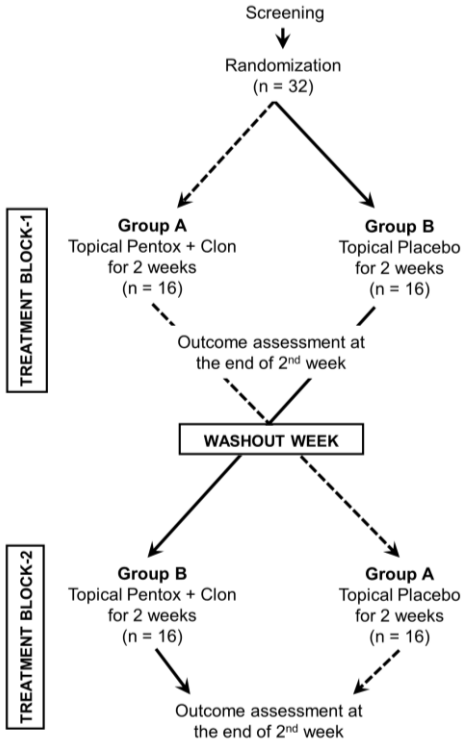

Figure 23. Study flow chart.

8.5.2 Study setting

This is a single-center study taking place in the Alan Edwards Pain Management Unit at the Montreal General Hospital, Montreal, Quebec. The study has been authorized by the McGill University Health Center Research Ethics Board (TTNP: 2018-3906) and Health Canada (control number: 201880). Moreover, it is registered with NIH's US National Library of Medicine database: ClinicalTrials.gov (Identifier: NCT03342950).

8.5.3 Study Process

A summary of the study process is outlined in Table 2. The first screening visit will entail: obtaining informed consent; reviewing patients' medical history, including the patients' current medications; performing sensory tests for mechanical hypersensitivity; collecting blood samples for liver and kidney function; and obtaining urine samples from pre-menopausal women for a pregnancy test. Patients will be provided with a pain diary to record their daily pain intensity for the seven days that follow the screening visit. Patients with normal laboratory test results of liver and kidney function will be contacted over the phone. After the assessment of pain diaries for pain intensity, patients that meet the eligibility requirements of the study (see Section 8.5.4), will be provided with study drugs according to the treatment regimen assigned for the first two weeks of the trial. Regular medications used before enrolment into the study will be continued.

Throughout each treatment period and during the wash-out, patients will be asked to record their average pain scores in a pain diary, once daily in the evening. At the beginning and end of each treatment block, sensory testing will be performed to determine the degree of dynamic mechanical allodynia and area of punctate hyperalgesia. At the end of each treatment block, patients will be asked to score the degree of their overall pain relief. Adverse events will also be captured at the end of each treatment block and after the washout week.

Table 2. Study process

		STUDY PERIOD					
			Screening week	Rx Block-1	Washout week	Rx Block-2	Close -out
	Time point (Weeks)	-1	0	2	3	5	
ENROLLMENT	Informed consent	◆					
	Blood tests *	◆					
	Urine pregnancy test	◆					
	Medical history & physical exam	◆					
	Vital signs	◆		◆	◆	◆	
Rx	Topical pentox + clon		◆	◆	◆	◆	
	Placebo		◆	◆	◆	◆	
OUTCOME	VAS on pain diary	◆	◆	◆	◆	◆	
	Sensory testing (DMA & PHA)	◆		◆	◆	◆	
	Pain relief score			◆		◆	
	Adverse events			◆	◆	◆	
	Database completion & lockdown						◆

Rx: treatment, pentx: pentoxifylline, clon: clonidine, DMA: dynamic mechanical allodynia, PHA: area of punctate hyperalgesia, * Blood tests: constitute liver transaminases, serum creatinine, and blood urea nitrogen

8.5.4 Eligibility criteria

Patients will have to fulfill all the following inclusion criteria:

1. Female or male patients, aged 18-70 yrs
2. An average spontaneous pain level of at least 4 on an 11-point numerical rating pain score (0 = no pain, 10 = worst pain possible) on at least 3 days during the week before the study
3. The existence of tactile allodynia, as a sign of neuropathic pain, following a traumatic peripheral nerve injury
4. Ability to communicate in English or French
5. Willing and able to sign an informed consent
6. Stable pain disease with no anticipated change in treatment for the next 5 weeks.
7. Female subjects of childbearing age must agree to use an effective method of contraception during the study period. Female subjects who utilize a hormonal contraceptive as one of their birth control methods must have consistently used the same method for at least three months

before study drug dosing. Effective methods of contraception include double barrier methods [male condom (with spermicide), female condom (with spermicide), diaphragm with spermicide, cervical cap with spermicide or sponge with spermicide] or hormonal methods [oral contraceptives (either combined or progesterone only), injectable progesterone, subdermal contraceptive implant, transdermal contraceptive patch, contraceptive vaginal ring].

Any of the following conditions will exclude patients from this study:

1. Diabetes mellitus necessitating antihyperglycemic treatment or any other endocrine disease
2. Any liver disease, resulting in liver transaminase enzyme levels greater than three times the normal values
3. Chronic renal failure or kidney disease, resulting in serum creatinine levels > 133 µmol/L
4. Hypertension or taking of anti-hypertensive medication
5. Malignant disease or taking of chemotherapeutic agents
6. Known diagnosis of angina pectoris, arrhythmias, congestive heart failure or peripheral arterial disease
7. Pregnancy or breastfeeding. Female patients of childbearing age must have a negative urine pregnancy test
8. Known allergic reaction to clonidine or pentoxifylline
9. Presence of a medical condition known to affect peripheral circulation (intermittent claudication, peripheral arterial disease, Raynaud's syndrome)
10. Medication that can potentially interact with clonidine or pentoxifylline [cardiovascular drugs such as angiotensin-converting enzyme inhibitors, alpha-blockers (prazosin, terazosin or doxazosin), beta-blockers (atenolol, metoprolol, propranolol), neuroleptics (butyrophenones, phenothiazines, thioxanthenes), calcium channel blockers (verapamil, diltiazem) and non-cardiovascular drugs such as diuretics, thyroxine, monoamine oxidase inhibitors, and selective serotonin reuptake inhibitors, as well as vitamin K antagonists and blood thinners like warfarin]
11. Any medical condition that might be impacted by clonidine or pentoxifylline, such as cardiovascular disease, cardiac rhythm disorders (sinus node dysfunction, atrial-ventricular blockade or other conduction abnormalities), orthostatic regulation disturbances, disorders of cerebral perfusion
12. A cerebral and/or retinal hemorrhage (in the last 5 years)

8.5.5 Interventions

All participants will be randomized to treatment with either active drug or placebo in one of the two 2-week long treatment blocks of the study. The active drug is an anhydrous topical solution containing 0.1% weight/volume (W/V) clonidine and 5% (W/V) pentoxifylline. The placebo is a blank version of the anhydrous solution with constituents identical with the active drug except omitting the therapeutic agents, i.e. clonidine and pentoxifylline. The stability of the topical solutions at room temperature has been ascertained with mass spectrometric studies done upon the request of Health Canada. The exact composition of the active drug and placebo is as outlined in Table 3.

Both the active drug and placebo solutions are colorless and will be packaged in identical Topi-Pump® dose-metered applicators that dispense 1 ml of topical solution with each pump. Participants will be instructed to apply one pump or 1 ml of the topical solution three times daily on an eight-hourly basis. They will also be asked to return the emptied pumps after use so adherence to treatment can be monitored.

Table 3. Composition of 100 ml of the active drug topical solution with 0.1 % (W/V) clonidine and 5% (W/V) pentoxifylline:

Solid Ingredients		**Amount (g)**
1	Clonidine	0.1
2	Pentoxifylline	5.0
Liquid Ingredients		**Amount (ml)**
3	Anhydrous ethanol	6.2
4	Polyethylene glycol 400	18.9
5	Oleyl alcohol	50.8
6	Propylene glycol	18.9

8.5.6 Outcomes

Primary outcome measures of the study are as follows:

1) The average daily pain intensity level is recorded by the patient at home in an REB approved pain diary that contains a visual analog scale (VAS) displaying the words “no pain” and “worst pain possible” on the left and right of a 100 mm line, respectively.

2) The degree of dynamic mechanical allodynia (DMA) is determined by stroking the most painfully sensitive area of the skin once at a velocity of 1-2 cm/s with a soft brush (bristle length

by width: 20 x 15 mm). The patient then indicates the amount of pain evoked on an REB approved data collection sheet with a 100-mm VAS. This test will be stopped immediately at the request of the patient if it is too painful.

Secondary outcome measures of the study are as follows:

1) Pain relief scores are recorded on a 6-point categorical pain relief scale (0: worse pain to 5: complete pain relief).

2) Area of punctate hyperalgesia (PHA) is measured using punctate stimulation with a 26 g von Frey monofilament (Semmes Weinstein Monofilament Kit; Stoelting, Wood Dale, IL). Stimulation commences from outside the sensitive area toward the center of the sensitive area, along eight approximately evenly spaced radii, while asking the subject to report when the sensation becomes unpleasant or frankly painful. The points at which the sensations change will be marked, and the markings gently transferred onto a lightweight transparent plastic sheet. The marked sheet is subsequently photographed with a ruler to calibrate the number of pixels per unit length during image analysis and area calculation.

8.5.7 Sample size

Thirty-two patients will be recruited for this trial. This number of patients is chosen based on the assumption that during the active topical drug treatment period patients will experience a reduction of either of the primary outcome measures (spontaneous pain or intensity of DMA) of 20 mm or more on the 100 mm VAS (standard deviation assumed: 25, i.e. Cohen's d=0.8, alpha=0.025 corrected for multiple testing; power=95%). Based on a power calculation for two groups using a cross-over design, the estimated required sample size is 27, and we expect that a sample size of 32 is sufficient to detect changes in pain levels even if up to 18% of patients do not complete the study.

8.5.8 Recruitment & Informed consent

The thirty-two patients, females and males, with post-traumatic peripheral neuropathic pain will be recruited from the Quebec Pain Registry (more information available at www.quebecpainregistry.com) and the Alan Edwards Pain Management Unit at the Montreal General Hospital.

Informed consent is obtained by research study staff that have obtained Good Clinical Practice (GCP) clinical research training certification by the Research Institute of the McGill

University Health Centre. Also, the research study staff have receive training on general standard operating procedures for clinical trials and specific tasks for conducting the study.

8.5.9 Randomization and Blinding

Randomization for treatment order (placebo - active drug/active drug - placebo) is performed by a coin flip done by a research coordinator assigned for this task who is not involved in patient interaction and outcome assessment. The same coordinator also ensures blinding by assigning a three-digit number code, from a computer-generated random list, to the Topi-Pumps used in each treatment block. The list of number codes and the identity of their corresponding treatment is only shared with the compounding pharmacy. The blinding applies to the patient, the study coordinators performing patient assessments and the dispensing pharmacy.

8.5.10 Data collection, management & confidentiality

Data will initially be collected on paper-based source documents filled out at every visit. Data will then be transferred promptly into a spreadsheet stored in a password protected drive. Data entry and management will be completed and cross-checked independently by two study coordinators and the sponsor will not have access to the data. All source documents and data sheets used in the clinical trial will not have patient identifying information, instead, patients will be assigned a study code number with the key securely stored to ensure confidentiality. All information pertaining to the study will be retained by the principal investigator for 25 years after completion as per the guidelines of Health Canada.

8.5.11 Statistical methods

Statistical analysis will be performed using an unpaired t-test to evaluate between-group differences in the 2 sequence groups, as is recommended for analyses in cross-over designs [31]. The summarized VAS pain scores during the second week of treatment will be compared between treatment groups adjusting for period and order. We will also use unpaired t-tests to assess carry-over effects by comparing baseline and wash-out period measures. DMA pain scores at the end of each treatment period will also be compared between treatment periods. Secondary analyses of pain relief and the area of punctate hyperalgesia will also be conducted using unpaired t-tests.

8.5.12 Safety and adverse event outcomes

Both clonidine and pentoxifylline have a history of safe systemic use in humans and there is evidence that topical application of these agents produces extremely low to undetectable plasma

levels [27]. As no negative effects were observed in animals or adverse effects detected in our trial in healthy volunteers, we do not anticipate any adverse side-effects with our topical treatment [25, 28].

According to the FDA, rare events in patients taking pentoxifylline (although not established as caused by the drug) include anaphylactic reactions [32]. Subjects will be asked to immediately report any signs of anaphylaxis (itchy rash, lip or throat swelling) to the study coordinator, who will instruct the patient to go immediately to a hospital emergency room.

Risks involved in taking clonidine orally are dizziness, light-headedness, drowsiness, tiredness and vision problems, which are caused by a decrease in blood pressure [33]. Patients will be asked to immediately report any of these symptoms to the study coordinator, who will instruct the patient to see the study physician, who will perform a medical evaluation, including assessment of blood pressure. If any subject's systolic pressure is found to be below 90 mmHg, they will be asked to stop the treatments and will be removed from the study. Abrupt discontinuation of clonidine can occasionally result in a withdraw syndrome, characterized by a rebound increase in blood pressure and other symptoms and signs of sympathetic activity like restlessness, increased heart rate and increased irritability [34]. This usually occurs after cessation of large oral doses and in some instances after transdermal treatment. There is minimal risk for this syndrome with our study drug, as the dose of clonidine used is much lower than oral or transdermal preparations. Patients will be asked to report to the study coordinator if any of these symptoms occur during the washout period of the study and the few days following completion of the study. Clinical evaluation and appropriate medical care will accordingly be provided.

All adverse events will be tabulated, summarized and reviewed by the study physician. Serious adverse drug reactions, i.e. reactions causing hospitalization; persistent or significant disability; that is life-threatening or resulting in death, will be immediately reported to the study sponsor who is the primary investigator and the research ethics coordinator for the study site. Serious adverse drug reaction reporting will follow International Council for Harmonization (ICH) guidelines.

8.5.13 Endpoints & post-trial care

Eligible patients are expected to have stable disease with no alteration to their treatment regimen in the 5 weeks preceding enrollment. If a patient develops uncontrolled pain with a clinical

indication for a new treatment during the study period, the participation of the patient in the trial will be discontinued and the new treatment commenced. If any subject's systolic pressure is found to drop below 90 mmHg during any section of the trial, they will be asked to stop the treatments and they will be removed from the study. Finally, in the event of an anaphylactic reaction to any of the topical treatments used in the study, the patient's participation in the study will immediately be terminated. In both instances, the coordinator performing the randomization will send the study physician the information required to unblind him to the study drug the patient was taking so the information can be transferred to the medical team that will care for the patient.

Patients will be instructed to contact the study coordinator and/or physician if symptoms of clonidine withdrawal occur during wash-out or the week following completion of the study. In such an event, the study physician will perform a clinical evaluation of the patient and appropriate medical care will be provided. Unblinding and termination of participation in the study will depend on the study physician's assessment of the patient's clinical status.

8.5.14 Compensation

Participants will receive reimbursement of travel expenses up to a sum of 100 Canadian dollars. If participants fail to complete the study, the compensation they receive will be pro-rated amounts based on duration of participation.

8.6 Discussion

The peripheral neuropathic pain patient population is composed of a sizable number of patients that can benefit from avoiding the systemic anticonvulsants and antidepressants that are first-line treatments of the condition [3-5]. Given their typically older age and comorbid conditions, these patients are at greater risk of developing adverse events from these recommended systemic treatments [4, 5]. This clinical study is a required step in developing a locally-administered, potentially effective and tolerable analgesic alternative for the treatment of peripheral neuropathic pain.

As a phase-II clinical trial, this study is a proof-of-concept step to evaluate the potential efficacy and safety of the topical combination of clonidine and pentoxifylline in peripheral neuropathic pain [35]. To optimize the detection of hypothesized analgesic effects, the selection of the study population is restricted to a specific subgroup of neuropathic pain patients with an uncomplicated medical profile.

The cross-over design of the study was another step taken to increase the efficiency of the initial screening of the formulation's analgesic effects. The design allows the use of a relatively small number of patients by minimizing between-subject data variability thereby reserving resources and time [35, 36]. We believe the two-week duration of treatment is long enough to reveal the alleviation of neuropathic pain symptoms achieved. At the same time, the treatment is set to be brief enough to avoid long-term effects that can potentially change the underlying pathology preventing effective washout. The one-week long washout period is based on evidence obtained from the pharmacokinetics of constituent drugs and the length of analgesic effects measured from neuropathic rodent models and healthy volunteers treated with the formulation [25, 28, 33, 37]. It is expected that this time frame is sufficient in duration and carry-over effects are unlikely to occur.

Clinical trials with cross-over designs with relatively few patients have been used to successfully detect the efficacy of analgesics in painful diabetic neuropathy, post-herpetic neuralgia and post-traumatic neuropathic pain [38-40]. The cross-over design assures the patients that treatment with both placebo and the active drug will occur prompting them to think more objectively about their pain levels in each period. This has resulted in significantly lower placebo group response rates in cross-over trials as compared to parallel-group trials, as reported in a meta-analysis of neuropathic pain trials [41].

With randomization and blinding, we have tried to maintain the internal validity of the study. Outcome measures were chosen to pointedly assess the analgesic efficacy and solely focus on discovering the degree of relief from spontaneous and evoked pain [42]. A broader investigation of physical-functional outcome and overall improvement of quality of life is undoubtedly necessary, but will be reserved for subsequent study phases that will be performed after initial proof of efficacy has been obtained for the formulation [43].

The outcome measures used as comparators in this study are kept the same as what has been used in the healthy volunteer human-study performed with the topical combination [28]. The measures comprehensively assess both spontaneous and evoked pain. The VAS ratings employed for assessments of daily levels of spontaneous pain and the degree of dynamic mechanical allodynia have a proven high degree of resolution and sensitivity in clinical pain research [44].

To the best of our knowledge, this study is the first randomized, double-blind and placebo-controlled clinical trial to assess the analgesic efficacy of the topical combination of clonidine and pentoxifylline in neuropathic pain. Topical clonidine on its own has been investigated in this manner for the treatment of painful diabetic neuropathy and a positive outcome has been reported [27]. In complex regional pain syndrome, there have been clinical case reports of analgesic benefits achieved with the use of bare topical clonidine, and topical clonidine in combination with pentoxifylline, ketamine and dimethyl sulfoxide [26, 45]. However, the level of evidence from these studies does not allow a reliable conclusion to be drawn on the therapeutic benefit of the topical treatments.

Lastly, relevant clinical data is required to affirm the abundant preclinical evidence on the role of poor nerve oxygenation and blood supply in the genesis of peripheral neuropathic pain [10, 25, 46-48]. This study is a means of achieving that, above and beyond presenting a specific drug formulation with potential efficacy for the treatment of peripheral neuropathic pain. The results can, furthermore, potentially promote the investigation of other vasodilatory agents for analgesic effects, building a new collection of safe and effective drugs for the treatment of peripheral neuropathic pain.

8.7 Trial status

The study is currently actively recruiting and enrolling eligible participants. Recruitment started in January. This is protocol version 5, dated June 2018. MUHC-REB identification number: TTNP 2018-3906. If significant changes requiring the modification of this protocol are required, the changes will be submitted for approval to the MUHC-REB through its web-based platform Nagano.

8.8 Availability of data and materials

There are no plans to publicly share the raw data or materials used during this clinical trial. There are plans to communicate the results of the analyzed data through conference presentations and publications in relevant journals.

8.9 Ethical approval & consent participate

We confirm that informed consent will be obtained from all study participants according to regulations outlined by Health Canada and the McGill University Health Center Research Ethics Board.

8.10 References

1. Treede RD, Jensen TS, Campbell JN, Cruccu G, Dostrovsky JO, Griffin JW, et al. Neuropathic pain: redefinition and a grading system for clinical and research purposes. Neurology. 2008;70(18):1630-5.
2. Colloca L, Ludman T, Bouhassira D, Baron R, Dickenson AH, Yarnitsky D, et al. Neuropathic pain. Nat Rev Dis Primers. 2017;3:17002.
3. Attal N. Pharmacological treatments of neuropathic pain: The latest recommendations. Rev Neurol (Paris). 2019;175(1-2):46-50.
4. Haanpää ML, Gourlay GK, Kent JL, Miaskowski C, Raja SN, Schmader KE, et al. Treatment considerations for patients with neuropathic pain and other medical comorbidities. Mayo Clin Proc. 2010;85(3 Suppl):S15-S25.
5. Schmader KE, Baron R, Haanpaa ML, Mayer J, O'Connor AB, Rice AS, et al. Treatment considerations for elderly and frail patients with neuropathic pain. Mayo Clin Proc. 2010;85(3 Suppl):S26-32.
6. Cameron NE, Eaton SE, Cotter MA, Tesfaye S. Vascular factors and metabolic interactions in the pathogenesis of diabetic neuropathy. Diabetologia. 2001;44(11):1973-88.
7. Malik RA, Tesfaye S, Thompson SD, Veves A, Sharma AK, Boulton AJM, et al. Endoneurial localisation of microvascular damage in human diabetic neuropathy. Diabetologia. 1993;36(5):454-9.
8. Mackinnon SE, Dellon AL, Hudson AR, Hunter DA. Chronic human nerve compression--a histological assessment. Neuropathol Appl Neurobiol. 1986;12(6):547-65.
9. Lipowsky HH. Microvascular rheology and hemodynamics. Microcirculation. 2005;12(1):5-15.
10. Van Dam PS, Cotter MA, Bravenboer B, Cameron NE. Pathogenesis of diabetic neuropathy: Focus on neurovascular mechanisms. Eur J Pharmacol. 2013;719(1):180-6.
11. Cameron NE, Cotter MA. The neurocytokine, interleukin-6, corrects nerve dysfunction in experimental diabetes. Exp Neurol.. 2007;207(1):23-9.
12. Bruno RM, Ghiadoni L, Seravalle G, Dell'oro R, Taddei S, Grassi G. Sympathetic regulation of vascular function in health and disease. Front Physiol. 2012;3:284.
13. Bauer V, Sotníková R. Nitric oxide-the endothelium-derived relaxing factor and its role in endothelial functions. Gen Physiol Biophys. 2010;29(4):319.
14. Kass DA, Takimoto E, Nagayama T, Champion HC. Phosphodiesterase regulation of nitric oxide signaling. Cardiovasc Res. 2007;75(2):303-14.
15. Shafiee-Nick R, Afshari AR, Mousavi SH, Rafighdoust A, Askari VR, Mollazadeh H, et al. A comprehensive review on the potential therapeutic benefits of phosphodiesterase inhibitors on cardiovascular diseases. Biomed Pharmacother. 2017;94:541-56.
16. McCarty MF, O'Keefe JH, DiNicolantonio JJ. Pentoxifylline for vascular health: a brief review of the literature. Open Heart. 2016;3(1):e000365-e.
17. Perego MA, Sergio G, Artale F, Giunti P, Danese C. Haemorrheological improvement by pentoxifylline in patients with peripheral arterial occlusive disease. Curr Med Res Opin. 1986;10(2):135-8.
18. Ott E, Lechner H, Fazekas F. Hemorheological effects of pentoxifylline on disturbed flow behavior of blood in patients with cerebrovascular insufficiency. Eur Neurol. 1983;22 Suppl 1:105-7.
19. van Brummelen P, Jie K, van Zwieten PA. Alpha-adrenergic receptors in human blood vessels. Br J Clin Pharmacol. 1986;21 Suppl 1:33s-9s.

20. Szabo B, Hedler L, Starke K. Peripheral presynaptic and central effects of clonidine, yohimbine and rauwolscine on the sympathetic nervous system in rabbits. Naunyn Schmiedebergs Arch Pharmacol. 1989;340(6):648-57.
21. Figueroa XF, Poblete MI, Boric MP, Mendizabal VE, Adler-Graschinsky E, Huidobro-Toro JP. Clonidine-induced nitric oxide-dependent vasorelaxation mediated by endothelial alpha(2)-adrenoceptor activation. Br J Pharmacol. 2001;134(5):957-68.
22. Yagihashi S, Mizukami H, Sugimoto K. Mechanism of diabetic neuropathy: Where are we now and where to go? J Diabetes Investig. 2011;2(1):18-32.
23. Dyck PJ, Karnes JL, O'Brien P, Okazaki H, Lais A, Engelstad J. The spatial distribution of fiber loss in diabetic polyneuropathy suggests ischemia. Ann Neurol. 1986;19(5):440-9.
24. Peppin JF, Albrecht PJ, Argoff C, Gustorff B, Pappagallo M, Rice FL, et al. Skin Matters: A Review of Topical Treatments for Chronic Pain. Part One: Skin Physiology and Delivery Systems. Pain Ther. 2015;4(1):17-32.
25. Ragavendran JV, Laferriere A, Xiao WH, Bennett GJ, Padi SS, Zhang J, et al. Topical combinations aimed at treating microvascular dysfunction reduce allodynia in rat models of CRPS-I and neuropathic pain. J Pain. 2013;14(1):66-78.
26. Davis K, Treede R, Raja SN, Meyer R, Campbell JN. Topical application of clonidine relieves hyperalgesia in patients with sympathetically maintained pain. Pain. 1991;47(3):309-17.
27. Campbell CM, Kipnes MS, Stouch BC, Brady KL, Kelly M, Schmidt WK, et al. Randomized control trial of topical clonidine for treatment of painful diabetic neuropathy. Pain. 2012;153(9):1815-23.
28. Ragavendran JV, Laferriere A, Bennett GJ, Ware MA, Gandhi W, Bley K, et al. Effects of topical combinations of clonidine and pentoxifylline on capsaicin-induced allodynia and postcapsaicin tourniquet-induced pain in healthy volunteers: a double-blind, randomized, controlled study. Pain. 2016;157(10):2366-74.
29. Wang H, Bolognese J, Calder N, Baxendale J, Kehler A, Cummings C, et al. Effect of morphine and pregabalin compared with diphenhydramine hydrochloride and placebo on hyperalgesia and allodynia induced by intradermal capsaicin in healthy male subjects. J Pain. 2008;9(12):1088-95.
30. Wallace MS, Schulteis G. Effect of chronic oral gabapentin on capsaicin-induced pain and hyperalgesia: a double-blind, placebo-controlled, crossover study. Clin J Pain. 2008;24(6):544-9.
31. Wellek S, Blettner M. On the proper use of the crossover design in clinical trials: part 18 of a series on evaluation of scientific publications. Dtsch Arztebl Int. 2012;109(15):276-81.
32. Pharmaceuticals V. Trental: Labeling-Package Insert, Reference ID: 3873773 2016; https://www.accessdata.fda.gov/scripts/cder/daf/index.cfm?event=BasicSearch
33. Keränen A, Nykänen S, Taskinen J. Pharmacokinetics and side-effects of clonidine. Eur J Clin Pharmacol. 1978;13(2):97-101.
34. Hunyor SN, Hansson L, Harrison TS, Hoobler SW. Effects of clonidine withdrawal: possible mechanisms and suggestions for management. Br Med J. 1973;2(5860):209-11.
35. Gewandter JS, Dworkin RH, Turk DC, McDermott MP, Baron R, Gastonguay MR, et al. Research designs for proof-of-concept chronic pain clinical trials: IMMPACT recommendations. Pain. 2014;155(9):1683-95.
36. Senn SS. Cross-over trials in clinical research. 2nd ed. West Sussex: John Wiley & Sons; c2002. p. 5-9.

37. Ward A, Clissold SP. Pentoxifylline. A review of its pharmacodynamic and pharmacokinetic properties, and its therapeutic efficacy. Drugs. 1987;34(1):50-97.
38. Raja SN, Haythornthwaite JA, Pappagallo M, Clark MR, Travison TG, Sabeen S, et al. Opioids versus antidepressants in postherpetic neuralgia: a randomized, placebo-controlled trial. Neurology. 2002;59(7):1015-21.
39. Jenkins TM, Smart TS, Hackman F, Cooke C, Tan KK. Efficient assessment of efficacy in post-traumatic peripheral neuropathic pain patients: pregabalin in a randomized, placebo-controlled, crossover study. J Pain Res. 2012;5:243-50.
40. Gilron I, Bailey JM, Tu D, Holden RR, Jackson AC, Houlden RL. Nortriptyline and gabapentin, alone and in combination for neuropathic pain: a double-blind, randomised controlled crossover trial. Lancet. 2009;374(9697):1252-61.
41. Katz J, Finnerup NB, Dworkin RH. Clinical trial outcome in neuropathic pain: relationship to study characteristics. Neurology. 2008;70(4):263-72.
42. Wallace MS, Rowbotham MC, Katz NP, Dworkin RH, Dotson RM, Galer BS, et al. A randomized, double-blind, placebo-controlled trial of a glycine antagonist in neuropathic pain. Neurology. 2002;59(11):1694-700.
43. Mehta P, Claydon L, Hendrick P, Winser S, Baxter GD. Outcome measures in randomized-controlled trials of neuropathic pain conditions: a systematic review of systematic reviews and recommendations for practice. Clin J Pain. 2015;31(2):169-76.
44. Younger J, McCue R, Mackey S. Pain outcomes: a brief review of instruments and techniques. Curr Pain Headache Rep. 2009;13(1):39-43.
45. Russo MA, Santarelli DM. A Novel Compound Analgesic Cream (Ketamine, Pentoxifylline, Clonidine, DMSO) for Complex Regional Pain Syndrome Patients. Pain Pract. 2016;16(1):E14-E20.
46. Lim TK, Shi XQ, Johnson JM, Rone MB, Antel JP, David S, et al. Peripheral Nerve Injury Induces Persistent Vascular Dysfunction and Endoneurial Hypoxia, Contributing to the Genesis of Neuropathic Pain. J Neurosci. 2015;35(8):3346-59.
47. Kirchmair R, Tietz AB, Panagiotou E, Walter DH, Silver M, Yoon Y-S, et al. Therapeutic angiogenesis inhibits or rescues chemotherapy-induced peripheral neuropathy: taxol-and thalidomide-induced injury of vasa nervorum is ameliorated by VEGF. Mol Ther. 2007;15(1):69-75.
48. Fulas OA, Laferriere A, Stein RS, Bohle DS, Coderre TJ. Topical combination of meldonium and N-acetyl cysteine relieves allodynia in rat models of CRPS-1 and peripheral neuropathic pain by enhancing NO-mediated tissue oxygenation. J Neurochem. 2020;152(5):570-84.

9. COMPREHENSIVE DISCUSSION

9.1 Combating of local tissue hypoxia with topical meldonium-NAC alleviates mechanical hypersensitivity in rat models of PNP & CRPS

Topical treatment with meldonium-NAC effectively relieved mechanical allodynia in the CPIP rat model of CRPS, as well as the CCI and CIPN rat models of PNP. This effect was especially robust in the CPIP rats where the PWT elevations produced by the drug brought the thresholds of the rats close to their pre-CPIP injury level. The anti-allodynic efficacy of topical meldonium-NAC can be put in a better perspective by comparing the magnitude of its effects to that published by our laboratory following the systemic administration of antidepressants, anticonvulsants and opioids [284]. In the study, we reported the PWT responses of CPIP rats to low, medium and high systemic doses of amitriptyline, pregabalin and morphine. The extent of PWT increment produced by topical meldonium-NAC matched what was reported for the highest doses of the three systemically administered drugs. A similar observation can be made when comparing the effect of topical meldonium-NAC in alleviating mechanical sensitivity in CCI rats to the one reported by our laboratory after systemic treatment of the rats with gabapentin [354]. In the study, the treatment of CCI rats with a high dose of intraperitoneal gabapentin produced a significant PWT elevation, but the magnitude of change achieved was lower than what we currently recorded for topical meldonium-NAC. This is remarkable considering meldonium-NAC is a previously untested topically administered agent with local anti-hypoxic effects, while the three high-dose systemic agents are routinely used analgesics administered for PNP and CRPS, with broad CNS modulatory activities. Although there are some reservations to comparing these independent experiments that are widely spaced in time, this comparison is reliable as the strain and sex of rats used were similar, and the CPIP and CCI procedures and the PWT measurements were performed in the same laboratory using identical protocols.

The systemic administration of meldonium has previously been reported to prevent the development of painful peripheral neuropathy in rats with streptozotocin-induced diabetes [355]. Likewise, a structural analog of meldonium, propionyl L-carnitine, has alleviated pain in patients with chronic critical limb ischemia after two weeks of intravenous administration [356]. Propionyl L-carnitine also bears mechanistic similarity with meldonium in increasing endothelial NO concentration and vasodilation [356]. On the other hand, systemic administration NAC to CPIP

rats has been demonstrated to dose-dependently ameliorate mechanical hypersensitivity in CPIP rats [109, 141]. Other free radical scavengers and antioxidants including 4-hydroxy-2,2,6,6-tetra-methyl-piperydine-1-oxyl (TEMPOL), mangiferin, and vitamin C and E have also resulted in prevention or alleviation of mechanical sensitivity in CPIP rats [109, 141, 357, 358].

Our studies indicate that a change in local tissue oxygenation plays a significant role in the anti-allodynic effects produced by topical meldonium-NAC. A rise in percutaneous hind paw oxygen saturation values was observed in CPIP rats after topical treatment with meldonium-NAC. The boost in hind paw tissue oxygenation occurred at the time point of peak anti-allodynic effects, creating a link between the effect of the drug on PWT and tissue oxygenation. The anti-allodynic effects, produced by systemic administration of pentoxifylline in CPIP rats, have been previously linked with its impact on restoring the defective post-occlusive hyperemic response in the injured hind paw of the rats [359]. This indicated the anti-allodynic effects of the drug occurred via the alleviation of microvascular dysfunction and tissue hypoxia [359]. Similarly, the anti-nociceptive effects of a NO-releasing topical hydrogel in rats with inflammatory pain was mediated by enhancement of dermal blood flow, as measured by laser Doppler flowmetry (LDF) [360]. In the first of these two studies, the LDF used to assess tissue microvascular (dys)function based its measurements from the flux of red blood cells through the microvascular lumen [361]. In the meldonium-NAC experiment, a visible-light-based oxygen saturation measurement was used and it calculated spectral absorption difference between oxy- and deoxy-hemoglobin to reflect oxygenation of the tissue microvascular bed [340]. Tissue oxygen saturation is more specific in indicating the oxygenation status of tissue by reflecting the balance between oxygen delivery and consumption [362]. Regardless, previous studies on the human skin have revealed a strong association exists between measures of blood flow using LDF and that of oxygen saturation using visible-light spectroscopy, leading to the conclusion that both can be used with the same level of confidence in assessing microvascular impairment [362]. Therefore, the result of these studies can be taken to cumulatively imply the targeting of microvascular impairment and tissue hypoxia using vasoactive drugs like pentoxifylline and meldonium-NAC can be an effective approach for achieving analgesia in different pain conditions.

Further evidence for the critical involvement of tissue oxygenation in the anti-allodynic effects of topical meldonium-NAC was obtained from the experiments that combined the topical

treatment with hyperbaric oxygen. Administration of hyperbaric oxygen has been reported to alleviate mechanical allodynia in CIPN and CCI rat models of PNP [349, 363, 364]. The duration of treatment used in these studies varied from a single 60-minute long session to seven consecutive days' treatment [349, 363, 364]. Mechanical and cold hypersensitivities were attenuated in CIPN rats after one or four consecutive days of treatment with hyperbaric oxygen [363]. Likewise, an increase in mechanical withdrawal threshold and prolonged thermal withdrawal latency were reported in CCI rats after the administration of 5-7 days of hyperbaric oxygen therapy [349, 364].

In the CPIP rats that were topically treated with a sub-therapeutic dose of topical meldonium-NAC, the addition of a 30-minutes long treatment with hyperbaric oxygen resulted in a significant elevation in PWT. This indicated that optimal alleviation of tissue hypoxia, through both pharmacological and non-pharmacological means, can significantly impact the pain hypersensitivities exhibited by the CPIP rats. Clinical studies of CRPS patients have also provided preliminary evidence for the analgesic benefits of hyperbaric oxygen treatment [232].

9.2 The anti-allodynic effects of topical meldonium-NAC in CPIP rats occur through enhancement of NO-mediated local tissue oxygenation

Both meldonium and NAC have been reported to have vasodilatory effects through their stimulation of endothelial NO production [365, 366]. NO is a key regulator of microvascular flow and oxygen delivery to all tissues in the body, and interventions that increase the level and activity of NO in the microvasculature have been shown to be therapeutic for vascular diseases that cause tissue ischemia [367]. In the CPIP rats treated with topical meldonium-NAC, the targeted disease process was tissue hypoxia, and our studies hypothesized that the manipulation of microvascular NO availability would alleviate mechanical hypersensitivity. After demonstrating analgesia and increased oxygenation, experiments were designed to test the involvement of NO in both the anti-allodynia effects and the increase of local tissue oxygenation produced by topical meldonium-NAC. The first one of these experiments assessed whether systemic pre-administration of the NOS inhibitor (L-NAME) influenced the anti-allodynia produced by topical treatment with meldonium NAC in CPIP rats. The results revealed that endothelial NO synthesis is key to the anti-allodynic effects produced by topical meldonium-NAC. Thus, inhibition of NO synthesis with L-NAME pre-treatment significantly diminished the PWT elevation previously observed with topical administration of meldonium-NAC in CPIP rats. Systemic administration of meldonium has

previously been shown to increase blood, myocardial and cortical levels of NO in rats [366, 368]. Co-administration of a NOS-inhibitor, Nω-nitro L-arginine, with meldonium eliminated the observed rise in tissue NO [366]. The impact of meldonium on vascular NO occurs through its enhancement of the levels of ϒ-butyrobetaine esters which are cholinomimetics that can potentially stimulate endothelial NOS activity via acetylcholine receptors [366, 369].

In addition to the expected impact of meldonium on tissue NO, the potent antioxidant NAC, which constitutes half of the topical formulation, has the potential to further enhance NO availability by sparing ROS consumption of NO [370]. ROS are highly reactive with NO and together produce peroxynitrites and other RNS [121]. CPIP rats, not unlike CRPS patients, have been shown to have excess ROS, and show evidence of oxidative stress, in their injured hind paw [141]. Therefore, it is conceivable that any therapeutic effort to increase NO production in a ROS overloaded microenvironment like CPIP would yield RNS, sparing little free NO for vasodilatory effects. The composition of topical meldonium-NAC, using an antioxidant that has potent ROS quenching effects, was strategized to avoid such a conversion of microvascular NO into RNS production [371]. The success of the approach can be observed from the results of the EPR experiment that directly measured plantar muscle NO in CPIP rats treated with topical meldonium-NAC. The elevated level of plantar muscle NO measured from the drug-treated CPIP rats was an indication that ROS quenching of NO was minimized to spare enough microvascular NO in the hind paw tissue to produce increased blood flow and tissue oxygenation.

Our findings with topical meldonium-NAC are in line with some studies that have reported the analgesic effect of local treatment with NO substrates. We have previously published work demonstrating linsidomine, a NO donor, produces a dose-dependent alleviation of mechanical sensitivity after topical administration to CPIP rats [215, 222]. Its systemic administration to CPIP rats also produced similar effects [372]. In combination with other vasodilators, linsidomine synergistically reduced mechanical sensitivity not only in CPIP rats but also in CCI and CIPN rat models of PNP [215]. Topical administration of a gel containing S-nitrosoglutathione, a NO donor, has been shown to reduce mechanical hypersensitivity in rats with inflammatory pain [360]. Moreover, local application of a cream containing isosorbide dinitrate and the NO donor *S*-nitroso-*N*-acetylpenicillamine reduced mechanical allodynia in rat models of incisional pain [373]. Oral

administration of Nicorandil, a NO donor antianginal drug, has also been reported to produce anti-nociceptive effects in mice with formaldehyde-induced pain [374].

In clinical studies, the topical use of an ointment formulation containing isosorbide dinitrate alleviated pain in patients with CRPS [103]. Likewise, sprays of isosorbide dinitrate and a similar compound, glyceryl trinitrate, have shown local analgesic effects in patients with painful diabetic neuropathy [220, 375]. Local application of nitrate patches has also been shown to reduce ischemic rest pain in patients with severe peripheral vascular disease, with greater impact in those with primary small vessel disease [376]. Nitrates serve as exogenous precursors of NO that are metabolized by vascular smooth muscle cells to produce increased endothelial NO and resultant vasodilation [377]. Treatment with topical meldonium produced the same effects of enhanced microvascular NO and tissue oxygenation in CPIP rats, by stimulating endothelial NOS and reducing NO consumption by oxidants.

9.3 Repeated topical treatment with meldonium-NAC produces prolonged anti-allodynic effects in CPIP & CCI rats

There is a limit to the absorptive capacity of the skin after a single topical administration of a drug, and the length of therapeutic effects is constrained by the quantity of active ingredient that can reach the diseased tissue [378]. Subsequent experiments were therefore designed to assess the impact of repeated topical administration of meldonium-NAC on its duration of anti-allodynic effects in CPIP and CCI rats. A six-weeks long repeated systemic treatment with meldonium has previously been shown to prevent the development of painful diabetic neuropathy in rats, whereas, a six day-long oral supplementation of NAC to rats has been found to prevent thermal hyperalgesia and nerve degeneration following CCI of the sciatic nerve [355, 379]. We have demonstrated that meldonium-NAC, after a single topical application, produces immediate anti-allodynic effects that lasts for more than 2 hours in both CPIP and CCI rats. After twelve consecutive days of topical treatment with meldonium-NAC, both CPIP and CCI rats started exhibiting a prolonged anti-allodynic response to the drug with significantly raised PWTs lasting for nearly 2 days. One possible explanation for this prolonged effect is the occurrence of a depot effect, where the drug accumulates in the layers of the skin and subcutaneous fatty tissue, which serve as a reservoir for sustained local effects of the drug [378]. This has been observed with the topical application of the NSAID diclofenac for the treatment of inflammatory arthritis [380]. The methyl nicotinate assay

was used in this study, where a colorimetric evaluation of methyl nicotinate-induced skin erythema was performed after diclofenac application [380]. The extent of attenuation in skin redness was used to relatively quantify the amount of diclofenac absorbed into the skin after topical administration [380]. Further investigations of this type are needed to assess if repeated administration of topical meldonium-NAC results in the formation of a drug reservoir in the skin prolonging its effect. This depends on several factors including the lipid solubility of the drug; its protein-binding capacity and percutaneous absorption [378].

An alternate explanation for the prolonged effect of meldonium-NAC is that the persistent alleviation of tissue hypoxia may produce lasting tissue reparative changes in PNP and CRPS rats. Although it needs to be experimentally validated, the enhancement in tissue oxygen saturation that occurred after a single administration of topical meldonium-NAC likely continued to take place with repeated treatments with the drug. Local tissue hypoxia has multifactorial contributions to the altered microenvironment of peripheral sensory nerves causing their activation and sensitization. Among these contributory factors are impaired high energy phosphate metabolism and ATP deficit; local tissue acidosis due to increased anaerobic respiration; and mitochondrial dysfunction and ROS generation with consequent inflammation [37, 49, 91, 144, 381]. Endoneurial hypoxia with altered metabolic indices including increased oxygen consumption, increased glycolysis, and mitochondrial bioenergetic dysfunction has been reported in mice with PNP after partial sciatic nerve ligation [37, 144]. Increased tissue acidosis, inflammation and oxidative damage have also been demonstrated in the hind paw of CPIP rats [141]. Repeated and prolonged treatments with anti-hypoxic drugs like meldonium-NAC have the potential to remove the root cause of these cascades of maladaptive events, possibly resulting in a longer-lasting recovery of the sensitized sensory nerve endings.

9.4 Mechanochemical synthesis can be used to generate antioxidant-nutraceutical salts & co-crystal of pentoxifylline, clonidine & linsidomine

Some of the work reported in this thesis described a novel means of fortifying the topical analgesic effect of the anti-hypoxic vasoactive agents pentoxifylline, clonidine, and linsidomine for the treatment of PNP and CRPS. These drugs, given either individually or in combination, produce anti-allodynic effects after topical administration in rat models of PNP and CRPS [215, 222]. Novel salts and co-crystals of the drugs were synthesized by pairing them with nutraceutical

antioxidants in efforts to further enhance their local anti-allodynic effects. The new products were aimed to simultaneously alleviate tissue hypoxia and oxidative stress and consequently dampen sensory nerve hyperactivity and pain.

A mechanochemical technique, LAG, was utilized to synthesize the anti-hypoxic-antioxidant salts and co-crystals by pairing clonidine with α-lipoic acid, linsidomine with caffeic acid and pentoxifylline with protocatechuic acid. The successful formation of these salts and co-crystals was an outcome of reciprocal hydrogen bonding and proton exchange between the nitrogen-containing basic chemical moieties of the anti-hypoxic drugs' and the acidic carboxylic acid groups of the antioxidant nutraceuticals. The pairing of these compatible chemical groups required optimization of the substrates' stoichiometric ratio and the LAG reaction conditions, including the type of milling jars and balls and the duration of milling. A combination of crystallographic, spectroscopic and thermal analysis techniques was used to determine the successful formation of the novel salts and co-crystals. These experiments revealed the salts and co-crystals to be novel products of unique molecular arrangement, solid-state structure and physical properties that are distinct from their individual constituent substrates, or their plain combination. There are reports of salt and co-crystal synthesis with some of the drug and nutraceutical substrates used in this project. Pentoxifylline has been co-crystallized with the diuretic furosemide [382]. Co-crystals of gallic acid with the anti-bacterial, isoniazid, has also been reported [383]. The nootropic, oxiracetam, has been co-crystallized with gallic and protocatechuic acid [384]. Caffeic acid has also been co-crystallized with nicotinamide (vitamin B_3) [383]. Clonidine and linsidomine are commercially available as hydrochloride salts. The hydrochloric acid used for the formation of these salts is added as an inactive excipient to create a modification that makes the drugs more crystalline and water-soluble. The synthesis of the nutraceutical salts of clonidine and linsidomine necessitated removing their pharmacologically inactive anion of hydrochloric acid and replacing it with that of the antioxidant carboxylic acids.

These mechanistically targeted, multi-component salts and co-crystals reported in this thesis are some of the first demonstrations of solid-state synthesis for the production of effective analgesics for chronic pain. The chemical synthesis of drug-drug co-crystals with analgesic potential has been reported for meloxicam with aspirin, aceclofenac with paracetamol, and duloxetine with naproxen [300, 385, 386]. Other than the demonstration of enhanced solubility

from experiments done on the bench, these agents have not been tested for biological effects [300, 385, 386]. There are also published patents reporting the synthesis of co-crystals of tramadol with paracetamol and naproxen [300]. As compared to these products, the salts and co-crystals reported in this thesis have progressed a step further from chemical synthesis and analysis on the bench to *in vivo* testing of their analgesic effects in rat models of CRPS.

More effective analgesic drugs can be developed with this approach of synthesizing multi-functional salts and co-crystals from multiple APIs targeting different analgesic mechanisms. The drug-drug co-crystal synthesized from the opioid, tramadol, and the NSAID, celecoxib, has demonstrated superior efficacy to its constituent drugs for the alleviation of moderate to severe acute post-surgical oral pain [335]. Likewise, a co-crystal of the NSAID, etodolac, and lidocaine has been formulated as a topical patch and is in clinical testing for the treatment of acute inflammatory shoulder pain [387]. The novel anti-hypoxic-antioxidant salts and co-crystals reported in this thesis expand on this relatively new approach of generating multifunctional analgesics to discover better treatment options for PNP and CRPS.

9.5 The anti-allodynic effects of pentoxifylline, clonidine & linsidomine are enhanced by co-crystallization & salt formation with antioxidant nutraceuticals

The CPIP rat model of CRPS was used to assess the anti-allodynic effects of the novel anti-hypoxic-antioxidant salts and co-crystals because local tissue hypoxia and oxidative stress, are strongly evident, with widespread tissue involvement, in the injured hind paw of these rats [141]. The topical anti-allodynic effects of the salts and co-crystals were tested at a few different doses in parallel with their respective individual constituents to determine the efficacy and potency of the new synthetic efforts. Topical clonidine, pentoxifylline and linsidomine have previously demonstrated anti-allodynic effects in CPIP rats [215]. The study also examined the anti-allodynic effects of topical combinations made by pairing pentoxifylline with clonidine, linsidomine with lisofylline and S-nitroso-N-acetyl penicillamine with lisofylline as plain mixtures of varying W/W concentrations [215]. The topical combinations exhibited significantly enhanced potency in alleviating mechanical hypersensitivity in CPIP rats as compared to the single agents contained in them [215]. The constitution of the topical preparations, the dose ranges of the drugs and the testing paradigm reported in this study were adopted with some modifications for the experiments done

in this project. The dose-dependent alleviation of mechanical sensitivity recorded following topical treatment with the drugs in our single-agent experiments was comparable to the results reported in the study. In the current experiments, we determined how the anti-allodynic dose-response curves of these anti-hypoxic vasoactive drugs changed after the drugs were paired with nutraceutical antioxidants through salt and co-crystal formation. All of the three salts and co-crystal made by pairing clonidine with α-lipoic acid, linsidomine with caffeic acid, and pentoxifylline with protocatechuic acid exhibited anti-allodynic effects of greater potency in CPIP rats as compared to their parent constituent drugs. The dose-response comparisons were made by first calculating the stoichiometric content of the anti-hypoxic and antioxidants agents contained in the salts and co-crystals to obtain a matching dose-range for testing the constituent agents singularly. The anti-allodynic effects produced by the constituent agents were then plotted against that of their respective salts and co-crystals. The CPIP rats showed no direct anti-allodynic response to any of the topical doses of the antioxidants. This indicated that the superior potency of the new salts and co-crystals is not resulting from an additive effect between the anti-hypoxic and antioxidant drug groups used to synthesize the novel products.

It is possible that the antioxidant activity of the nutraceuticals, in eliminating ROS that propagates microvascular injury and mitochondrial dysfunction, acted to synergistically boost the tissue oxygenation achieved by the local vascular effects of pentoxifylline, clonidine and linsidomine. A previous study has reported the systemic anti-allodynic effects of clonidine, pentoxifylline and clonidine, in CPIP rats, are enhanced by the co-administration of mangiferin, a plant extract with antioxidant and anti-inflammatory properties [388]. In this study, sub-effective intraperitoneal doses of clonidine, pentoxifylline and linsidomine were combined with oral mangiferin to yielding a significant alleviation mechanical hypersensitivity in CPIP rats [388]. The authors implicate the interactive role of tissue hypoxia, acidosis and oxidative stress in triggering nociceptor hypersensitivity in CPIP [388]. Hypoxia and ROS-evoked sensitization of nociceptor transduction ion channels have been reported from behavioral assays done in CPIP mice [389]. Moreover, hypoxic and ROS exposure of cell cultures expressing the same transduction channels has been shown to evoke hyperactivation [389]. The novel salts or co-crystal may have been more effective in alleviating mechanical hypersensitivity in the CPIP rats due to their simultaneous impact in reducing tissue hypoxia, oxidative stress and consequently nociceptor hyperactivity.

In addition to their bifunctional constitution, these novel salts and co-crystal may have newly acquired physicochemical properties that favorably impacted their pharmacokinetics resulting in better analgesic effects. Although this needs experimental validation, altered properties like solubility, tissue binding, and bioavailability, acquired through salt and co-crystal formation, can result in superior pharmacological activity. Such transformations have been reported for some drugs of analgesic applications like indomethacin, aceclofenac, and carbamazepine [390-392]. In particular, the co-crystallization of the NSAID, aceclofenac, with chitosan, a cationic polysaccharide has been shown to result in a marked increase in dissolution rate which translated to enhanced serum levels after oral dosing and rapid analgesic effects in rodent models of inflammatory pain [390].

9.6 The pairing of the anti-hypoxic drugs & antioxidants nutraceuticals through salt & co-crystal synthesis may aid clinical translation

The probability of a biologically active agent achieving clinical translation is dependent on multiple factors that affect the expenses of preclinical and clinical investigations for the development of the drug [393]. Data obtained from publicly available databases reveal that the cost of developing an average drug approximates to 900 million American dollars with the preclinical cost accounting for 45% of the total estimate [393].

Effective pharmacological treatment of complex pathologies like PNP and CRPS requires the use of multi-targeted drug combinations [394]. In addition to improving drug-load and patient compliance, the development of these combinations as single-entity salts and co-crystals is significantly less expensive as compared to developing the drugs as plain mixtures [334]. This is mainly because multicomponent salts and co-crystals can be unit-dosed and studied for safety and efficacy as single agents, while the same drugs combined as plain drug mixtures require multiple dosing rounds with the constituent agents and their combinations in different ratios [300, 395, 396]. Such drug combinations also have less managerial and production costs related to packaging and prescriptions [300]. Furthermore, even though salts and co-crystals may be composed of pharmacological agents existing in the market, they are recognized as new solid forms with novel physicochemical properties deeming them patentable by regulatory bodies [301]. Once their safety and efficacy are proven, salts and co-crystals are given the status of ‘new active substances’ [300].

The three novel salts and co-crystal made by pairing clonidine with α-lipoic acid, linsidomine with caffeic acid, and pentoxifylline with protocatechuic acid are the first mechanochemically synthesized products of anti-hypoxic and antioxidant properties to be proposed for the alleviation of pain in PNP and CRPS. The novelty of the products is characterized by their chemical formulation (i.e. as salts and co-crystals), their technique of synthesis, the specific combination of local mechanisms they target, and their intended clinical application. These attributes collectively make the products patentable and therefore more investable, hastening their possible clinical translation for use as analgesics for in PNP and CRPS.

9.7 The co-crystal of pentoxifylline & protocatechuic acid alleviates local tissue hypoxia & mitochondrial dysfunction

The co-crystal combining pentoxifylline and protocatechuic acid (pentx-pca) is a prototype of the mechanochemically synthesized anti-hypoxic-antioxidant salts and co-crystals. It has demonstrated potent topical anti-allodynic effects in CPIP rats that significantly exceeded that of its individual constituents. In subsequent experiments, its mechanism of action was investigated with a focus on changes in tissue oxygenation and downstream metabolic activity. The co-crystal contains equimolar quantities of pentoxifylline and protocatechuic acid. The assays of local tissue oxygen measurement and mitochondrial function were designed to assess the effects of both constituents. Being the anti-hypoxic component, pentoxifylline likely has a more direct impact on tissue perfusion and oxygenation. Its systemic administration to CPIP rats has been shown to alleviate the impairment of microvascular reserve in the rats as observed from their hind paw LDF measurement of post-occlusive hyperemic response [359]. But the role of the antioxidant protocatechuic acid in reducing hypoxia could also be inferred from the assays of tissue oxygenation and mitochondrial function [397]. Protocatechuic acid has been shown to have free radical scavenging activity that can effectively neutralize the ROS, superoxide and hydrogen peroxide, which are major players in ROS-induced microvascular injury [320, 398]. In addition, polyphenols, many of which are precursors of protocatechuic acid, are reported to induce endothelium-dependent vasodilation through their activating effect on endothelial NOS that occurs via phosphorylation [399, 400]. *In vitro*, protocatechuic acid has been shown to alleviate hydrogen-peroxide induced mitochondrial dysfunction in human neuronal cell lines [401].

Assays that are routinely used to measure oxidative stress usually determine the extent of oxidative damage to different parts of a tissue [339]. Therapeutic reversals of such tissue damage occur over time. A seven-day course of treatment with linagliptin was administered to CPIP rats to demonstrate the antioxidant mechanism of the drug in alleviating mechanical hypersensitivity using an assay that measured lipid peroxidation in the hind paw tissue of the rats [402]. These tests of tissue oxidative damage were unsuitable for assessing the antioxidant effects of topical pentx-pca considering the relatively immediate anti-allodynia it produced. The antioxidant effects of protocatechuic acid in the anti-allodynia produced by topical pentx-pca likely occurred through its impact on more readily reversible processes like microvascular injury, endothelial dysfunction and ROS consumption of vasodilatory NO [399, 400, 403].

Percutaneous measurement of oxygen saturation with visible-light based oximetry was used to assess local tissue oxygenation in the hind paw of the CPIP rats treated with topical pentx-pca. The results obtained were informative of both the pathology in the CPIP hind paw and the impact of topical treatment with pentx-pca. CPIP injury caused a significant decline in hind paw tissue oxygen saturation measured five days post-procedure, which occurred when the rats exhibited maximal mechanical allodynia. This data assessed the local tissue hypoxia that results from CPIP injury-induced microvascular impairment and provided a link between these processes and mechanical hypersensitivity exhibited by the animals. Previous work in CPIP rats has shown evidence of poor microvascular reserve in the animals' injured hind paws since they exhibited a lower degree of post-occlusive reactive hyperemia [359]. An alternate approach to assessing tissue hypoxia has been reported in a study that examined endoneurial hypoxia in mice with partial sciatic nerve ligation using an exogenously administered probe that is activated under conditions of hypoxia (Hypoxyprobe-1) [37]. Endoneurial hypoxia was ascertained after the abundant detection of the activated probe using immunohistochemistry on the injured sciatic nerve sections [37]. Similarly, an increase in the level of the hypoxia-inducible transcription factor (HIF-1), in sciatic nerve samples from these mice, has been used as a marker of endoneurial hypoxia [37]. The percutaneous SaO_2 measurement done on the CPIP rats in this thesis is a non-invasive and non-terminal approach to acquiring similar data on local tissue hypoxia.

Consequences of poor tissue oxygenation have also been reported in the CPIP hind paw, including elevated lactate accumulation and mitochondrial dysfunction [141]. *In vivo* experiments

have reported elevated lactate and diminished ATP levels in the serum and skeletal muscle of dogs after a prolonged arterial occlusion of the posterior limb followed by reperfusion [404]. Clinical studies using non-invasive assessment of oxygen saturation have repeatedly demonstrated local tissue hypoxia in CRPS limbs [38, 114, 341]. The current data, demonstrating low oxygen saturation in the CPIP hind paws, provided further validation of the model in mimicking the local pathologic processes seen in CRPS patients.

In CRPS, the local tissue hypoxia produces downstream metabolic and inflammatory changes that ultimately result in peripheral nociceptor activation/sensitization and pain [49, 381]. Having obtained evidence for the local anti-hypoxic effects of pentx-pca, subsequent experiments investigated the impact of improved oxygenation on hind-paw muscle mitochondrial function. Previous studies in our laboratory have reported hind paw muscle extracted from CPIP rats exhibit hypoxia-driven mitochondrial dysfunction [141, 222]. In these studies, hind paw muscle slices and homogenates were incubated with triphenyl tetrazolium chloride (TTC), a substrate of oxygen-dependent mitochondrial dehydrogenases, that is converted into a red-pigmented product, formazan, whose concentration is spectrophotometrically measurable [141, 222]. Increased formazan levels were measured from the hind paw skin and muscle samples of CPIP rats topically treated with an anti-hypoxic drug combination containing apraclonidine and lisofylline [222]. Using an identical technique, the current study investigated the effect of topical pentx-pca on CPIP-induced mitochondrial dysfunction in the hind-paw muscle. Tissue extraction was performed at a time point when the peak PWT increasing effects of pentx-pca were previously recorded, allowing assessment of the correlation between the effect of the drug on mitochondrial dysfunction and the anti-allodynic effects it produced. Topical treatment with pentx-pca significantly alleviated CPIP-induced mitochondrial dysfunction in the hind paw muscle. A similar tetrazolium salt-based assay has previously been used to demonstrate the impact of treatment with protocatechuic acid in enhancing mitochondrial function in human neuronal cell culture lines [222]. As the TTC assay measures the activity of oxygen-dependent mitochondrial enzymes, the reversal of mitochondrial dysfunction produced with the pentx-pca treatment can be taken as a downstream effect of the drug in enhancing hind paw tissue oxygenation. Improvement in the hind paw tissue mitochondrial function is bound to enhance high energy phosphate metabolism and retard lactic acid generating anaerobic pathways. The accumulation of lactate in CPIP muscle has been previously reported to directly correlate with the degree of mechanical allodynia exhibited

by CPIP rats [141]. Similarly, increased tissue lactate is a feature observed in CRPS affected limbs [114]. Reduction of serum lactate levels along with improved metabolic and circulatory recovery have been reported in rat models of cardiac arrest that received bolus administration of pentoxifylline [405].

In the weeks that follow the CPIP injury, local microvascular dysfunction, and resulting tissue hypoxia gradually resolve and cease to be significant contributors to the allodynia exhibited by the rats [141]. The contrasting anti-allodynic effects of topical pentx-pca at one week versus six weeks post-CPIP provided a further indication that local tissue hypoxia is the therapeutic target of the drug. While topical treatment with pentx-pca alleviated mechanical allodynia in CPIP rats at one week after injury, the drug had no effect at six weeks. In a parallel experiment, oxygen-dependent mitochondrial function was assayed from CPIP hind paw muscle obtained at 1- week versus 6- weeks post-procedure. While the CPIP tissue obtained at 1 week showed significantly impaired oxygen-dependent mitochondrial respiration, such impairment was not evident at 6 weeks post-injury. These results showed a parallel between the resolution of hypoxia driven local processes in CPIP and the attenuation of anti-allodynic responses to topical pentx-pca.

In previous work published by our laboratory, CPIP rats, in the first week after injury, exhibited a decline in microvascular reserve of their affected hind paw along with a robust anti-allodynic response to an anti-hypoxic topical combination made from apraclonidine and lisofylline while showing no analgesic response to systemic pregabalin [222]. After 18 days post-injury, the CPIP-induced decline in microvascular reserve resolved and the anti-allodynic response to the anti-hypoxic topical combination was lost while an anti-allodynic response to systemic pregabalin emerged [222]. This indicated mechanical hypersensitivity in CPIP rats is driven by local ischemic-hypoxia at early time points post-injury, and that it resolves gradually possibly giving way to central mechanisms at later time points. Therefore, it could be asserted that it is local ischemic hypoxia and downstream changes in CPIP rats that are impacted by topical pentx-pca to produce anti-allodynic effects at 1-week post-injury, but not at 6-weeks post-procedure.

Deep tissue ischemic-hypoxia and downstream peripheral processes are reported to contribute to the pain pathology of all phases and subtypes of CRPS, but the severity of peripheral processes vary, as do the presenting clinical features [9]. The resolution of local tissue hypoxia and downstream metabolic changes in CPIP rats, after a few weeks post-procedure, is a feature

specific to this experimental model. Having data that indicates topical pentx-pca is most effective as an analgesic in the presence of local tissue hypoxia provides valuable information for the targeted selection of patients to be treated with this anti-hypoxic/antioxidant drug.

9.8 The development of topical treatment with pentoxifylline & clonidine for clinical use as an analgesic for PNP

The clinical trial protocol on the combination of pentoxifylline and clonidine, for the topical treatment of PNP, is presented as a practical demonstration of the translatability of locally targeting tissue hypoxia as means of achieving analgesia in PNP. Although the two drugs are most commonly administered systemically, the accumulated evidence for the peripheral anti-hypoxic mechanisms of action and safe clinical use of systemic pentoxifylline and clonidine was what led to the selection of the drugs as an analgesic topical combination.

Pentoxifylline alleviates deep tissue hypoxia through a combination of effects it has on both the vascular wall and the blood cells, all summating to facilitate microvascular flow and tissue perfusion. Pentoxifylline has a direct vasodilatory effect on the luminal diameter of both resting small arteries and those pre-constricted with norepinephrine [406]. There is also considerable evidence that pentoxifylline improves the red blood cell (RBC) flow by decreasing viscosity, increasing RBC deformability, and decreasing the aggregation of inflammatory cells [316]. Its therapeutic and preventive efficacy has been reported in vascular conditions like peripheral occlusive arterial disease and cerebrovascular transient ischemic attacks [407]. Clonidine, on the other hand, is a centrally acting antihypertensive with additional peripheral vasodilatory effects, as it inhibits the vasoconstrictive effects of sympathetic efferents innervating the vasculature [217]. It has also been reported to stimulate endothelial NO release to induce vasodilation [319]. Although the contribution of its vascular effects was not implicated, topical clonidine has been repeatedly used as an analgesic for painful diabetic neuropathy, where peripheral nerve vascular impairment is a prominent feature [219]. Considering the complementary mechanisms by which clonidine enhances arterial blood flow, while pentoxifylline increases capillary blood flow to improve tissue oxygenation, their use in combination was hypothesized to efficiently alleviate the ischemic-hypoxia in the peripheral nerves in PNP and potentially result in analgesia [173].

The proposed clinical study for the analgesic use of the topical combination of pentoxifylline and clonidine, in PNP patients, has been preceded by extensive preclinical animal

studies and a healthy human volunteer study that tested the effectiveness of the treatment. The combination has demonstrated efficacy in rat models of post-traumatic, chemotherapy-induced and diabetic PNP [215]. In an experimentally-induced neuropathic pain condition, using a cohort of healthy volunteers, the topical treatment has been shown to alleviate pain and reduce mechanical hypersensitivity [228]. The proposed follow-up phase-2 clinical trial assesses the therapeutic analgesic potential of the topical combination in PNP patients. A recently published case series described the analgesic effect of a compound cream containing pentoxifylline, clonidine, ketamine and DMSO in patients with CRPS [293]. The study reported an audit conducted on 13 CRPS patients treated with the cream. The outcome measures used were patient-reported pain scores, qualitative description of treatment benefit and frequency of concurrent need for additional analgesic medications [293]. In contrast to this open-label study, our study protocol describes a double-blind RCT testing the efficacy of a topical formulation containing pentoxifylline and clonidine in a cohort of post-traumatic PNP patients.

Several considerations have gone into the planning of this clinical trial, all aimed at optimizing the detection of analgesic benefits from the formulation. The study has adopted a double-blind, randomized and placebo-controlled design which is the gold standard for the clinical testing of therapeutic efficacy [408]. Furthermore, confounds that could be introduced by variabilities in patients' disease status have been minimized by putting in place inclusion and exclusion criteria that select for a relatively uniform patient population. The cross-over design of the study, where each patient serves as his/her own control, was also a measure taken to reduce between-subject data variability [409]. Finally, the outcome measures used in the study assess both spontaneous and evoked pain giving a comprehensive assessment of the formulation's analgesic effect.

10. CONCLUSIONS

10.1 Contribution to original knowledge

10.1.1 The topical combination of meldonium-NAC as an effective analgesic for PNP & CRPS

This thesis has introduced a novel topical treatment made from a combination of the drugs meldonium and NAC, characterizing its anti-allodynic effect in rat models of PNP and CRPS. This was a first demonstration of the use of this combination as a topical analgesic. Meldonium has previously been reported to prevent the development of painful peripheral neuropathy in diabetic rats after a long-term systemic administration [355]. Although there are reports of pain-relieving effects seen with systemically administered NAC in CRPS patients, this thesis is the first to study its topical use as an analgesic, alone and in combination with meldonium [16]. The work presented has also discovered a mechanistic explanation for the topical anti-allodynic effects of meldonium-NAC. The experiments uncovered a NO-mediated enhancement in tissue oxygenation produced by the drug combination. This finding has strengthened the concept of pain-alleviation in PNP and CRPS by relieving tissue hypoxia via the manipulation of local tissue NO levels using topical treatments.

10.1.2 Use of mechanochemistry to synthesize anti-hypoxic-antioxidant salts & co-crystals for the treatment of PNP & CRPS

The work presented in this thesis introduces mechanochemistry as a means of synthesizing multicomponent-multitargeted analgesics for PNP and CRPS by combining agents of anti-hypoxic and antioxidant activity into salts and co-crystals. We have reported three novel nutraceutical analgesic salts and co-crystals made by pairing pentoxifylline with protocatechuic acid, clonidine with α-lipoic acid and linsidomine with caffeic acid. This thesis is the first not only to report the successful mechanochemical synthesis of these products, but also to demonstrate the enhanced potency of their topical anti-allodynic effect in a rat model of CRPS. Additionally, the anti-allodynic mechanisms of one of the novel products, the co-crystal of pentoxifylline and protocatechuic, have been revealed to occur through the enhancement of local tissue oxygen saturation and oxygen-dependent mitochondrial function.

10.1.3 The analgesic testing of a topical combination of pentoxifylline & clonidine in patients with post-traumatic PNP

The clinical trial protocol presented in this thesis details the proceedings of the first clinical study assessing the analgesic effects of the topical combination of clonidine and pentoxifylline in patients with PNP. Although a case report summary on early CRPS patients has described the analgesic benefits of a compounded cream containing pentoxifylline, clonidine, ketamine and DMSO, the two anti-hypoxic drugs in combinations have not been tested in patients with PNP [293]. Furthermore, the proposed study is a double-blind, placebo-controlled, RCT with an optimal cross-over design that will potentially provide data to support the clinical use of this topical combination in patients with PNP.

10.2 Limitations and potential mitigations

10.2.1 Animal models and pain outcome measures

The work presented in this thesis mainly utilized the CPIP rat model of CRPS. The underlying mechanism for the CRPS-like phenotype in these rats is an IRI-induced deep tissue ischemic-hypoxia, oxidative stress and inflammation. This is unlikely to represent all CRPS patients, given the heterogeneity in this patient population due to the variable contributary peripheral and central pathologic processes [9]. Additional investigations using another rodent model of CRPS may help bridge this gap. The fracture and cast model of CRPS, in its chronic stages, reflects central mechanisms implicated in CRPS including aberrant microglial activation in the spinal cord, and neuroplastic changes in the brain manifested with structural alterations and impaired emotional and cognitive function [410, 411]. Other features of CRPS involving bone loss and autoimmune changes can also be addressed using this model [412, 413].

Most of the experiments on the mechanism of action studies for the topical agents developed for PNP and CRPS in this thesis were done with the CPIP rat model of CRPS. While it was important to replicate the findings reported in the rat models of PNP, the methods used were too limited in sensitivity to detect the proposed changes in tissue oxygenation and oxidative stress in the endoneurial space of the affected nerves, given the small size of these nerves. Nonetheless, the mechanisms of action of the new topical agents are anticipated to be similar to in both PNP and CRPS given the targeted processes, tissue hypoxia and oxidative stress, occur similarly in the

two conditions barring the extent of tissue involvement [37, 91, 262]. A detailed *ex vivo* examination of the bioenergetic profiles of rodent nerves can be performed with the use of an extracellular flux analyzer and mitochondrial toxins [144]. Such a study on sciatic nerve samples extracted from the partial sciatic nerve ligation mouse models of PNP has revealed enhanced oxygen consumption, increased glycolysis, and evidence of mitochondrial dysfunction like impaired ATP synthase activity and electron transport chain activity in the injured nerves of the mice [144]. The use of such methods could allow for further investigation of the effects of our treatments on metabolic improvement in nerve injury models.

The pain outcome measures utilized in this thesis were evoked tests of mechanical sensitivity. This was mainly because this measure gave more reliable, consistent and easily reproducible behavioral data in the topical analgesic drug testing carried out. While the data obtained provided enough starting evidence required for the therapeutic potential of the mechanisms targeted, the drugs used, and the route of drug administration employed, confirmatory studies would help achieve a well-rounded behavioral characterization of the drug effects. Change in facial expression coded by facial grimace scales and analysis of ultrasonic vocalizations can be utilized to assess the impact of the topical treatments on the emotional aspects of rodent pain levels [414, 415]. There are also free-choice tests like the temperature preference test, where rodents are allowed to freely move on plates of different surface temperature to indicate thermal hypersensitivity, and the conditioned place preference test, where an increase in preference shown by an animal to a previously analgesic-paired chamber is taken as an indicator of relief from ongoing pain [416, 417]. While the reported mechanical sensitivity testing has determined the impact of the proposed treatments on the sensory-discriminative aspects of pain, the use of a combination of some of these non-evoked pain outcome measures will help assess the drugs' effects on the emotional and affective dimensions of pain [240]. While most of these measures of spontaneous or operant behaviors have been completed in studies of systemic drugs, a scale of facial grimace and frequency of vocalization have been reported to accurately and reliably assess the analgesic efficacy of a topical anesthetic treatment in rabbits undergoing ear tattooing [418].

Some sections of this thesis deal with head-to-head comparisons between the dose-response curves of analgesic salts and co-crystals with their individual components. At least three different doses of a parent drug and its salt/co-crystal were utilized to prepare the dose-response

curves and get an estimate of how the pairs compared. While these served the purpose of getting the general picture of how the dose-response profile of the two agents compared, the ideal approach requires the utilization of isobolographic analysis. Although normally used in trials with systemic analgesics, this analysis has been implemented to uncovered analgesic synergy between topical combinations of local anesthetics and opioids in rodents [419, 420]. It is a time and resource-intensive analysis that requires the determination of the effects produced by various doses of the agents to accurately plot and calculate dose-effect relationships that will conclusively define the comparison between the agents of interest [394, 421].

In all the experiments performed for this thesis, the rats utilized were of the male sex. Considering the female predominance in the chronic pain patient population, there is a clear need to include both sexes in both pre-clinical and clinical studies in the field. Rats of both male and female sexes were not used in our experiments due to limitations in resource and time. Regardless, results from a study that assessed sex-differences in CPIP rodents suggest the anti-hypoxic-antioxidant topical combinations proposed in this thesis could potentially be equally effective in female rats. In the study, female CPIP mice exhibited higher serum levels of inflammatory and oxidative stress markers, and lower levels of the antioxidant enzyme superoxide dismutase [422]. These results suggest that oxidative stress and inflammation are underlying mechanisms for CPIP allodynia in both sexes. As the topical agents proposed in this thesis target these processes, there is likely no sex difference in the analgesic effects they produce, if not greater effects in females.

10.2.2 Limits to the applicability of findings

This thesis has proposed topical analgesic treatments for PNP and CPRS that are targeted at the peripheral processes of tissue hypoxia and oxidative stress. The practical implementation of these treatments as single therapies mainly applies to patients that have a reasonably localized disease. Topical analgesics can still serve as adjunct therapies to other treatment modalities in patients with widespread pain symptoms and signs. Given the variability in the extent of disease in PNP and CRPS, there will be a need for careful selection of patients to maximize the benefit of these topical treatments.

Moreover, multiple peripheral pathologic processes variably contribute to the chronic pain symptoms and signs of patients with PNP and CRPS. This makes the topical treatments introduced

in this thesis more useful for the subset of these patient populations with a syndrome subtype that prominently involves microvascular compromise, tissue hypoxia and oxidative stress.

10.3 Final remarks and future directions

The work in this thesis is focused on locally targeting the peripheral processes, tissue hypoxia and oxidative stress, to discover effective topical analgesics for PNP and CRPS. In the first project, a novel topical combination made of the drugs meldonium and NAC was introduced with data that demonstrated its anti-allodynic effects in rat models of PNP and CRPS. The topical combination produced a NO-dependent enhancement in local tissue oxygenation. In the subsequent project, studies were designed to mechanochemically synthesize new salts and co-crystals of anti-hypoxic-antioxidant composition by pairing pentoxifylline with protocatechuic acid, clonidine with α-lipoic acid and linsidomine with caffeic acid. The products exhibited potent topical anti-allodynic effects in CPIP rat models of CRPS. Follow up mechanisms of action studies showed a prototype of the new products, pentx-pca, alleviated local tissue hypoxia and consequent mitochondrial dysfunction. The potential for clinical advancement of the approaches detailed in this work is demonstrated by the phase-II clinical trial protocol included to detail the proceedings of a study assessing the analgesic effects of an anti-hypoxic topical combination of clonidine and pentoxifylline in PNP patients.

The approaches discussed herein have paved the way for further exploration of peripheral mechanisms that can serve as therapeutic targets for PNP and CRPS. Given the multiple interactive neural and non-neural peripheral processes in the pathology of PNP and CRPS, there are more complementary disease mechanisms involving inflammatory mediators, cytokines, neurotrophins, neuropeptides, transduction receptors and channels that can serve as targets for effecting analgesia. In addition, since analgesics with peripheral targets are best administered topically, these endeavors are likely to yield a greater selection of topical analgesic treatments for PNP and CRPS that have a much-improved side effect profiles.

The thesis also gives a practical demonstration of a refined method for preparing multi-targeted analgesic drug combinations. With the use of mechanochemical synthesis more multi-functional and multi-component analgesic salts and co-crystals of better efficacy and potency can be developed. These products will have higher prospects of clinical translation due to the unique opportunities salts and co-crystals offer in terms of simplifying the process of pharmacological

research and development, and investability by providing opportunities for obtaining intellectual rights. Finally, the clinical trial protocol outlining the analgesic testing of a mechanistically diverse anti-hypoxic topical combination in PNP has the potential to yield evidence for the practical translatability of the approaches outlined in this thesis.

11. REFERENCES

** References listed below are for sections 1, 3, 5, 7, 9 and 10.*

1. Treede RD, Rief W, Barke A, Aziz Q, Bennett MI, Benoliel R, et al. Chronic pain as a symptom or a disease: the IASP Classification of Chronic Pain for the International Classification of Diseases (ICD-11). Pain. 2019;160(1):19-27.
2. Goldberg DS, McGee SJ. Pain as a global public health priority. BMC Public Health. 2011;11:770.
3. Enright A, Goucke R. The global burden of pain: the tip of the iceberg? Anesth Analg. 2016; 123(3):529-530.
4. van Hecke O, Austin SK, Khan RA, Smith BH, Torrance N. Neuropathic pain in the general population: A systematic review of epidemiological studies. Pain. 2014;155(4):654-62.
5. Colloca L, Ludman T, Bouhassira D, Baron R, Dickenson AH, Yarnitsky D, et al. Neuropathic pain. Nat Rev Dis Primers. 2017;3:17002.
6. Sloan G, Shillo P, Selvarajah D, Wu J, Wilkinson ID, Tracey I, et al. A new look at painful diabetic neuropathy. Diabetes Research and Clinical Practice. 2018;144:177-91.
7. Hopkins HL, Duggett NA, Flatters SJL. Chemotherapy-induced painful neuropathy: pain-like behaviours in rodent models and their response to commonly used analgesics. Curr Opin Support Palliat Care. 2016;10(2):119-28.
8. Birklein F, Dimova V. Complex regional pain syndrome–up-to-date. Pain Reports. 2017;2(6):e624.
9. Bruehl S. Complex regional pain syndrome. Br Med J. 2015;351:h2730.
10. Bussa M, Mascaro A, Cuffaro L, Rinaldi S. Adult Complex Regional Pain Syndrome Type I: A Narrative Review. PM&R. 2017;9(7):707-19.
11. Bussa M, Guttilla D, Lucia M, Mascaro A, Rinaldi S. Complex regional pain syndrome type I: a comprehensive review. Acta anaesthesiologica Scandinavica. 2015;59(6):685-97.
12. Urits I, Shen AH, Jones MR, Viswanath O, Kaye AD. Complex Regional Pain Syndrome, Current Concepts and Treatment Options. Curr Pain Headache Rep. 2018;22(2):10.
13. Goh EL, Chidambaram S, Ma D. Complex regional pain syndrome: a recent update. Burns & trauma. 2017;5:2.
14. Bruehl S, Ph.D. An Update on the Pathophysiology of Complex Regional Pain Syndrome. Anesthesiology: The Journal of the American Society of Anesthesiologists. 2010;113(3):713-25.
15. Harden RN, Bruehl S, Galer BS, Saltz S, Bertram M, Backonja M, et al. Complex regional pain syndrome: are the IASP diagnostic criteria valid and sufficiently comprehensive? Pain. 1999;83(2):211-9.
16. Perez RS, Zuurmond WW, Bezemer PD, Kuik DJ, van Loenen AC, de Lange JJ, et al. The treatment of complex regional pain syndrome type I with free radical scavengers: a randomized controlled study. Pain. 2003;102(3):297-307.
17. Birklein F, Riedl B, Claus D, Neundorfer B. Pattern of autonomic dysfunction in time course of complex regional pain syndrome. Clin Auton Res. 1998;8(2):79-85.
18. Marinus J, Moseley L, Birklein F, Baron R, Maihöfner C, et al. Clinical features and pathophysiology of complex regional pain syndrome. Lancet neurology. 2011;10:637-48.

19. Treede RD, Jensen TS, Campbell JN, Cruccu G, Dostrovsky JO, Griffin JW, et al. Neuropathic pain: redefinition and a grading system for clinical and research purposes. Neurology. 2008;70(18):1630-5.
20. Scholz J, Finnerup NB, Attal N, Aziz Q, Baron R, Bennett MI, et al. The IASP classification of chronic pain for ICD-11: chronic neuropathic pain. Pain. 2019;160(1):53-9.
21. Failde I, Dueñas M, Ribera MV, Gálvez R, Mico JA, Salazar A, et al. Prevalence of central and peripheral neuropathic pain in patients attending pain clinics in Spain: factors related to intensity of pain and quality of life. J Pain Res. 2018;11:1835-47.
22. Freynhagen R, Bennett MI. Diagnosis and management of neuropathic pain. Br Med J. 2009;339:b3002.
23. Colvin LA, Dougherty PM. Peripheral neuropathic pain: signs, symptoms, mechanisms, and causes: are they linked? Br J Anaesth. 2014;114(3):361-3.
24. Attal N, Fermanian C, Fermanian J, Lanteri-Minet M, Alchaar H, Bouhassira D. Neuropathic pain: are there distinct subtypes depending on the aetiology or anatomical lesion? Pain. 2008;138(2):343-53.
25. Dworkin RH. An overview of neuropathic pain: syndromes, symptoms, signs, and several mechanisms. Clin J Pain. 2002;18(6):343-9.
26. Freynhagen R, Baron R, Gockel U, Tolle TR. painDETECT: a new screening questionnaire to identify neuropathic components in patients with back pain. Curr Med Res Opin. 2006;22(10):1911-20.
27. Bouhassira D, Attal N, Alchaar H, Boureau F, Brochet B, Bruxelle J, et al. Comparison of pain syndromes associated with nervous or somatic lesions and development of a new neuropathic pain diagnostic questionnaire (DN4). Pain. 2005;114(1-2):29-36.
28. Hansson P. Neuropathic pain: clinical characteristics and diagnostic workup. Eur J Pain. 2002;6(SA):47-50.
29. Cruccu G, Truini A. Tools for assessing neuropathic pain. PLoS Med. 2009;6(4):e1000045.
30. Fine PG. Long-term consequences of chronic pain: mounting evidence for pain as a neurological disease and parallels with other chronic disease states. Pain Med. 2011;12(7):996-1004.
31. Attal N, Lanteri-Minet M, Laurent B, Fermanian J, Bouhassira D. The specific disease burden of neuropathic pain: Results of a French nationwide survey. Pain. 2011;152(12):2836-43.
32. Costigan M, Scholz J, Woolf CJ. Neuropathic pain: a maladaptive response of the nervous system to damage. Ann Rev Neurosci. 2009;32:1-32.
33. Meacham K, Shepherd A, Mohapatra DP, Haroutounian S. Neuropathic Pain: Central vs. Peripheral Mechanisms. Curr Pain Headache Rep. 2017;21(6):28.
34. Ludwig J, Baron R. Complex regional pain syndrome: an inflammatory pain condition? Drug Discov Today Dis Mech. 2004;1(4):449-55.
35. Ellis A, Bennett D. Neuroinflammation and the generation of neuropathic pain. British Journal of Anaesthesia. 2013;111(1):26-37.
36. Ostergaard L, Terkelsen AJ, Finnerup NB, Knudsen L, Drasbek KR, Jespersen SN, et al. Capillary dysfunction and impaired tissue oxygenation in complex regional pain syndrome: a hypothesis. Pain. 2014;155(10):1922-6.
37. Lim TK, Shi XQ, Johnson JM, Rone MB, Antel JP, David S, et al. Peripheral Nerve Injury Induces Persistent Vascular Dysfunction and Endoneurial Hypoxia, Contributing to the Genesis of Neuropathic Pain. J Neurosci. 2015;35(8):3346-59.

38. Koban M, Leis S, Schultze-Mosgau S, Birklein F. Tissue hypoxia in complex regional pain syndrome. Pain. 2003;104(1-2):149-57.
39. Oaklander AL, Rissmiller JG, Gelman LB, Zheng L, Chang Y, Gott R. Evidence of focal small-fiber axonal degeneration in complex regional pain syndrome-I (reflex sympathetic dystrophy). Pain. 2006;120(3):235-43.
40. Geertzen JH, Bodde MI, van den Dungen JJ, Dijkstra PU, den Dunnen WF. Peripheral nerve pathology in patients with severely affected complex regional pain syndrome type I. Int J Rehabil Res. 2015;38(2):121-30.
41. Sieweke N, Birklein F, Riedl B, Neundorfer B, Handwerker HO. Patterns of hyperalgesia in complex regional pain syndrome. Pain. 1999;80(1-2):171-7.
42. Mickle AD, Shepherd AJ, Mohapatra DP. Sensory TRP channels: the key transducers of nociception and pain. Prog Mol Biol Transl Sci. 2015;131:73-118.
43. Devor M. Sodium channels and mechanisms of neuropathic pain. J Pain. 2006;7(1):S3-S12.
44. Luo ZD, Chaplan SR, Higuera ES, Sorkin LS, Stauderman KA, Williams ME, et al. Upregulation of dorsal root ganglion (alpha)2(delta) calcium channel subunit and its correlation with allodynia in spinal nerve-injured rats. J Neurosci. 2001;21(6):1868-75.
45. Cohen SP, Mao J. Neuropathic pain: mechanisms and their clinical implications. Br Med J. 2014;348.
46. Hoppa MB, Lana B, Margas W, Dolphin AC, Ryan TA. α2δ expression sets presynaptic calcium channel abundance and release probability. Nature. 2012;486(7401):122-5.
47. Tsantoulas C, McMahon SB. Opening paths to novel analgesics: the role of potassium channels in chronic pain. Trends Neurosci. 2014;37(3):146-58.
48. Evans JA. Sympathectomy for reflex sympathetic dystrophy; report of twenty-nine cases. J Am Med Assoc. 1946;132(11):620-3.
49. Coderre TJ, Bennett GJ. A hypothesis for the cause of complex regional pain syndrome-type I (reflex sympathetic dystrophy): pain due to deep-tissue microvascular pathology. Pain Med. 2010;11(8):1224-38.
50. Coderre TJ. Complex regional pain syndrome: what's in a name? J Pain. 2011;12(1):2-12.
51. Harden RN, Duc TA, Williams TR, Coley D, Cate JC, Gracely RH. Norepinephrine and epinephrine levels in affected versus unaffected limbs in sympathetically maintained pain. Clin J Pain. 1994;10(4):324-30.
52. Wasner G, Schattschneider J, Heckmann K, Maier C, Baron R. Vascular abnormalities in reflex sympathetic dystrophy (CRPS I): mechanisms and diagnostic value. Brain. 2001;124(3):587-99.
53. Bussa M, Lucia M, Mascaro A, Cuffaro L, Bontà T, Rinaldi S. Pathophysiology of Complex Re-gional Pain Syndrome Type I: Update. Int J Anesth Res. 2015;3(7):141-6.
54. Cepeda MS, Lau J, Carr DB. Defining the therapeutic role of local anesthetic sympathetic blockade in complex regional pain syndrome: a narrative and systematic review. Clin J Pain. 2002;18(4):216-33.
55. De Mos M, Sturkenboom MCJM, Huygen FJPM. Current Understandings on Complex Regional Pain Syndrome. Pain Pract. 2009;9(2):86-99.
56. Knudsen LF, Terkelsen AJ, Drummond PD, Birklein F. Complex regional pain syndrome: a focus on the autonomic nervous system. Clin Auton Res. 2019;29(4):457-67.
57. Finch PM, Drummond ES, Dawson LF, Phillips JK, Drummond PD. Up-regulation of cutaneous alpha1 -adrenoceptors in complex regional pain syndrome type I. Pain Medicine. 2014;15(11):1945-56.

58. Sato J, Perl ER. Adrenergic excitation of cutaneous pain receptors induced by peripheral nerve injury. Science. 1991;251(5001):1608.
59. Drummond PD, Morellini N, Finch PM, Birklein F, Knudsen LF. Complex regional pain syndrome: intradermal injection of phenylephrine evokes pain and hyperalgesia in a subgroup of patients with upregulated α: 1:-adrenoceptors on dermal nerves. Pain. 2018;159(11):2296-305.
60. Calvo M, Dawes JM, Bennett DLH. The role of the immune system in the generation of neuropathic pain. The Lancet Neurology. 2012;11(7):629-42.
61. Pinho-Ribeiro FA, Verri WA, Jr., Chiu IM. Nociceptor Sensory Neuron-Immune Interactions in Pain and Inflammation. Trends Immunol. 2017;38(1):5-19.
62. Zhang J. Neuroinflammation: New Insights into Beneficial and Detrimental Functions. 1st ed. Hoboken (NJ). c2015. Chapter 7, Peripheral and Central Immune Mechanisms in Neuropathic Pain; p. 107-21.
63. Moalem G, Xu K, Yu L. T lymphocytes play a role in neuropathic pain following peripheral nerve injury in rats. Neuroscience. 2004;129(3):767-77.
64. Matsushita K, Tozaki-Saitoh H, Kojima C, Masuda T, Tsuda M, Inoue K, et al. Chemokine (C-C motif) receptor 5 is an important pathological regulator in the development and maintenance of neuropathic pain. Anesthesiology. 2014;120(6):1491-503.
65. Zhang J, Shi XQ, Echeverry S, Mogil JS, De Koninck Y, Rivest S. Expression of CCR2 in both resident and bone marrow-derived microglia plays a critical role in neuropathic pain. J Neurosci. 2007;27(45):12396-406.
66. Uceyler N, Eberle T, Rolke R, Birklein F, Sommer C. Differential expression patterns of cytokines in complex regional pain syndrome. Pain. 2007;132(1-2):195-205.
67. Schinkel C, Gaertner A, Zaspel J, Zedler S, Faist E, Schuermann M. Inflammatory mediators are altered in the acute phase of posttraumatic complex regional pain syndrome. Clin J Pain. 2006;22(3):235-9.
68. Alexander GM, van Rijn MA, van Hilten JJ, Perreault MJ, Schwartzman RJ. Changes in cerebrospinal fluid levels of pro-inflammatory cytokines in CRPS. Pain. 2005;116(3):213-9.
69. Ritz BW, Alexander GM, Nogusa S, Perreault MJ, Peterlin BL, Grothusen JR, et al. Elevated blood levels of inflammatory monocytes (CD14+ CD16+) in patients with complex regional pain syndrome. Clin Exp Immunol. 2011;164(1):108-17.
70. Huygen FJ, Ramdhani N, van Toorenenbergen A, Klein J, Zijlstra FJ. Mast cells are involved in inflammatory reactions during Complex Regional Pain Syndrome type 1. Immunol Lett. 2004;91(2-3):147-54.
71. Dallos A, Kiss M, Polyánka H, Dobozy A, Kemény L, Husz S. Effects of the neuropeptides substance P, calcitonin gene-related peptide, vasoactive intestinal polypeptide and galanin on the production of nerve growth factor and inflammatory cytokines in cultured human keratinocytes. Neuropeptides. 2006;40(4):251-63.
72. Leis S, Weber M, Isselmann A, Schmelz M, Birklein F. Substance-P-induced protein extravasation is bilaterally increased in complex regional pain syndrome. Exp Neurol. 2003;183(1):197-204.
73. Holzer P. Neurogenic vasodilatation and plasma leakage in the skin. Gene Pharmacol Vascul Syst. 1998;30(1):5-11.
74. Maihöfner C, Handwerker HO, Neundörfer B, Birklein F. Mechanical hyperalgesia in complex regional pain syndrome: A role for TNF-α? Neurology. 2005;65(2):311-3.

75. Goebel A, Leite MI, Yang L, Deacon R, Cendan CM, Fox-Lewis A, et al. The passive transfer of immunoglobulin G serum antibodies from patients with longstanding Complex Regional Pain Syndrome. Eur J Pain. 2011;15(5):504.e1-6.
76. Staiculescu MC, Foote C, Meininger GA, Martinez-Lemus LA. The role of reactive oxygen species in microvascular remodeling. Int J Mol Sci. 2014;15(12):23792-835.
77. Martinez-Lemus LA. The dynamic structure of arterioles. Basic Clin Pharmacol Toxicol. 2012;110(1):5-11.
78. Stanton-Hicks M. Anatomy and Physiology Related to Peripheral Nerve Stimulation. In: Neuromodulation: Comprehensive Textbook of Principles, Technologies, and Therapies. 2nd ed. Vol 1. London. Elsevier Academic Press. c2018.; p. 723-7.
79. Yagihashi S, Mizukami H, Sugimoto K. Mechanism of diabetic neuropathy: Where are we now and where to go? J Diabetes Investig. 2011;2(1):18-32.
80. Zochodne DW. Local blood flow in peripheral nerves and their ganglia: Resurrecting key ideas around its measurement and significance. Muscle & Nerve. 2018;57(6):884-95.
81. Feletou M. The Endothelium: Part 1: Multiple Functions of the Endothelial Cells-Focus on Endothelium-Derived Vasoactive Mediators. In: Integrated Systems Physiology: from Molecule to Function to Disease. San Rafael (CA): Morgan & Claypool Life Sciences; 2011.
82. Galley HF, Webster NR. Physiology of the endothelium. Br J Anaesth. 2004;93(1):105-13.
83. Palmer RM, Ashton D, Moncada S. Vascular endothelial cells synthesize nitric oxide from L-arginine. Nature. 1988;333(6174):664-6.
84. Deanfield JE, Halcox JP, Rabelink TJ. Endothelial function and dysfunction: testing and clinical relevance. Circulation. 2007;115(10):1285-95.
85. Mitchell JA, Ali F, Bailey L, Moreno L, Harrington LS. Role of nitric oxide and prostacyclin as vasoactive hormones released by the endothelium. Exp Physiol. 2008;93(1):141-7.
86. Alonso D, Radomski MW. The nitric oxide-endothelin-1 connection. Heart Fail Rev. 2003;8(1):107-15.
87. Mackinnon SE, Dellon AL, Hudson AR, Hunter DA. Chronic human nerve compression--a histological assessment. Neuropathol Appl Neurobiol. 1986;12(6):547-65.
88. Sunderland S. The nerve lesion in the carpal tunnel syndrome. J Neurol Neurosurg Psychiatry. 1976;39(7):615-26.
89. Schreiber AK, Nones CFM, Reis RC, Chichorro JG, Cunha JM. Diabetic neuropathic pain: Physiopathology and treatment. World J Diabetes. 2015;6(3):432-44.
90. Sommer C, Myers RR. Vascular Pathology in CCL Neuropathy: A Quantitative Temporal Study. Exp Neurol. 1996;141(1):113-9.
91. Cameron NE, Eaton SE, Cotter MA, Tesfaye S. Vascular factors and metabolic interactions in the pathogenesis of diabetic neuropathy. Diabetologia. 2001;44(11):1973-88.
92. Malik RA, Tesfaye S, Thompson SD, Veves A, Sharma AK, Boulton AJM, et al. Endoneurial localisation of microvascular damage in human diabetic neuropathy. Diabetologia. 1993;36(5):454-9.
93. Quattrini C, Harris ND, Malik RA, Tesfaye S. Impaired skin microvascular reactivity in painful diabetic neuropathy. Diabetes Care. 2007;30(3):655-9.
94. Malik RA, Tesfaye S, Newrick PG, Walker D, Rajbhandari SM, Siddique I, et al. Sural nerve pathology in diabetic patients with minimal but progressive neuropathy. Diabetologia. 2005;48(3):578-85.
95. Kihara M, Zollman PJ, Smithson IL, Lagerlund TD, Low PA. Hypoxic effect of exogenous insulin on normal and diabetic peripheral nerve. Am J physiol. 1994;266(6 Pt 1):E980-5.

96. Newrick PG, Wilson AJ, Jakubowski J, Boulton AJ, Ward JD. Sural nerve oxygen tension in diabetes. BMJ (Clin Res Ed). 1986;293(6554):1053-4.
97. Ibrahim S, Harris ND, Radatz M, Selmi F, Rajbhandari S, Brady L, et al. A new minimally invasive technique to show nerve ischaemia in diabetic neuropathy. Diabetologia. 1999;42(6):737-42.
98. Van Dam PS, Cotter MA, Bravenboer B, Cameron NE. Pathogenesis of diabetic neuropathy: Focus on neurovascular mechanisms. Eur J Pharmacol. 2013;719(1):180-6.
99. Maxfield EK, Cameron NE, Cotter MA. Effects of diabetes on reactivity of sciatic vasa nervorum in rats. J Diabetes Complications. 1997;11(1):47-55.
100. Zajączkowska R, Kocot-Kępska M, Leppert W, Wrzosek A, et al. Mechanisms of Chemotherapy-Induced Peripheral Neuropathy. Int J Mol Sci. 2019;20(6):1451.
101. Kirchmair R, Walter DH, Ii M, Rittig K, Tietz AB, Murayama T, et al. Antiangiogenesis mediates cisplatin-induced peripheral neuropathy: attenuation or reversal by local vascular endothelial growth factor gene therapy without augmenting tumor growth. Circulation. 2005;111(20):2662-70.
102. Kirchmair R, Tietz AB, Panagiotou E, Walter DH, Silver M, Yoon Y-S, et al. Therapeutic angiogenesis inhibits or rescues chemotherapy-induced peripheral neuropathy: taxol-and thalidomide-induced injury of vasa nervorum is ameliorated by VEGF. Mol Ther. 2007;15(1):69-75.
103. Groeneweg G, Niehof S, Wesseldijk F, Huygen F, Zijlstra FJ. Vasodilative effect of lsosorbide dinitrate ointment in complex regional pain syndrome type 1. Clin J Pain. 2008;24(1):89-92.
104. Dayan L, Salman S, Norman D, Vatine JJ, Calif E, Jacob G. Exaggerated vasoconstriction in complex regional pain syndrome-1 is associated with impaired resistance artery endothelial function and local vascular reflexes. J Rheumatol. 2008;35(7):1339-45.
105. Groeneweg JG, Huygen FJ, Heijmans-Antonissen C, Niehof S, Zijlstra FJ. Increased endothelin-1 and diminished nitric oxide levels in blister fluids of patients with intermediate cold type complex regional pain syndrome type 1. BMC Musculoskelet Disord. 2006;7:91.
106. Schattschneider J, Hartung K, Stengel M, Ludwig J, Binder A, Wasner G, et al. Endothelial dysfunction in cold type complex regional pain syndrome. Neurology. 2006;67(4):673-5.
107. Berghoff M, Kathpal M, Kilo S, Hilz MJ, Freeman R. Vascular and neural mechanisms of ACh-mediated vasodilation in the forearm cutaneous microcirculation. J Appl Physiol (1985). 2002;92(2):780-8.
108. Van der Laan L, Ter Laak H, Gabreels-Festen A, Gabreels F, Goris R. Complex regional pain syndrome type I (RSD): pathology of skeletal muscle and peripheral nerve. Neurology. 1998;51(1):20-5.
109. Coderre TJ, Xanthos DN, Francis L, Bennett GJ. Chronic post-ischemia pain (CPIP): a novel animal model of complex regional pain syndrome-Type I (CRPS-I; reflex sympathetic dystrophy) produced by prolonged hindpaw ischemia and reperfusion in the rat. Pain. 2004;112(1–2):94-105.
110. Albrecht PJ, Hines S, Eisenberg E, Pud D, Finlay DR, Connolly MK, et al. Pathologic alterations of cutaneous innervation and vasculature in affected limbs from patients with complex regional pain syndrome. Pain. 2006;120(3):244-66.
111. Matsumura H, Jimbo Y, Watanabe K. Haemodynamic changes in early phase reflex sympathetic dystrophy. Scand J Plast Reconstr Surg Hand Surg. 1996;30(2):133-8.

112. Tan EC, Ter Laak HJ, Hopman MT, van Goor H, Goris RJA. Impaired oxygen utilization in skeletal muscle of CRPS I patients. J Surg Res. 2012;173(1):145-52.
113. Heerschap A, Hollander JAD, Reynen H, Goris RJA. Metabolic changes in reflex sympathetic dystrophy: A 31P NMR spectroscopy study. Muscle Nerve. 1993;16(4):367-73.
114. Birklein F, Weber M, Neundörfer B. Increased skin lactate in complex regional pain syndrome: Evidence for tissue hypoxia? Neurology. 2000;55(8):1213-5.
115. Birklein F, Weber M, Ernst M, Riedl B, Neundorfer B, Handwerker HO. Experimental tissue acidosis leads to increased pain in complex regional pain syndrome (CRPS). Pain. 2000;87(2):227-34.
116. Basbaum AI, Bautista DM, Scherrer G, Julius D. Cellular and Molecular Mechanisms of Pain. Cell. 2009;139(2):267-84.
117. Immke DC, McCleskey EW. Protons open acid-sensing ion channels by catalyzing relief of Ca2+ blockade. Neuron. 2003;37(1):75-84.
118. Immke D, McCleskey E. Lactate enhances the acid-sensing Na+ channel on ischemia-sensing neurons. Nature Neuroscience. 2001;4(9):869-70.
119. Birben E, Sahiner UM, Sackesen C, Erzurum S, Kalayci O. Oxidative stress and antioxidant defense. World Allergy Organ J. 2012;5(1):9-19.
120. Figueroa-Romero C, Sadidi M, Feldman EL. Mechanisms of disease: the oxidative stress theory of diabetic neuropathy. Rev Endocr Metab Disord. 2008;9(4):301-14.
121. Martinez MC, Andriantsitohaina R. Reactive nitrogen species: molecular mechanisms and potential significance in health and disease. Antioxid. Redox Signal. 2009;11(3):669-702.
122. Halliwell B. How to Characterize a Biological Antioxidant. Free Radic Res Commun. 1990;9(1):1-32.
123. Forman HJ, Zhang H, Rinna A. Glutathione: overview of its protective roles, measurement, and biosynthesis. Mol Aspects Med. 2009;30(1-2):1-12.
124. Naik AK, Tandan SK, Dudhgaonkar SP, Jadhav SH, Kataria M, Prakash VR, et al. Role of oxidative stress in pathophysiology of peripheral neuropathy and modulation by N-acetyl-L-cysteine in rats. Eur J Pain. 2006;10(7):573-9.
125. Areti A, Yerra VG, Naidu V, Kumar A. Oxidative stress and nerve damage: role in chemotherapy induced peripheral neuropathy. Redox Biol. 2014;2:289-95.
126. Janes K, Doyle T, Bryant L, Esposito E, Cuzzocrea S, Ryerse J, et al. Bioenergetic deficits in peripheral nerve sensory axons during chemotherapy-induced neuropathic pain resulting from peroxynitrite-mediated post-translational nitration of mitochondrial superoxide dismutase. Pain. 2013;154(11):2432-40.
127. Ding R, Jiang H, Sun B, Wu X, Li W, Zhu S, et al. Advanced oxidation protein products sensitized the transient receptor potential vanilloid 1 via NADPH oxidase 1 and 4 to cause mechanical hyperalgesia. Redox Biol. 2016;10:1-11.
128. Chuang H-h, Lin S. Oxidative challenges sensitize the capsaicin receptor by covalent cysteine modification. PNAS. 2009;106(47):20097.
129. Zhang X, Guan Z, Wang X, Sun D, Wang D, et al. Curcumin Alleviates Oxaliplatin-Induced Peripheral Neuropathic Pain through Inhibiting Oxidative Stress-Mediated Activation of NF-kappaB and Mitigating Inflammation. Biol Pharm Bull. 2020;43(2):348-55.
130. Jain K. Drug-induced neurological disorders. 3rd ed. Toronto. Hogrefe Publishing; c2012. Chapter 11, Drug-induced peripheral neuropathies; p:237-62.
131. Low PA, Nickander KK, Tritschler HJ. The roles of oxidative stress and antioxidant treatment in experimental diabetic neuropathy. Diabetes. 1997;46:S38-42.

132. Vincent AM, Russell JW, Low P, Feldman EL. Oxidative Stress in the Pathogenesis of Diabetic Neuropathy. Endocr Rev. 2004;25(4):612-28.
133. Wang X, Zhang G, Qiao Y, Feng C, Zhao X. Crocetin attenuates spared nerve injury-induced neuropathic pain in mice. J Pharmacol Sci. 2017;135(4):141-7.
134. Tan EC, Janssen AJ, Roestenberg P, van den Heuvel LP, Goris RJ, Rodenburg RJ. Mitochondrial dysfunction in muscle tissue of complex regional pain syndrome type I patients. Eur J Pain. 2011;15(7):708-15.
135. Eisenberg E, Shtahl S, Geller R, Reznick AZ, Sharf O, Ravbinovich M, et al. Serum and salivary oxidative analysis in Complex Regional Pain Syndrome. Pain. 2008;138(1):226-32.
136. Taha R, Blaise GA. Update on the pathogenesis of complex regional pain syndrome: role of oxidative stress. Can J Anaesth. 2012;59(9):875-81.
137. Lejay A, Meyer A, Schlagowski AI, Charles AL, Singh F, Bouitbir J, et al. Mitochondria: mitochondrial participation in ischemia-reperfusion injury in skeletal muscle. Int J Biochem Cell Biol. 2014;50:101-5.
138. Zollinger PE, Tuinebreijer WE, Breederveld RS, Kreis RW. Can vitamin C prevent complex regional pain syndrome in patients with wrist fractures? A randomized, controlled, multicenter dose-response study. The Journal of Bone and Joint Surgery. 2007;89(7):1424-31.
139. O'Connell NE, Wand BM, McAuley J, Marston L, Moseley GL. Interventions for treating pain and disability in adults with complex regional pain syndrome. The Cochrane database of Systematic Reviews. 2013(4):Cd009416.
140. Van der Laan L, Kapitein P, Verhofstad A, Hendriks T, Goris R. Clinical signs and symptoms of acute reflex sympathetic dystrophy in one hindlimb of the rat, induced by infusion of a free-radical donor. Acta Orthop Belg. 1998;64(2):210-7.
141. Laferrière A, Millecamps M, Xanthos DN, Xiao WH, Siau C, de Mos M, et al. Cutaneous tactile allodynia associated with microvascular dysfunction in muscle. Mol Pain. 2008;4:49.
142. Groeneweg G, Huygen FJ, Coderre TJ, Zijlstra FJ. Regulation of peripheral blood flow in complex regional pain syndrome: clinical implication for symptomatic relief and pain management. BMC Musculoskeletal Disorders. 2009;10:116.
143. Cameron NE, Eaton SEM, Cotter MA, Tesfaye S. Vascular factors and metabolic interactions in the pathogenesis of diabetic neuropathy. Diabetologia. 2001;44(11):1973-88.
144. Lim TK, Rone MB, Lee S, Antel JP, Zhang J. Mitochondrial and bioenergetic dysfunction in trauma-induced painful peripheral neuropathy. Mol Pain. 2015;11(1):58.
145. Finnerup NB, Attal N, Haroutounian S, McNicol E, Baron R, Dworkin RH, et al. Pharmacotherapy for neuropathic pain in adults: a systematic review and meta-analysis. Lancet Neurol. 2015;14(2):162-73.
146. Attal N. Pharmacological treatments of neuropathic pain: The latest recommendations. Rev Neurol (Paris). 2019;175(1-2):46-50.
147. Alles SRA, Smith PA. Etiology and Pharmacology of Neuropathic Pain. Pharmacol Rev. 2018;70(2):315-47.
148. Attal N, Bouhassira D. Pharmacotherapy of neuropathic pain: which drugs, which treatment algorithms? Pain. 2015;156 Suppl 1:S104-14.
149. Chincholkar M. Analgesic mechanisms of gabapentinoids and effects in experimental pain models: a narrative review. Br J Anaesth. 2018;120(6):1315-34.
150. Chincholkar M. Gabapentinoids: pharmacokinetics, pharmacodynamics and considerations for clinical practice. Br J Pain. 2020:2049463720912496.

151. Baron R, Allegri M, Correa-Illanes G, Hans G, Serpell M, Mick G, et al. The 5% Lidocaine-Medicated Plaster: Its Inclusion in International Treatment Guidelines for Treating Localized Neuropathic Pain, and Clinical Evidence Supporting its Use. Pain Ther. 2016;5(2):149-69.
152. Sommer C, Cruccu G. Topical Treatment of Peripheral Neuropathic Pain: Applying the Evidence. J Pain Symptom Manage. 2017;53(3):614-29.
153. Edlund MJ, Martin BC, Russo JE, DeVries A, Braden JB, Sullivan MD. The role of opioid prescription in incident opioid abuse and dependence among individuals with chronic noncancer pain: the role of opioid prescription. The Clinical Journal of Pain. 2014;30(7):557-64.
154. Bannister K, Sachau J, Baron R, Dickenson AH. Neuropathic Pain: Mechanism-Based Therapeutics. Annu Rev Pharmacol Toxicol. 2020;60:257-74.
155. Attal N, de Andrade DC, Adam F, Ranoux D, Teixeira MJ, Galhardoni R, et al. Safety and efficacy of repeated injections of botulinum toxin A in peripheral neuropathic pain (BOTNEP): a randomised, double-blind, placebo-controlled trial. Lancet Neurol. 2016;15(6):555-65.
156. Turner-Stokes L, Goebel A. Complex regional pain syndrome in adults: concise guidance. Clin Med (Lond). 2011;11(6):596-600.
157. Perez RS, Zollinger PE, Dijkstra PU, Thomassen-Hilgersom IL, Zuurmond WW, Rosenbrand KC, et al. Evidence based guidelines for complex regional pain syndrome type 1. BMC Neurol. 2010;10:20.
158. Harden RN, Oaklander AL, Burton AW, Perez RS, Richardson K, Swan M, et al. Complex regional pain syndrome: practical diagnostic and treatment guidelines, 4th edition. Pain Med. 2013;14(2):180-229.
159. Livingstone JA, Atkins RM. Intravenous regional guanethidine blockade in the treatment of post-traumatic complex regional pain syndrome type 1 (algodystrophy) of the hand. J Bone Joint Surg Br. 2002;84(3):380-6.
160. O'Connell NE, Wand BM, Gibson W, Carr DB, Birklein F, Stanton TR. Local anaesthetic sympathetic blockade for complex regional pain syndrome. Cochrane Database Syst Rev. 2016;7:Cd004598.
161. Poetker DM, Reh DD. A comprehensive review of the adverse effects of systemic corticosteroids. Otolaryngol. Clin. North Am. 2010;43(4):753-68.
162. Rand SE, Basu S, Khalid S. Complex Regional Pain Syndrome: Current Diagnostic and Treatment Considerations. Curr Sports Med Rep. 2019;18(9):325-9.
163. Jensen TS, Backonja M-M, Jiménez SH, Tesfaye S, Valensi P, Ziegler D. New perspectives on the management of diabetic peripheral neuropathic pain. Diab Vasc Dis Res. 2006;3(2):108-19.
164. Schmader KE, Baron R, Haanpaa ML, Mayer J, O'Connor AB, Rice AS, et al. Treatment considerations for elderly and frail patients with neuropathic pain. Mayo Clin Proc. 2010;85(3 Suppl):S26-32.
165. Haanpää ML, Gourlay GK, Kent JL, Miaskowski C, Raja SN, Schmader KE, et al. Treatment considerations for patients with neuropathic pain and other medical comorbidities. Mayo Clin Proc. 2010;85(3 Suppl):S15-S25.
166. Sindrup SH, Finnerup NB, Jensen TS. Tailored treatment of peripheral neuropathic pain. Pain. 2012;153(9):1781-2.
167. Woolf CJ, Bennett GJ, Doherty M, Dubner R, Kidd B, Koltzenburg M, et al. Towards a mechanism-based classification of pain? Pain. 1998;77(3):227-9.

168. Walsh DA, McWilliams DF. Pain in rheumatoid arthritis. Curr Pain Headache Rep. 2012;16(6):509-17.
169. Woolf Clifford J, M.D., Ph.D., Max Mitchell B, M.D. Mechanism-based Pain Diagnosis: Issues for Analgesic Drug Development. Anesthesiology. 2001;95(1):241-9.
170. Campbell CM, Kipnes MS, Stouch BC, Brady KL, Kelly M, Schmidt WK, et al. Randomized control trial of topical clonidine for treatment of painful diabetic neuropathy. Pain. 2012;153(9):1815-23.
171. Jensen TS, Finnerup NB. Allodynia and hyperalgesia in neuropathic pain: clinical manifestations and mechanisms. Lancet Neurol. 2014;13(9):924-35.
172. Richards N, McMahon SB. Targeting novel peripheral mediators for the treatment of chronic pain. Br J Anaesth. 2013;111(1):46-51.
173. Coderre TJ. Topical drug therapeutics for neuropathic pain. Expert Opin Pharmacother. 2018;19(11):1211-20.
174. Mick G, Baron R, Finnerup NB, Hans G, Kern KU, Brett B, et al. What is localized neuropathic pain? A first proposal to characterize and define a widely used term. Pain Manag. 2012;2(1):71-7.
175. Leffler A, Fischer MJ, Rehner D, Kienel S, Kistner K, Sauer SK, et al. The vanilloid receptor TRPV1 is activated and sensitized by local anesthetics in rodent sensory neurons. J Clin Invest. 2008;118(2):763-76.
176. Brau ME, Vogel W, Hempelmann G. Fundamental properties of local anesthetics: half-maximal blocking concentrations for tonic block of Na+ and K+ channels in peripheral nerve. Anesth Analg. 1998;87(4):885-9.
177. Nagy I, Friston D, Valente JS, Torres Perez JV, Andreou AP. Pharmacology of the capsaicin receptor, transient receptor potential vanilloid type-1 ion channel. Prog Drug Res. 2014;68:39-76.
178. Anand P, Bley K. Topical capsaicin for pain management: therapeutic potential and mechanisms of action of the new high-concentration capsaicin 8% patch. Br J Anaesth. 2011;107(4):490-502.
179. Kopsky DJ, Keppel Hesselink JM. Phenytoin Cream for the Treatment for Neuropathic Pain: Case Series. Pharmaceuticals (Basel). 2018;11(2).
180. Kern KU, Weiser T. Topical ambroxol for the treatment of neuropathic pain. An initial clinical observation. Schmerz. 2015;29 Suppl 3:S89-96.
181. Hiom S, Patel GK, Newcombe RG, Khot S, Martin C. Severe postherpetic neuralgia and other neuropathic pain syndromes alleviated by topical gabapentin. Br J Dermatol. 2015;173(1):300-2.
182. Kopsky DJ, Keppel Hesselink JM, Bhaskar A, Hariton G, Romanenko V, Casale R. Analgesic effects of topical ketamine. Minerva Anestesiol. 2015;81(4):440-9.
183. Ushida T, Tani T, Kanbara T, Zinchuk VS, Kawasaki M, Yamamoto H. Analgesic effects of ketamine ointment in patients with complex regional pain syndrome type 1. Reg Anesth Pain Med. 2002;27(5):524-8.
184. Sawynok J. Topical and peripheral ketamine as an analgesic. Anesth Analg. 2014;119(1):170-8.
185. Quan D, Wellish M, Gilden DH. Topical ketamine treatment of postherpetic neuralgia. Neurology. 2003;60(8):1391-2.
186. Gammaitoni A, Gallagher RM, Welz-Bosna M. Topical ketamine gel: possible role in treating neuropathic pain. Pain Medicine. 2000;1(1):97-100.

187. Durham MJ, Mekhjian HS, Goad JA, Lou M, Ding M, Richeimer SH. Topical Ketamine in the Treatment of Complex Regional Pain Syndrome. Int J Pharm Compd. 2018;22(2):172-5.
188. Stander S, Gunzer M, Metze D, Luger T, Steinhoff M. Localization of mu-opioid receptor 1A on sensory nerve fibers in human skin. Regul Pept. 2002;110(1):75-83.
189. Guindon J, Beaulieu P. The role of the endogenous cannabinoid system in peripheral analgesia. Curr Mol Pharmacol. 2009;2(1):134-9.
190. Casale R, Symeonidou Z, Bartolo M. Topical Treatments for Localized Neuropathic Pain. Current Pain and Headache Reports. 2017;21(3):15.
191. Thompson DF, Brooks KG. Systematic review of topical amitriptyline for the treatment of neuropathic pain. J Clin Pharm Ther. 2015;40(5):496-503.
192. Liebregts R, Kopsky DJ, Hesselink JM. Topical amitriptyline in post-traumatic neuropathic pain. J Pain Symptom Manage. 2011;41(4):e6-7.
193. Kopsky DJ, Hesselink JM. High doses of topical amitriptyline in neuropathic pain: two cases and literature review. Pain Pract. 2012;12(2):148-53.
194. McCleane G. Topical application of doxepin hydrochloride can reduce the symptoms of complex regional pain syndrome: a case report. Injury. 2002;33(1):88-9.
195. McCleane G. Topical application of doxepin hydrochloride, capsaicin and a combination of both produces analgesia in chronic human neuropathic pain: a randomized, double-blind, placebo-controlled study. Br J Clin Pharmacol. 2000;49(6):574-9.
196. Kopsky DJ, Keppel Hesselink JM. Neuropathic pain as a result of acromegaly, treated with topical baclofen cream. J Pain Symptom Manage. 2013;46(4):e4-5.
197. Phan NQ, Siepmann D, Gralow I, Stander S. Adjuvant topical therapy with a cannabinoid receptor agonist in facial postherpetic neuralgia. J Dtsch Dermatol Ges. 2010;8(2):88-91.
198. Lynch ME, Clark AJ, Sawynok J, Sullivan MJ. Topical amitriptyline and ketamine in neuropathic pain syndromes: an open-label study. J Pain. 2005;6(10):644-9.
199. Sawynok J, Zinger C. Topical amitriptyline and ketamine for post-herpetic neuralgia and other forms of neuropathic pain. Expert Opin Pharmacother. 2016;17(4):601-9.
200. Barton DL, Wos EJ, Qin R, Mattar BI, Green NB, Lanier KS, et al. A double-blind, placebo-controlled trial of a topical treatment for chemotherapy-induced peripheral neuropathy: NCCTG trial N06CA. Support Care Cancer. 2011;19(6):833-41.
201. Mao J, Gold MS, Backonja MM. Combination drug therapy for chronic pain: a call for more clinical studies. J Pain. 2011;12(2):157-66.
202. Sommer C, Leinders M, Uceyler N. Inflammation in the pathophysiology of neuropathic pain. Pain. 2018;159(3):595-602.
203. Lin C-R, Amaya F, Barrett L, Wang H, Takada J, Samad TA, et al. Prostaglandin E2 receptor EP4 contributes to inflammatory pain hypersensitivity. J Pharmacol Exp Ther. 2006;319(3):1096-103.
204. Mason L, Moore RA, Edwards JE, Derry S, McQuay HJ. Topical NSAIDs for chronic musculoskeletal pain: systematic review and meta-analysis. BMC Musculoskeletal Disord. 2004;5(1):28.
205. De Benedittis G, Lorenzetti A. Topical aspirin/diethyl ether mixture versus indomethacin and diclofenac/diethyl ether mixtures for acute herpetic neuralgia and postherpetic neuralgia: a double-blind crossover placebo-controlled study. Pain. 1996;65(1):45-51.
206. Bareggi SR, Pirola R, De Benedittis G. Skin and plasma levels of acetylsalicylic acid: a comparison between topical aspirin/diethyl ether mixture and oral aspirin in acute herpes zoster and postherpetic neuralgia. Eur J Clin Pharmacol. 1998;54(3):231-5.

207. Ahmed SU, Zhang Y, Chen L, Cohen A, St Hillary K, Vo T, et al. Effect of 1.5% Topical Diclofenac on Clinical Neuropathic Pain. Anesthesiology. 2015;123(1):191-8.
208. Mensah-Nyagan AG, Meyer L, Schaeffer V, Kibaly C, Patte-Mensah C. Evidence for a key role of steroids in the modulation of pain. Psychoneuroendocrinology. 2009;34:S169-S77.
209. Devor M, Govrin-Lippmann R, Raber P. Corticosteroids suppress ectopic neural discharge originating in experimental neuromas. Pain. 1985;22(2):127-37.
210. Cushman DM, Ofek E, Syed RH, Clements N, Gardner JE, Sams JM, et al. Comparison of Varying Corticosteroid Type, Dose, and Volume for the Treatment of Pain in Small- and Intermediate-Size Joint Injections: A Narrative Review. PM R. 2019;11(7):758-70.
211. Bellamy N, Campbell J, Robinson V, Gee T, Bourne R, Wells G. Intraarticular corticosteroid for treatment of osteoarthritis of the knee. Cochrane Database Syst Rev. 2006;2:Cd005328.
212. Chesterton LS, Blagojevic-Bucknall M, Burton C, Dziedzic KS, Davenport G, Jowett SM, et al. The clinical and cost-effectiveness of corticosteroid injection versus night splints for carpal tunnel syndrome (INSTINCTS trial): an open-label, parallel group, randomised controlled trial. Lancet. 2018;392(10156):1423-33.
213. Johansson A, Bennett GJ. Effect of local methylprednisolone on pain in a nerve injury model. A pilot study. Reg Anesth. 1997;22(1):59-65.
214. Xanthos DN, Bennett GJ, Coderre TJ. Norepinephrine-induced nociception and vasoconstrictor hypersensitivity in rats with chronic post-ischemia pain. Pain. 2008;137(3):640-51.
215. Ragavendran JV, Laferriere A, Xiao WH, Bennett GJ, Padi SS, Zhang J, et al. Topical combinations aimed at treating microvascular dysfunction reduce allodynia in rat models of CRPS-I and neuropathic pain. J Pain. 2013;14(1):66-78.
216. Li C, Sekiyama H, Hayashida M, Takeda K, Sumida T, Sawamura S, et al. Effects of topical application of clonidine cream on pain behaviors and spinal Fos protein expression in rat models of neuropathic pain, postoperative pain, and inflammatory pain. Anesthesiology. 2007;107(3):486-94.
217. Szabo B, Hedler L, Starke K. Peripheral presynaptic and central effects of clonidine, yohimbine and rauwolscine on the sympathetic nervous system in rabbits. Naunyn Schmiedebergs Arch Pharmacol. 1989;340(6):648-57.
218. Davis K, Treede R, Raja SN, Meyer R, Campbell JN. Topical application of clonidine relieves hyperalgesia in patients with sympathetically maintained pain. Pain. 1991;47(3):309-17.
219. Wrzosek A, Woron J, Dobrogowski J, Jakowicka-Wordliczek J, Wordliczek J. Topical clonidine for neuropathic pain. Cochrane Database Syst Rev. 2015;8(9):CD010967.
220. Yuen KC, Baker NR, Rayman G. Treatment of chronic painful diabetic neuropathy with isosorbide dinitrate spray: a double-blind placebo-controlled cross-over study. Diabetes Care. 2002;25(10):1699-703.
221. Agrawal RP, Choudhary R, Sharma P, Sharma S, Beniwal R, Kaswan K, et al. Glyceryl trinitrate spray in the management of painful diabetic neuropathy: a randomized double blind placebo controlled cross-over study. Diabetes Res Clin Pract. 2007;77(2):161-7.
222. Laferriere A, Abaji R, Tsai CY, Ragavendran JV, Coderre TJ. Topical combinations to treat microvascular dysfunction of chronic postischemia pain. Anesth Analg. 2014;118(4):830-40.
223. Rendell M, Bamisedun O. Skin blood flow and current perception in pentoxifylline-treated diabetic neuropathy. Angiology. 1992;43(10):843-51.

224. Hosseini F, Mohammadbeigi A, Aghaali M, Borujerdi R, Parham M. Effect of pentoxifylline on diabetic distal polyneuropathy in type 2 diabetic patients: A randomized trial. J Res Med Sci. 2019;24:89.
225. Wang L, Chopp M, Szalad A, Lu X, Jia L, Lu M, et al. Tadalafil Promotes the Recovery of Peripheral Neuropathy in Type II Diabetic Mice. PloS One. 2016;11(7):e0159665-e.
226. Wang L, Chopp M, Szalad A, Jia L, Lu X, Lu M, et al. Sildenafil ameliorates long term peripheral neuropathy in type II diabetic mice. PloS One. 2015;10(2):e0118134.
227. Ogihara T, Nakagawa T, Hayashi M, Koyanagi M, Yonezawa A, Omura T, et al. Improvement of peripheral vascular impairment by a phosphodiesterase type 5 inhibitor tadalafil prevents oxaliplatin-induced peripheral neuropathy in mice. Journal of Pharmacological Sciences. 2019;141(4):131-8.
228. Ragavendran JV, Laferriere A, Bennett GJ, Ware MA, Gandhi W, Bley K, et al. Effects of topical combinations of clonidine and pentoxifylline on capsaicin-induced allodynia and postcapsaicin tourniquet-induced pain in healthy volunteers: a double-blind, randomized, controlled study. Pain. 2016;157(10):2366-74.
229. Sutherland AM, Clarke HA, Katz J, Katznelson R. Hyperbaric Oxygen Therapy: A New Treatment for Chronic Pain? Pain Pract. 2016;16(5):620-8.
230. Thompson CD, Uhelski ML, Wilson JR, Fuchs PN. Hyperbaric oxygen treatment decreases pain in two nerve injury models. Neurosci Res. 2010;66(3):279-83.
231. Mychaskiw G, 2nd, Pan J, Shah S, Zubkov A, Clower B, Badr A, et al. Effects of hyperbaric oxygen on skin blood flow and tissue morphology following sciatic nerve constriction. Pain Physician. 2005;8(2):157-61.
232. Kiralp MZ, Yildiz S, Vural D, Keskin I, Ay H, Dursun H. Effectiveness of hyperbaric oxygen therapy in the treatment of complex regional pain syndrome. J Int Med Res. 2004;32(3):258-62.
233. Zuurmond WW, Langendijk PN, Bezemer PD, Brink HE, de Lange JJ, van loenen AC. Treatment of acute reflex sympathetic dystrophy with DMSO 50% in a fatty cream. Acta Anaesthesiol Scand. 1996;40(3):364-7.
234. Kumar A, Kaundal RK, Iyer S, Sharma SS. Effects of resveratrol on nerve functions, oxidative stress and DNA fragmentation in experimental diabetic neuropathy. Life Sci. 2007;80(13):1236-44.
235. Inkster ME, Cotter MA, Cameron NE. Treatment with the xanthine oxidase inhibitor, allopurinol, improves nerve and vascular function in diabetic rats. Eur J Pharmacol. 2007;561(1-3):63-71.
236. Kim HK, Zhang YP, Gwak YS, Abdi S. Phenyl N-tert-butylnitrone, a free radical scavenger, reduces mechanical allodynia in chemotherapy-induced neuropathic pain in rats. Anesthesiology. 2010;112(2):432-9.
237. Carvalho LF, Silva AMF, Carvalho AA. The use of antioxidant agents for chemotherapy-induced peripheral neuropathy treatment in animal models. Clin Exp Pharmacol Physiol. 2017;44(10):971-9.
238. Ziegler D, Nowak H, Kempler P, Vargha P, Low PA. Treatment of symptomatic diabetic polyneuropathy with the antioxidant α-lipoic acid: a meta-analysis. Diabetic Med. 2004;21(2):114-21.
239. Sisignano M, Baron R, Scholich K, Geisslinger G. Mechanism-based treatment for chemotherapy-induced peripheral neuropathic pain. Nat Rev Neurol. 2014;10(12):694-707.

240. Sewell RDE. Neuropathic pain models and outcome measures: a dual translational challenge. Ann Transl Med. 2018;6(Suppl 1):S42-S.
241. Asato F, Butler M, Blomberg H, Gordh T. Variation in rat sciatic nerve anatomy: implications for a rat model of neuropathic pain. J. Peripher. Nerv. Syst.. 2000;5(1):19-21.
242. Savastano LE, Laurito SR, Fitt MR, Rasmussen JA, Gonzalez Polo V, Patterson SI. Sciatic nerve injury: a simple and subtle model for investigating many aspects of nervous system damage and recovery. J Neurosci Methods. 2014;227:166-80.
243. Seltzer Ze, Dubner R, Shir Y. A novel behavioral model of neuropathic pain disorders produced in rats by partial sciatic nerve injury. Pain. 1990;43(2):205-18.
244. Bennett GJ, Xie YK. A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. Pain. 1988;33(1):87-107.
245. Kim SH, Chung JM. An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. Pain. 1992;50(3):355-63.
246. Decosterd I, Woolf CJ. Spared nerve injury: an animal model of persistent peripheral neuropathic pain. Pain. 2000;87(2):149-58.
247. Gazelius B, Cui JG, Svensson M, Meyerson B, Linderoth B. Photochemically induced ischaemic lesion of the rat sciatic nerve. A novel method providing high incidence of mononeuropathy. NeuroReport. 1996;7(15-17):2619-23.
248. DeLeo JA, Coombs DW, Willenbring S, Colburn RW, Fromm C, Wagner R, et al. Characterization of a neuropathic pain model: sciatic cryoneurolysis in the rat. Pain. 1994;56(1):9-16.
249. Sousa AM, Lages GV, Pereira CL, Slullitel A. Experimental models for the study of neuropathic pain. Revista Dor. 2016;17:27-30.
250. Jaggi AS, Jain V, Singh N. Animal models of neuropathic pain. Fundam Clin Pharmacol. 2011;25(1):1-28.
251. Drel VR, Mashtalir N, Ilnytska O, Shin J, Li F, Lyzogubov VV, et al. The leptin-deficient (ob/ob) mouse: a new animal model of peripheral neuropathy of type 2 diabetes and obesity. Diabetes. 2006;55(12):3335-43.
252. Fleetwood-Walker SM, Quinn JP, Wallace C, Blackburn-Munro G, Kelly BG, Fiskerstrand CE, et al. Behavioural changes in the rat following infection with varicella-zoster virus. J Gen Virol. 1999;80 (Pt 9):2433-6.
253. Chen SR, Pan HL. Effect of systemic and intrathecal gabapentin on allodynia in a new rat model of postherpetic neuralgia. Brain Res. 2005;1042(1):108-13.
254. Coderre TJ, Laferrière A. The emergence of animal models of chronic pain and logistical and methodological issues concerning their use. J Neural Transm (Vienna). 2019.
255. Challa SR. Surgical animal models of neuropathic pain: Pros and Cons. Int J Neurosci. 2015;125(3):170-4.
256. Wagner R, Janjigian M, Myers RR. Anti-inflammatory interleukin-10 therapy in CCI neuropathy decreases thermal hyperalgesia, macrophage recruitment, and endoneurial TNF-α expression. Pain. 1998;74(1):35-42.
257. Bridges D, Thompson SW, Rice AS. Mechanisms of neuropathic pain. Br J Anaesth. 2001;87(1):12-26.
258. Myers RR, Yamamoto T, Yaksh TL, Powell HC. The role of focal nerve ischemia and Wallerian degeneration in peripheral nerve injury producing hyperesthesia. Anesthesiology. 1993;78(2):308-16.

259. Okamoto K, Martin DP, Schmelzer JD, Mitsui Y, Low PA. Pro- and Anti-inflammatory Cytokine Gene Expression in Rat Sciatic Nerve Chronic Constriction Injury Model of Neuropathic Pain. Exp Neurol. 2001;169(2):386-91.
260. Shibayama M, Kuniyoshi K, Suzuki T, Yamauchi K, Ohtori S, Takahashi K. The effects of locally injected triamcinolone on entrapment neuropathy in a rat chronic constriction injury model. J Hand Surg Am. 2014;39(9):1714-21.
261. Dale AM, Harris-Adamson C, Rempel D, Gerr F, Hegmann K, Silverstein B, et al. Prevalence and incidence of carpal tunnel syndrome in US working populations: pooled analysis of six prospective studies. Scand J Work Environ Health. 2013;39(5):495-505.
262. Flatters SJL, Bennett GJ. Studies of peripheral sensory nerves in paclitaxel-induced painful peripheral neuropathy: Evidence for mitochondrial dysfunction. Pain. 2006;122(3):245-57.
263. Kelling J, Sullivan K, Wilson L, Jordan MA. Suppression of centromere dynamics by Taxol® in living osteosarcoma cells. Cancer Res. 2003;63(11):2794-801.
264. Balmaceda C, Peterson K, Seidman AD, Brasher P, Deangelis LM. Prospective study of paclitaxel-induced peripheral neuropathy with quantitative sensory testing. J Neurooncol. 1997;35(1):47-53.
265. Sahenk Z, Barohn R, New P, Mendell JR. Taxol neuropathy: electrodiagnostic and sural nerve biopsy findings. Arch Neurol. 1994;51(7):726-9.
266. Seretny M, Currie GL, Sena ES, Ramnarine S, Grant R, MacLeod MR, et al. Incidence, prevalence, and predictors of chemotherapy-induced peripheral neuropathy: A systematic review and meta-analysis. Pain. 2014;155(12):2461-70.
267. Gupta R, Bhaskar A. Chemotherapy-induced peripheral neuropathic pain. BJA Educ. 2015;16(4):115-9.
268. Polomano RC, Mannes AJ, Clark US, Bennett GJ. A painful peripheral neuropathy in the rat produced by the chemotherapeutic drug, paclitaxel. Pain. 2001;94(3):293-304.
269. Dina OA, Chen X, Reichling D, Levine JD. Role of protein kinase Cepsilon and protein kinase A in a model of paclitaxel-induced painful peripheral neuropathy in the rat. Neuroscience. 2001;108(3):507-15.
270. Cliffer KD, Siuciak JA, Carson SR, Radley HE, Park JS, Lewis DR, et al. Physiological characterization of Taxol-induced large-fiber sensory neuropathy in the rat. Ann Neurol. 1998;43(1):46-55.
271. Authier N, Gillet JP, Fialip J, Eschalier A, Coudore F. Description of a short-term Taxol-induced nociceptive neuropathy in rats. Brain Res. 2000;887(2):239-49.
272. Duggett NA, Griffiths LA, Flatters SJL. Paclitaxel-induced painful neuropathy is associated with changes in mitochondrial bioenergetics, glycolysis, and an energy deficit in dorsal root ganglia neurons. Pain. 2017;158(8):1499-508.
273. Zheng H, Xiao WH, Bennett GJ. Functional deficits in peripheral nerve mitochondria in rats with paclitaxel- and oxaliplatin-evoked painful peripheral neuropathy. Exp Neurol. 2011;232(2):154-61.
274. Duggett NA, Griffiths LA, McKenna OE, de Santis V, Yongsanguanchai N, Mokori EB, et al. Oxidative stress in the development, maintenance and resolution of paclitaxel-induced painful neuropathy. Neuroscience. 2016;333:13-26.
275. Siau C, Xiao W, Bennett GJ. Paclitaxel- and vincristine-evoked painful peripheral neuropathies: Loss of epidermal innervation and activation of Langerhans cells. Exp Neurol. 2006;201(2):507-14.

276. Ohmichi Y, Sato J, Ohmichi M, Sakurai H, et al. Two-week cast immobilization induced chronic widespread hyperalgesia in rats. Eur J Pain. 2012;16(3):338-48.
277. Guo TZ, Offley SC, Boyd EA, Jacobs CR, Kingery WS. Substance P signaling contributes to the vascular and nociceptive abnormalities observed in a tibial fracture rat model of complex regional pain syndrome type I. Pain. 2004;108(1-2):95-107.
278. Li W-W, Sabsovich I, Guo T-Z, Zhao R, Kingery WS, Clark JD. The role of enhanced cutaneous IL-1beta signaling in a rat tibia fracture model of complex regional pain syndrome. Pain. 2009;144(3):303-13.
279. Guo TZ, Wei T, Huang TT, Kingery WS, Clark JD. Oxidative stress contributes to fracture/cast-induced inflammation and pain in a rat model of complex regional pain syndrome. J Pain. 2018;19(10):1147-56.
280. Terkelsen AJ, Flemming WB, Troels SJ. Experimental forearm immobilization in humans induces cold and mechanical hyperalgesia. Anesthesiology. 2008;109(2):297-307.
281. Siegel SM, Lee JW, Oaklander AL. Needlestick distal nerve injury in rats models symptoms of complex regional pain syndrome. Anesth Analg. 2007;105(6):1820-9.
282. Urbaniak JR, Seaber AV, Chen LE. Assessment of ischemia and reperfusion injury. Clin Orthop Relat Res. 1997(334):30-6.
283. Millecamps M, Laferriere A, Ragavendran JV, Stone LS, Coderre TJ. Role of peripheral endothelin receptors in an animal model of complex regional pain syndrome type 1 (CRPS-I). Pain. 2010;151(1):174-83.
284. Millecamps M, Coderre TJ. Rats with chronic post-ischemia pain exhibit an analgesic sensitivity profile similar to human patients with complex regional pain syndrome type I. Eur J Pharmacol. 2008;583(1):97-102.
285. Deuis JR, Dvorakova LS, Vetter I. Methods used to evaluate pain behaviors in rodents. Front Mol Neurosci. 2017;10:284-.
286. Santos-Nogueira E, Redondo Castro E, Mancuso R, Navarro X. Randall-Selitto test: a new approach for the detection of neuropathic pain after spinal cord injury. J Neurotrauma. 2012;29(5):898-904.
287. Woolfe G, MacDonald A. The evaluation of the analgesic action of pethidine hydrochloride (Demerol). J Pharmacol Exp Ther. 1944;80(3):300-7.
288. Tjolsen A, Rosland JH, Berge OG, Hole K. The increasing-temperature hot-plate test: an improved test of nociception in mice and rats. J Pharmacol Methods. 1991;25(3):241-50.
289. Hargreaves K, Dubner R, Brown F, Flores C, Joris J. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. Pain. 1988;32(1):77-88.
290. Deuis JR, Vetter I. The thermal probe test: A novel behavioral assay to quantify thermal paw withdrawal thresholds in mice. Temperature. 2016;3(2):199-207.
291. Vissers K, Meert T. A behavioral and pharmacological validation of the acetone spray test in gerbils with a chronic constriction injury. Anesth Analg. 2005;101(2):457-64.
292. Vierck CJ, Yezierski RP. Comparison of operant escape and reflex tests of nociceptive sensitivity. Neurosci Biobehav Rev. 2015;51:223-42.
293. Russo MA, Santarelli DM. A Novel Compound Analgesic Cream (Ketamine, Pentoxifylline, Clonidine, DMSO) for Complex Regional Pain Syndrome Patients. Pain Pract. 2016;16(1):E14-E20.
294. Hiom S, Khot S, Mogford S, Hart C, Patel G, Roberts G, et al. Topical delivery of gabapentin (Gaba Gel™) for neuropathic pain: a 'proof of concept' study. Int J Pharm Pract. 2015;23:46.

295. Ali G, Subhan F, Abbas M, Zeb J, Shahid M, Sewell RDE. A streptozotocin-induced diabetic neuropathic pain model for static or dynamic mechanical allodynia and vulvodynia: validation using topical and systemic gabapentin. Naunyn-Schmiedeberg's Arch Pharmacol. 2015;388(11):1129-40.
296. Bennett GJ. What Is Spontaneous Pain and Who Has It? J Pain. 2012;13(10):921-9.
297. Fulas OA, Laferriere A, Stein RS, Bohle DS, Coderre TJ. Topical combination of meldonium and N-acetyl cysteine relieves allodynia in rat models of CRPS-1 and peripheral neuropathic pain by enhancing NO-mediated tissue oxygenation. J Neurochem. 2020;152(5):570-84.
298. Karagianni A, Malamatari M, Kachrimanis K. Pharmaceutical Cocrystals: New Solid Phase Modification Approaches for the Formulation of APIs. Pharmaceutics. 2018;10(1):18.
299. Simon F. The trouble with making combination drugs. Nat Rev Drug Discov. 2006;5(11):881-2..
300. Thipparaboina R, Kumar D, Chavan RB, Shastri NR. Multidrug co-crystals: towards the development of effective therapeutic hybrids. Drug Discov Today. 2016;21(3):481-90.
301. Aitipamula S, Banerjee R, Bansal AK, Biradha K, Cheney ML, et al. Polymorphs, Salts, and Cocrystals: What's in a Name? Cryst Growth Des. 2012;12(5):2147-52.
302. Aakeröy CB, Fasulo ME, Desper J. Cocrystal or Salt: Does It Really Matter? Mol Pharm. 2007;4(3):317-22.
303. Tan D, Loots L, Friščić T. Towards medicinal mechanochemistry: evolution of milling from pharmaceutical solid form screening to the synthesis of active pharmaceutical ingredients (APIs). Chemical Communications. 2016;52(50):7760-81.
304. Sekhon BS. Drug-drug co-crystals. DARU J Pharm Sci. 2012;20(1):45-.
305. Karimi-Jafari M, Padrela L, Walker GM, Croker DM. Creating Cocrystals: A Review of Pharmaceutical Cocrystal Preparation Routes and Applications. Crystal Growth & Design. 2018;18(10):6370-87.
306. Friščić T, Mottillo C, Titi HM. Mechanochemistry for Synthesis. Angew Chem Int Ed Engl. 2020;59(3):1018-29.
307. James SL, Adams CJ, Bolm C, Braga D, Collier P, Friscic Friščić T, et al. Mechanochemistry: opportunities for new and cleaner synthesis. Chem Soc Rev. 2012;41(1):413-47.
308. Anastas P, Eghbali N. Green chemistry: principles and practice. Chem Soc Rev. 2010;39(1):301-12.
309. Pindelska E, Sokal A, Kolodziejski W. Pharmaceutical cocrystals, salts and polymorphs: Advanced characterization techniques. Adv Drug Deliv Rev. 2017;117:111-46.
310. Chauhan A, Chauhan P. Powder XRD technique and its applications in science and technology. J Anal Bioanal Tech. 2014;5(5):1.
311. Groom CR, Bruno IJ, Lightfoot MP, Ward SC. The Cambridge structural database. Acta Crystallogr B Struct Sci Cryst Eng Mater. 2016;72(2):171-9.
312. Chiu MH, Prenner EJ. Differential scanning calorimetry: An invaluable tool for a detailed thermodynamic characterization of macromolecules and their interactions. J Pharm Bioallied Sci. 2011;3(1):39-59.
313. Coats AW, Redfern JP. Thermogravimetric analysis. A review. Analyst. 1963;88(1053):906-24.
314. Bassenge E, Kukovetz WR. Molsidomine. Cardiovasc Drug Rev. 1984;2(1):177-91.
315. Rosenkranz B, Winkelmann BR, Parnham MJ. Clinical pharmacokinetics of molsidomine. Clin Pharmacokinet. 1996;30(5):372-84.

316. McCarty MF, O'Keefe JH, DiNicolantonio JJ. Pentoxifylline for vascular health: a brief review of the literature. Open Heart. 2016;3(1):e000365-e.
317. Perego MA, Sergio G, Artale F, Giunti P, Danese C. Haemorrheological improvement by pentoxifylline in patients with peripheral arterial occlusive disease. Curr Med Res Opin. 1986;10(2):135-8.
318. Ott E, Lechner H, Fazekas F. Hemorheological effects of pentoxifylline on disturbed flow behavior of blood in patients with cerebrovascular insufficiency. Eur Neurol. 1983;22 Suppl 1:105-7.
319. Figueroa XF, Poblete MI, Boric MP, Mendizabal VE, Adler-Graschinsky E, Huidobro-Toro JP. Clonidine-induced nitric oxide-dependent vasorelaxation mediated by endothelial alpha(2)-adrenoceptor activation. Br J Pharmacol. 2001;134(5):957-68.
320. Semaming Y, Pannengpetch P, Chattipakorn SC, Chattipakorn N. Pharmacological properties of protocatechuic Acid and its potential roles as complementary medicine. Evid Based Complement Alternat Med. 2015;2015:593902.
321. Koshihara Y, Neichi T, Murota S, Lao A, Fujimoto Y, Tatsuno T. Caffeic acid is a selective inhibitor for leukotriene biosynthesis. Biochim Biophys Acta. 1984;792(1):92-7.
322. Farbood Y, Sarkaki A, Hashemi S, Mansouri MT, Dianat M. The effects of gallic acid on pain and memory following transient global ischemia/reperfusion in Wistar rats. Avicenna J Phytomed. 2013;3(4):329-40.
323. Krzysztoforska K, Mirowska-Guzel D, Widy-Tyszkiewicz E. Pharmacological effects of protocatechuic acid and its therapeutic potential in neurodegenerative diseases: Review on the basis of in vitro and in vivo studies in rodents and humans. Nutr Neurosci. 2019;22(2):72-82.
324. Shin S, Cho SH, Park D, Jung E. Anti-skin aging properties of protocatechuic acid in vitro and in vivo. J Cosmet Dermatol. 2019.
325. Zhou Y, Fang SH, Ye YL, Chu LS, Zhang WP, Wang ML, et al. Caffeic acid ameliorates early and delayed brain injuries after focal cerebral ischemia in rats. Acta pharmacologica Sinica. 2006;27(9):1103-10.
326. Liang G, Shi B, Luo W, Yang J. The protective effect of caffeic acid on global cerebral ischemia-reperfusion injury in rats. Behav Brain Funct. 2015;11:18.
327. Chen JH, Ho C-T. Antioxidant Activities of Caffeic Acid and Its Related Hydroxycinnamic Acid Compounds. J Agric Food Chem. 1997;45(7):2374-8.
328. Mori H, Iwahashi H. Antioxidant Activity of Caffeic Acid through a Novel Mechanism under UVA Irradiation. J Clin Biochem Nutr. 2009;45(1):49-55.
329. Mard SA, Mojadami S, Farbood Y, Gharib Naseri MK. The anti-inflammatory and anti-apoptotic effects of gallic acid against mucosal inflammation- and erosions-induced by gastric ischemia-reperfusion in rats. Vet Res Forum. 2015;6(4):305-11.
330. Badavi M, Sadeghi N, Dianat M, Samarbafzadeh A. Effects of gallic Acid and cyclosporine a on antioxidant capacity and cardiac markers of rat isolated heart after ischemia/reperfusion. Iranian Red Crescent Med J. 2014;16(6).
331. Durrani AI, Schwartz H, Nagl M, Sontag G. Determination of free α-lipoic acid in foodstuffs by HPLC coupled with CEAD and ESI-MS. Food Chem. 2010;120(4):1143-8.
332. Laher I. Diabetes and Alpha Lipoic Acid. Front Pharmacol. 2011;2(69).
333. Nagamatsu M, Nickander KK, Schmelzer JD, Raya A, Wittrock DA, Tritschler H, et al. Lipoic acid improves nerve blood flow, reduces oxidative stress, and improves distal nerve conduction in experimental diabetic neuropathy. Diabetes Care. 1995;18(8):1160-7.

334. Thakuria R, Sarma B. Drug-Drug and Drug-Nutraceutical Cocrystal/Salt as Alternative Medicine for Combination Therapy: A Crystal Engineering Approach. Crystals. 2018;8(2).
335. Lopez-Cedrun J, Videla S, Burgueno M, Juarez I, Aboul-Hosn S, Martin-Granizo R, et al. Co-crystal of Tramadol-Celecoxib in Patients with Moderate to Severe Acute Post-surgical Oral Pain: A Dose-Finding, Randomised, Double-Blind, Placebo- and Active-Controlled, Multicentre, Phase II Trial. Drugs R D. 2018;18(2):137-48.
336. Wasner G. Vasomotor Disturbances in Complex Regional Pain Syndrome-A Review. Pain Med. 2010;11(8):1267-73.
337. Hosseini A, Abdollahi M. Diabetic neuropathy and oxidative stress: therapeutic perspectives. Oxid Med Cell Longev. 2013;2013:168039.
338. Areti A, Yerra VG, Naidu VGM, Kumar A. Oxidative stress and nerve damage: Role in chemotherapy induced peripheral neuropathy. Redox Biol. 2014;2:289-95.
339. Katerji M, Filippova M, Duerksen-Hughes P. Approaches and Methods to Measure Oxidative Stress in Clinical Samples: Research Applications in the Cancer Field. Oxid Med Cell Longev. 2019;2019:1279250.
340. Benaron DA, Parachikov IH, Friedland S, Soetikno R, Brock-Utne J, van der Starre PJ, et al. Continuous, noninvasive, and localized microvascular tissue oximetry using visible light spectroscopy. Anesthesiology. 2004;100(6):1469-75.
341. Bellingham GA, Smith RS, Morley-Forster P, Murkin JM. Use of near infrared spectroscopy to detect impaired tissue oxygen saturation in patients with complex regional pain syndrome type 1. Can J Anaesth. 2014;61(6):563-70.
342. Sinex JE. Pulse oximetry: Principles and limitations. Am J Emerg Med. 1999;17(1):59-66.
343. Watkin SL, Spencer SA, Dimmock PW, et al. A Comparison of Pulse Oximetry and Near Infrared Spectroscopy (NIRS) in the Detection of Hypoxaemia Occurring With Pauses in Nasal Airflow in Neonates. J Clin Monit Comput. 1999;15(7):441-7.
344. Swartz HM, Dunn JF. Measurements of Oxygen in Tissues: Overview and Perspectives on Methods. In: Oxygen Transport to Tissue XXIV. Boston(MA): Springer US; 2003: p. 1-12.
345. Douplik A, Saiko G, Schelkanova I, Tuchin VV. The response of tissue to laser light. In: Jelínková H, ed. Lasers for Medical Applications. Philadelphia: Woodhead Publishing; 2013: p. 47-109.
346. Fuhrmann DC, Brüne B. Mitochondrial composition and function under the control of hypoxia. Redox Biol. 2017;12:208-15.
347. Belkin M, Brown RD, Wright JG, LaMorte WW, Hobson RW. A new quantitative spectrophotometric assay of ischemia-reperfusion injury in skeletal muscle. Am J Surg. 1988;156(2):83-6.
348. Vivaldi M, Kloner R, Schoen F. Triphenyltetrazolium staining of irreversible ischemic injury following coronary artery occlusion in rats. Am J Pathol. 1985;121(3):522.
349. Ding Y, Yao P, Hong T, Li H, Zhu Y, Han Z, et al. The analgesic effect of early hyperbaric oxygen treatment in chronic constriction injury rats and its influence on nNOS & iNOS expression & inflammatory factor production. Mol Pain. 2018;14:1744806918765837.
350. Brewer AL, Shirachi DY, Quock RM, et al. Effect of hyperbaric oxygen on chemotherapy-induced neuropathy in male & female rats. Behav Pharmacol. 2020;31(1):61-72.
351. Quattrini C, Jeziorska M, Boulton AJ, Malik RA. Reduced vascular endothelial growth factor expression and intra-epidermal nerve fiber loss in human diabetic neuropathy. Diabetes Care. 2008;31(1):140-5.

352. Doupis J, Lyons TE, Wu S, Gnardellis C, Dinh T, Veves A. Microvascular reactivity and inflammatory cytokines in painful and painless peripheral diabetic neuropathy. J Clin Endocrinol Metab. 2009;94(6):2157-63.
353. Rayman G, Baker NR, Krishnan STM. Glyceryl Trinitrate Patches as an Alternative to Isosorbide Dinitrate Spray in the Treatment of Chronic Painful Diabetic Neuropathy. Diabetes Care. 2003;26(9):2697.
354. Coderre TJ, Kumar N, Lefebvre CD, Yu JS. Evidence that gabapentin reduces neuropathic pain by inhibiting the spinal release of glutamate. J Neurochem. 2005;94(4):1131-9.
355. Sokolovska J, Rumaks J, Karajeva N, Grinvalde D, Shapirova J, Klusa V, et al. [The influence of mildronate on peripheral neuropathy and some characteristics of glucose and lipid metabolism in rat streptozotocin-induced diabetes mellitus model]. Biomeditsinskaia khimiia. 2011;57(5):490-500.
356. De Marchi S, Zecchetto S, Rigoni A, Prior M, Fondrieschi L, Scuro A, et al. Propionyl-L-carnitine improves endothelial function, microcirculation and pain management in critical limb ischemia. Cardiovasc Drugs Ther. 2012;26(5):401-8.
357. Kim JH, Kim YC, Nahm FS, Lee PB. The therapeutic effect of vitamin C in an animal model of complex regional pain syndrome produced by prolonged hindpaw ischemia-reperfusion in rats. Int J Med Sci. 2017;14(1):97-101.
358. Garrido-Suarez BB, Garrido G, Castro-Labrada M, Pardo-Ruiz Z, Menendez AB, Spencer E, et al. Anti-allodynic effect of mangiferin in rats with chronic post-ischemia pain: a model of ccomplex regional pain syndrome type I. Front Pharmacol. 2018;9:14.
359. Ragavendran JV, Laferriere A, Coderre TJ, et al. Pentoxifylline reduces chronic post-ischaemia pain by alleviating microvascular dysfunction. Eur J Pain. 2014;18(3):406-14.
360. Vercelino R, Cunha TM, Ferreira ES, Cunha FQ, Ferreira SH, de Oliveira MG. Skin vasodilation and analgesic effect of a topical nitric oxide-releasing hydrogel. J Mater Sci Mater Med. 2013;24(9):2157-69.
361. Bonner R, Nossal R. Model for laser Doppler measurements of blood flow in tissue. Appl Opt. 1981;20(12):2097-107.
362. Kuliga KZ, McDonald EF, Gush R, Michel C, Chipperfield AJ, Clough GF. Dynamics of microvascular blood flow and oxygenation measured simultaneously in human skin. Microcirculation. 2014;21(6):562-73.
363. Zhang YM, Brewer AL, Nelson JT, Smith PT, Shirachi DY, Quock RM. Hyperbaric oxygen produces a nitric oxide synthase-regulated anti-allodynic effect in rats with paclitaxel-induced neuropathic pain. Brain Research. 2019;1711:41-7.
364. Hu Q, Fang L, Li F, Thomas S, Yang Z. Hyperbaric oxygenation treatment alleviates CCI-induced neuropathic pain and decreases spinal apoptosis. Eur J Pain. 2015;19(7):920-8.
365. Xia Z, Nagareddy PR, Guo Z, Zhang W, McNeill JH. Antioxidant N-acetylcysteine restores systemic nitric oxide availability and corrects depressions in arterial blood pressure and heart rate in diabetic rats. Free Radic Res. 2006;40(2):175-84.
366. Sjakste N, Gutcaits A, Kalvinsh I. Mildronate: an antiischemic drug for neurological indications. CNS Drug Rev. 2005;11(2):151-68.
367. Kurowska EM. Nitric oxide therapies in vascular diseases. Curr Pharm Des. 2002;8(3):155-66.
368. Sjakste N, Kleschyov AL, Boucher JL, Baumane L, Dzintare M, Meirena D, et al. Endothelium- and nitric oxide-dependent vasorelaxing activities of gamma-butyrobetaine

esters: possible link to the antiischemic activities of mildronate. Eur J Pharmacol. 2004;495(1):67-73.
369. Vilskersts R, Zharkova-Malkova O, Mezhapuke R, Grinberga S, Cirule H, Dambrova M. Elevated vascular γ-butyrobetaine levels attenuate the development of high glucose-induced endothelial dysfunction. Clin Exp Pharmacol Physiol. 2013;40(8):518-24.
370. Girouard H, Chulak C, Wu L, Lejossec M, de Champlain J. N-acetylcysteine improves nitric oxide and alpha-adrenergic pathways in mesenteric beds of spontaneously hypertensive rats. Am J Hypertens. 2003;16(7):577-84.
371. Aruoma OI, Halliwell B, Hoey BM, Butler J. The antioxidant action of N-acetylcysteine: its reaction with hydrogen peroxide, hydroxyl radical, superoxide, and hypochlorous acid. Free Radic Biol Med. 1989;6(6):593-7.
372. Xanthos DN, Coderre TJ. Sympathetic vasoconstrictor antagonism and vasodilatation relieve mechanical allodynia in rats with chronic postischemia pain. J Pain. 2008;9(5):423-33.
373. Prado WA, Schiavon VF, Cunha FQ. Dual effect of local application of nitric oxide donors in a model of incision pain in rats. Eur J Pharmacol. 2002;441(1):57-65.
374. Dutra M, Godin AM, Cesar IC, Nascimento EB, Menezes RR, Ferreira WC, et al. Activity of nicorandil, a nicotinamide derivative with a nitrate group, in the experimental model of pain induced by formaldehyde in mice. Pharmacol Biochem Behav. 2013;106:85-90.
375. Agrawal RP, Goswami J, Jain S, Kochar DK. Management of diabetic neuropathy by sodium valproate and glyceryl trinitrate spray: a prospective double-blind randomized placebo-controlled study. Diabetes Res Clin Pract. 2009;83(3):371-8.
376. Fletcher S, Wright M, Wilkinson A, Farr M, Sellars L. Locally applied transdermal nitrate patches for the treatment of ischaemic rest pain. Int J Clin Pract. 1997;51(5):324-5.
377. Tarkin JM, Kaski JC. Vasodilator Therapy: Nitrates and Nicorandil. Cardiovasc Drugs Ther. 2016;30(4):367-78.
378. Hagen M, Baker M. Skin penetration and tissue permeation after topical administration of diclofenac. Curr Med Res Opin. 2017;33(9):1623-34.
379. Wagner R, Heckman HM, Myers RR. Wallerian degeneration and hyperalgesia after peripheral nerve injury are glutathione-dependent. Pain. 1998;77(2):173-9.
380. Clijsen R, Baeyens JP, Barel AO, Clarys P. In vivo determination of the diclofenac skin reservoir: comparison between passive, occlusive, and iontophoretic application. Drug Des Devel Ther. 2015;9:835-40.
381. Kortekaas MC, Niehof SP, Stolker RJ, Huygen FJ. Pathophysiological Mechanisms Involved in Vasomotor Disturbances in Complex Regional Pain Syndrome and Implications for Therapy: A Review. Pain Pract. 2016;16(7):905-14.
382. Stepanovs D, Mishnev A. Multicomponent pharmaceutical cocrystals: furosemide and pentoxifylline. Acta Crystallogr Sect C-Struct Chem. 2012;68:O488-U279.
383. Sinha AS, Maguire AR, Lawrence SE. Cocrystallization of nutraceuticals. Cryst Growth Des. 2015;15(2):984-1009.
384. Wang Z-Z, Chen J-M, Lu T-B. Enhancing the hygroscopic stability of S-oxiracetam via pharmaceutical cocrystals. Cryst Growth Des. 2012;12(9):4562-6.
385. Cheney ML, Weyna DR, Shan N, Hanna M, Wojtas L, Zaworotko MJ. Co-former selection in pharmaceutical co-crystal development: a case study of a meloxicam aspirin co-crystal that exhibits enhanced solubility and pharmacokinetics. J Pharm Sci. 2011;100(6):2172-81.
386. Chandel N, Gupta V, Pandey A, Saxena S. Co-crystalization of aceclofenac and paracetamol and their characterization. Int J Pharm Life Sci. 2011;2(8).

387. Kuwabara Y, Hamamoto H, Hikake S, Miwa Y. A randomized, multi-Center, double-blind, placebo-controlled phase II/III trial to evaluate the efficacy, tolerability and safety of MRX-7EAT Etodolac-Lidocaine Topical Patch in the treatment of pain. J Pain. 2013;14(4):S73.
388. Garrido-Suárez BB, Garrido G, Castro-Labrada M, Pardo-Ruíz Z, Bellma Menéndez A, Spencer E, et al. Anti-allodynic Effect of Mangiferin in Rats With Chronic Post-ischemia Pain: A Model of Complex Regional Pain Syndrome Type I. Front Pharmacol. 2018;9(1119).
389. So K, Tei Y, Zhao M, Miyake T, Hiyama H, Shirakawa H, et al. Hypoxia-induced sensitisation of TRPA1 in painful dysesthesia evoked by transient hindlimb ischemia/reperfusion in mice. Sci Rep. 2016;6:23261-.
390. Mutalik S, Anju P, Manoj K, Usha AN. Enhancement of dissolution rate and bioavailability of aceclofenac: a chitosan-based solvent change approach. Int J Pharm. 2008;350(1-2):279-90.
391. Hickey MB, Peterson ML, Scoppettuolo LA, Morrisette SL, Vetter A, Guzman H, et al. Performance comparison of a co-crystal of carbamazepine with marketed product. Eur J Pharm Biopharm. 2007;67(1):112-9.
392. Basavoju S, Bostrom D, Velaga SP. Indomethacin-saccharin cocrystal: design, synthesis and preliminary pharmaceutical characterization. Pharm Res. 2008;25(3):530-41.
393. Adams CP, Brantner VV. Estimating The Cost Of New Drug Development: Is It Really $802 Million? Health Aff. 2006;25(2):420-8.
394. Raffa RB, Pergolizzi JV, Jr., Tallarida RJ. The determination and application of fixed-dose analgesic combinations for treating multimodal pain. J Pain. 2010;11(8):701-9.
395. Pourkavoos N. Unique Risks, Benefits, and Challenges of Developing Drug-Drug Combination Products in a Pharmaceutical Industrial Setting. Comb Prod Ther. 2012;2(1):2.
396. Desmeules J, Rollason V, Piguet V, Dayer P. Clinical pharmacology and rationale of analgesic combinations. Eur J Anaesthesiol. 2003;20(S28):7-12.
397. Bielli A, Scioli MG, Mazzaglia D, Doldo E, Orlandi A. Antioxidants and vascular health. Life Sci. 2015;143:209-16.
398. Zimmerman BJ, Grisham MB, Granger DN. Mechanisms of oxidant-mediated microvascular injury following reperfusion of the ischemic intestine. Basic Life Sci. 1988;49:881-6.
399. Yamagata K, Tagami M, Yamori Y. Dietary polyphenols regulate endothelial function and prevent cardiovascular disease. Nutrition. 2015;31(1):28-37.
400. Ndiaye M, Chataigneau M, Lobysheva I, Chataigneau T, Schini-Kerth VB. Red wine polyphenol-induced, endothelium-dependent NO-mediated relaxation is due to the redox-sensitive PI3-kinase/Akt-dependent phosphorylation of endothelial NO-synthase in the isolated porcine coronary artery. FASEB J. 2005;19(3):455-7.
401. Tarozzi A, Morroni F, Hrelia S, Angeloni C, Marchesi A, Cantelli-Forti G, et al. Neuroprotective effects of anthocyanins and their in vivo metabolites in SH-SY5Y cells. Neurosci Lett. 2007;424(1):36-40.
402. Hewedy WA. Linagliptin attenuates chronic post-ischemia pain: Possible anti-inflammatory and anti-oxidant mechanisms. Eur J Pharmacol. 2018;828:110-8.
403. Chen Q, Wang Q, Zhu J, Xiao Q, Zhang L. Reactive oxygen species: key regulators in vascular health and diseases. Br J Pharmacol. 2018;175(8):1279-92.
404. Kabaroudis A, Gerassimidis T, Karamanos D, Papaziogas B, Antonopoulos V, Sakantamis A. Metabolic alterations of skeletal muscle tissue after prolonged acute ischemia and reperfusion. J Invest Surg. 2003;16(4):219-28.

405. Bahlmann L, Pagel H, Klaus S, Heringlake M, Schmucker P, Wagner K. Pentoxifylline improves circulatory and metabolic recovery after cardiopulmonary resuscitation. Resuscitation. 2000;47(2):191-4.
406. Sonkin PL, Chen LE, Seaber AV, Hatchell DL. Vasodilator action of pentoxifylline on microcirculation of rat cremaster muscle. Angiology. 1992;43(6):462-9.
407. Frampton JE, Brogden RN. Pentoxifylline (Oxpentifylline). Drugs Aging. 1995;7(6):480-503.
408. Akobeng AK. Understanding randomised controlled trials. Arch Dis Child. 2005;90(8):840.
409. Gewandter JS, Dworkin RH, Turk DC, McDermott MP, Baron R, Gastonguay MR, et al. Research designs for proof-of-concept chronic pain clinical trials: IMMPACT recommendations. Pain. 2014;155(9):1683-95.
410. Tajerian M, Leu D, Zou YN, Sahbaie P, Li WW, Khan HD, et al. Brain neuroplastic changes accompany anxiety and memory deficits in a model of complex regional pain syndrome. Anesthesiology. 2014;121(4):852-65.
411. Kim JH, Park JS, Park D. Anti-allodynic effect of interleukin 10 in a mouse model of complex regional pain syndrome through reduction of NKI receptor expression of microglia in the spinal cord. J Pain Res. 2018;11:1729-41.
412. Wang LP, Guo TZ, Wei TP, Li WW, Shi XY, Clark JD, et al. Bisphosphonates inhibit pain, bone loss, and inflammation in a rat tibia fracture model of complex regional pain syndrome. Anesth Analg. 2016;123(4):1033-45.
413. Li WW, Guo TZ, Shi X, Czirr E, Stan T, Sahbaie P, et al. Autoimmunity contributes to nociceptive sensitization in a mouse model of complex regional pain syndrome. Pain. 2014;155(11):2377-89.
414. Sotocinal SG, Sorge RE, Zaloum A, Tuttle AH, Martin LJ, Wieskopf JS, et al. The Rat Grimace Scale: a partially automated method for quantifying pain in the laboratory rat via facial expressions. Mol Pain. 2011;7:55.
415. Kurejova M, Nattenmüller U, Hildebrandt U, Selvaraj D, Stösser S, Kuner R. An improved behavioural assay demonstrates that ultrasound vocalizations constitute a reliable indicator of chronic cancer pain and neuropathic pain. Mol pain. 2010;6:1744-8069-6-18.
416. Sufka KJ. Conditioned place preference paradigm: a novel approach for analgesic drug assessment against chronic pain. Pain. 1994;58(3):355-66.
417. Balayssac D, Ling B, Ferrier J, Pereira B, Eschalier A, Authier N. Assessment of thermal sensitivity in rats using the thermal place preference test: description and application in the study of oxaliplatin-induced acute thermal hypersensitivity and inflammatory pain models. Behav Pharmacol. 2014;25(2):99-111.
418. Keating SCJ, Thomas AA, Flecknell PA, Leach MC. Evaluation of EMLA Cream for Preventing Pain during Tattooing of Rabbits: Changes in Physiological, Behavioural and Facial Expression Responses. PloS One. 2012;7(9):11.
419. Kolesnikov YA, Cristea M, Pasternak GW. Analgesic synergy between topical morphine and butamben in mice. Anesthesia and Analgesia. 2003;97(4):1103-7.
420. Kolesnikov YA, Chereshnev I, Pasternak GW. Analgesic synergy between topical lidocaine and topical opioids. J Pharmacol Exp Ther. 2000;295(2):546-51.
421. Huang R-y, Pei L, Liu Q, Chen S, et al. Isobologram Analysis: A Comprehensive Review of Methodology and Current Research. Front Pharmacol. 2019;10(1222).
422. Tang C, Li J, Tai WL, et al. Sex difference in complex regional syndrome type 1 (CPRS-1) in mice. J Pain Res. 2017;10:1811-19.

12. APPENDIX

Supporting information for Manuscript 1

Topical combination of meldonium & N-acetyl cysteine relieves allodynia in rat models of CRPS-1 & PNP by enhancing NO-mediated tissue oxygenation

Supplementary data

Effect of topical meldonium & meldonium-NAC on the contralateral paw of CPIP rats

To check for systemic overflow of topically administered meldonium and meldonium-NAC, the drugs were topically applied on the uninjured contralateral paws of CPIP rats and the effects measured on the ipsilateral PWT. The PWT measured were no different from the ipsilateral topical vehicle application.

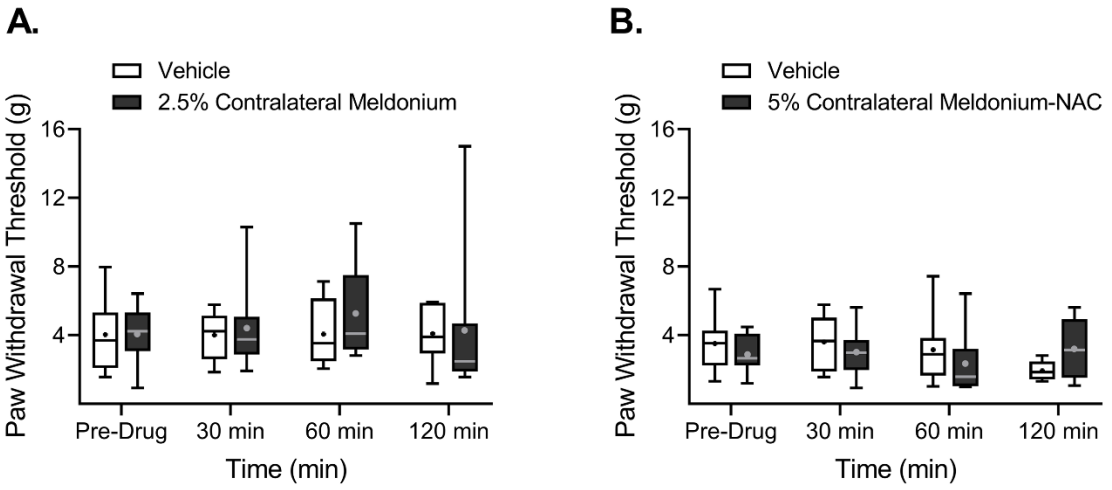

Figure S1.1 The effect of the application of topical meldonium and meldonium-NAC on the contralateral paw of CPIP rats. No change in PWT of the ipsilateral paw occurs post contralateral topical application of the drugs.

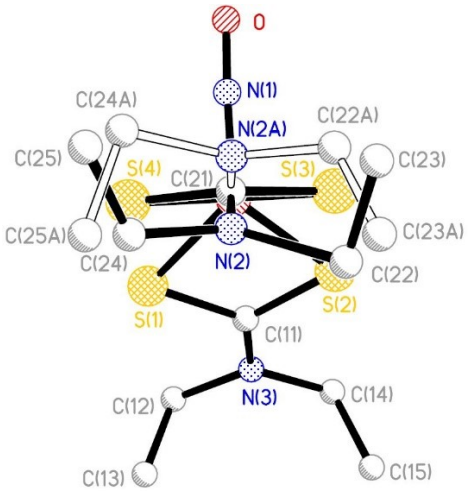

Figure S1.2 Crystal structure of mononitrosyl iron dithiocarbamate showing one of the diethylamino groups(N3) in a single ordered position and the second, N(2)/N(2A) as a disordered over two positions. Key metric parameters include; Fe-N(1) 1671(5); Fe-S(1) 2.2978(13); Fe-S(2) 2.3019(14); Fe-S(3) 2.2716(15); Fe-S(4) 2.2927(15), N(1)-O 1.134(6) Å. Fe-N(1)-O 175.1(5) °.

Supporting information for Manuscript 2

Drug-nutraceutical co-crystals & salts for making new & improved analgesics

Supplementary methods

^{15}N Solid State Nuclear Magnetic Resonance Spectroscopy (ssNMR)

^{15}N ssNMR on was performed using a Varian 400 MHz VNMRS wide-bore spectrometer operating at a ^{15}N frequency of 40.51 MHz. ^{15}N CPMAS spectra were acquired using a 7.5 mm double-resonance probe. Both **pentx** and the **pentx-pca** co-crystal spectra were acquired under spinning at 5 kHz using a recycle delay of 20 s and contact time of 4 ms and 2560 scans were acquired of **pentx** and 1024 scans were acquired of the co-crystal. ^{15}N spectra were referenced using glycine at 33.4 ppm.

^{1}H Solution Nuclear Magnetic Resonance Spectroscopy (NMR)

A solution ^{1}H NMR spectrum of **clon** was recorded on a Varian Mercury 300 MHz NMR spectrometer. Chemical shifts are reported relative to $CDCl_3$ (δ 7.26 ppm).

Supplementary data

Thermogravimetric Analysis

Thermogravimetric analysis of (pentx)(Hpca)

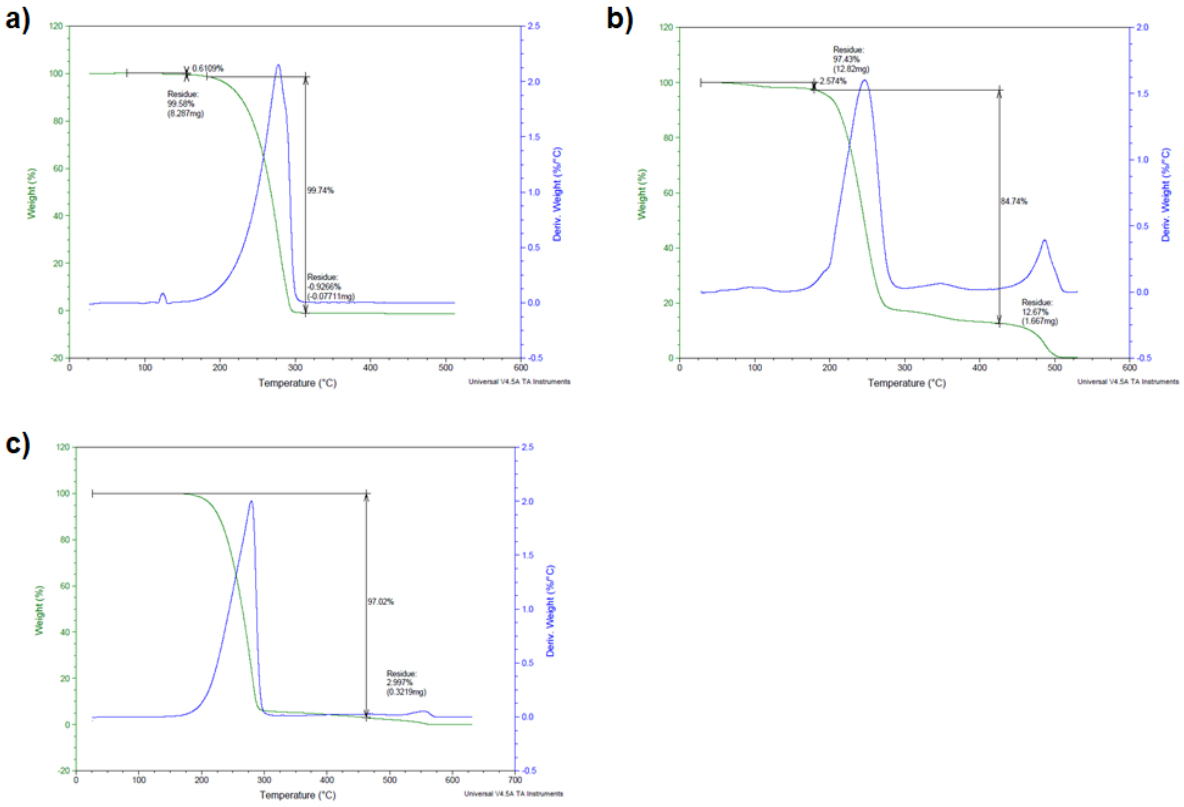

Figure S2.1 TGA thermogram for **pentx** (a), H**pca** (b) and **(pentx)(**H**pca)** (c)

Thermogravimetric analysis of (Hclon$^+$ala$^-$)

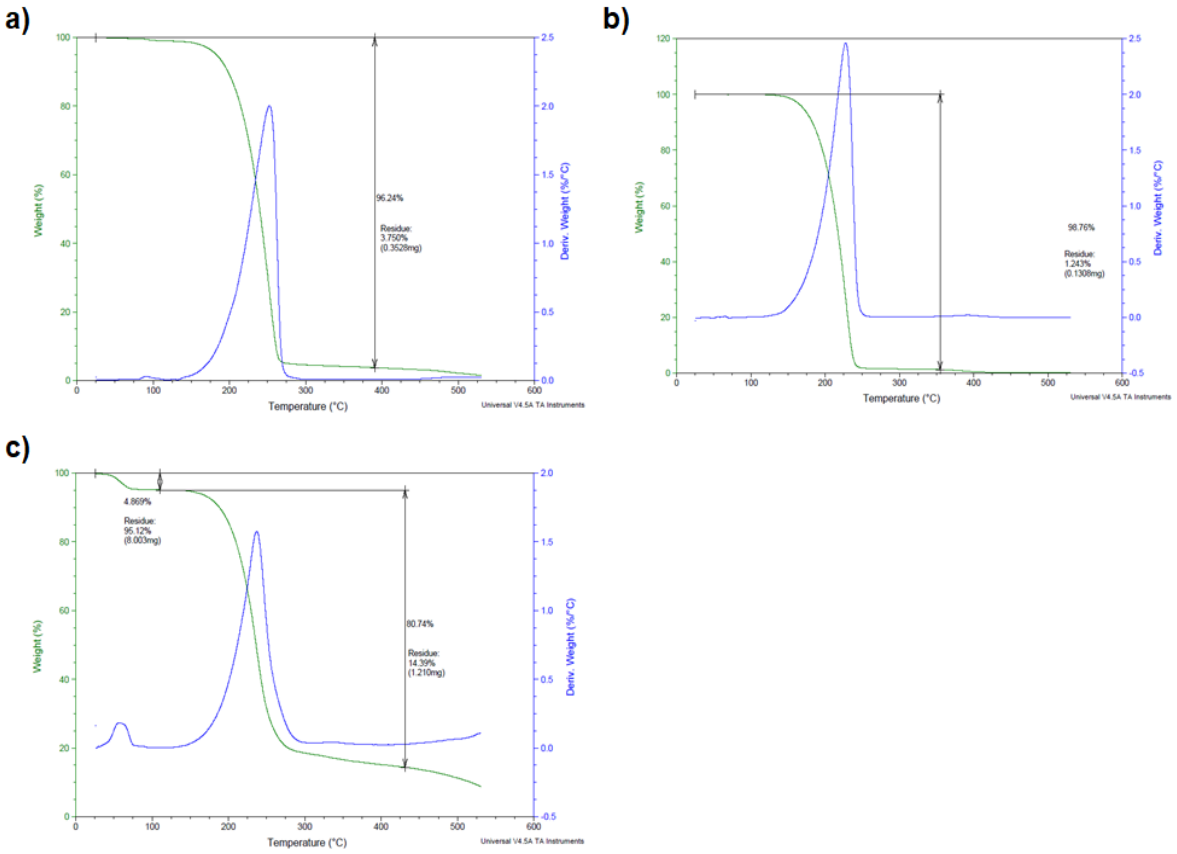

Figure S2.2 TGA thermogram for **clon** (a), H**ala** (b) and (H**clon**$^+$)(**ala**$^-$) (c)

Thermogravimetric analysis of (Hlin$^+$cafa$^-$)

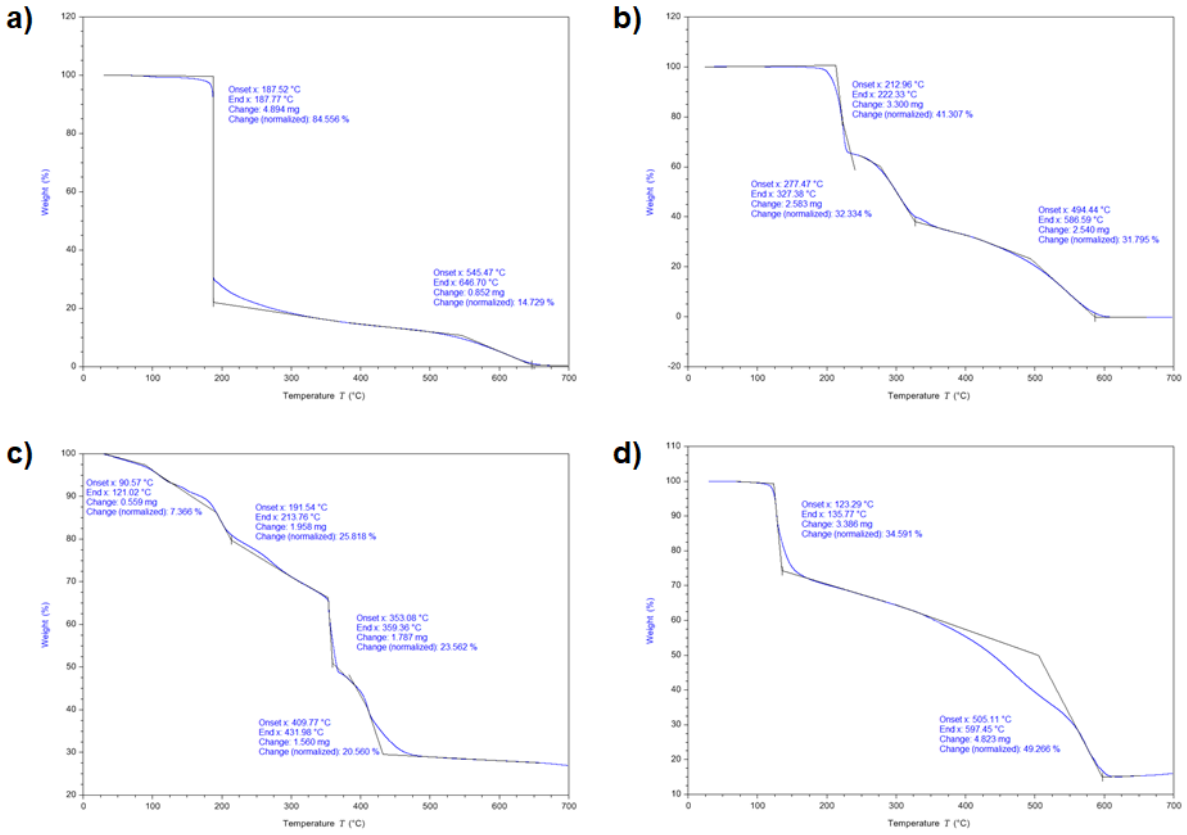

Figure S2.3 TGA thermogram for **(Hlin$^+$Cl$^-$)** (a), H**cafa** (b) (Na$^+$**cafa**$^-$) (c) and **(Hlin$^+$cafa$^-$)** (d)

Differential scanning calorimetry

Differential scanning calorimetry of (pentx)(Hpca)

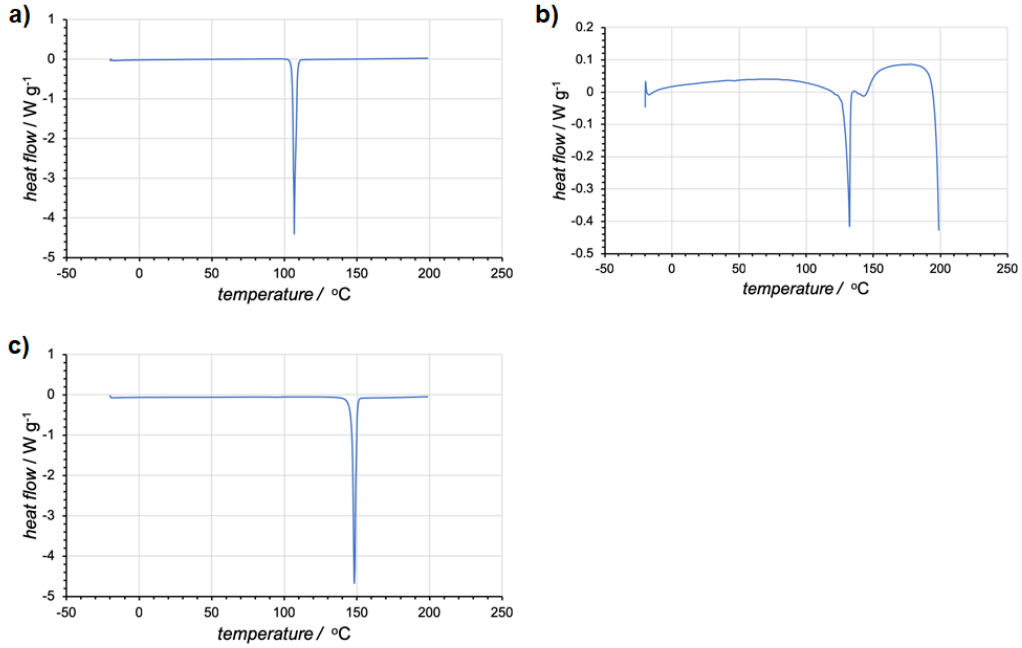

Figure S2.4 DSC thermogram for **pentx** (a), H**pca** (b) and **(pentx)(**H**pca)** (c)

Differential scanning calorimetry of (Hclon^{+}ala^{-})

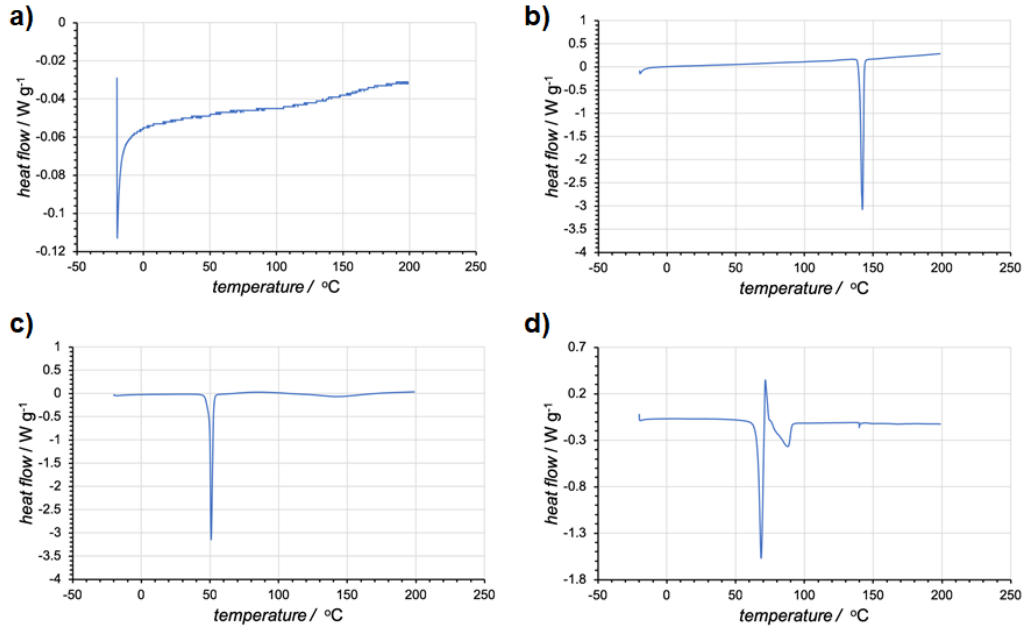

Figure S2.5 DSC thermogram for **(**H**clon**$^{+}$Cl^{-}**)** (a), **clon** (b), H**ala** (c) and **(**H**clon**$^{+}$**ala**$^{-}$**)** (d)

Differential scanning calorimetry of (Hlin$^+$cafa$^-$)

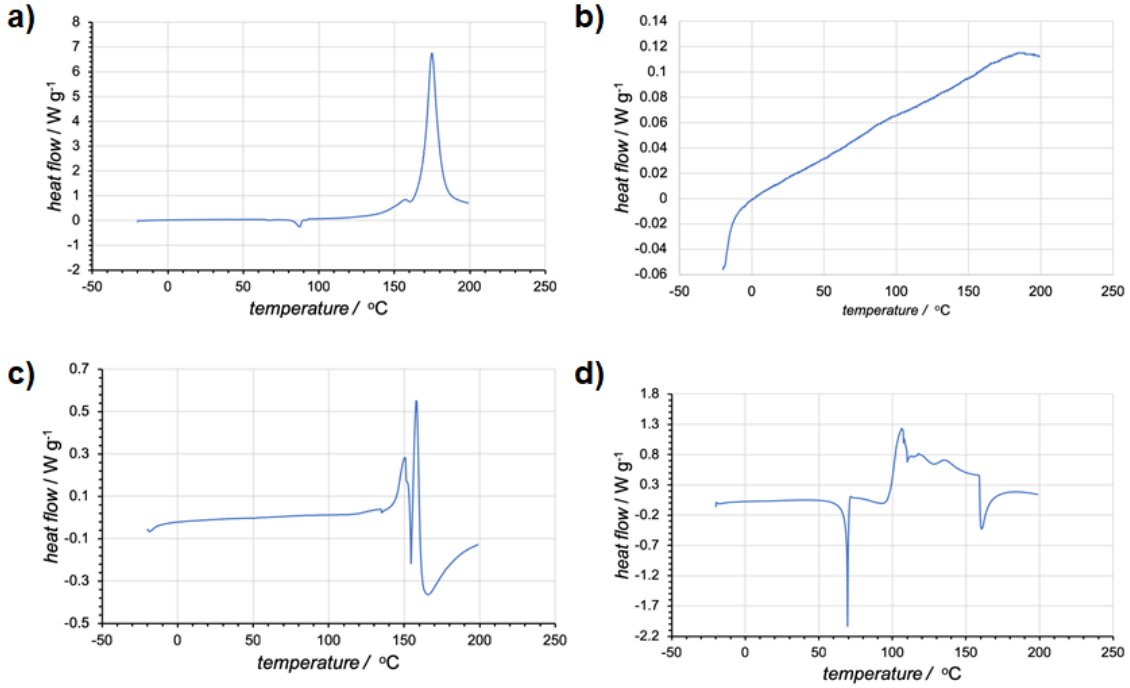

Figure S2.6 DSC thermogram of **(Hlin$^+$Cl$^-$)** (a), H**cafa** (b), **(Na$^+$cafa$^-$)** (c) and **(Hlin$^+$cafa$^-$)** (d)

15N Solid State Nuclear Magnetic Resonance Spectroscopy (ssNMR) of (pentx)(Hpca)

The largest chemical shift change is at the C=N site of **pentx** (217.7 ppm in the component and 230.3 ppm in the complex), which is the site that interacts most strongly with the Hpca, congruent to what is observed in the crystal structure.

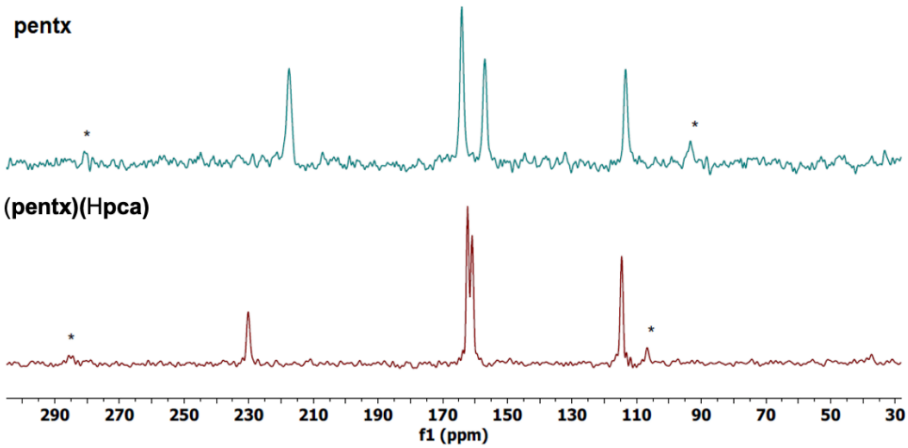

Figure S2.7 ^{15}N ssNMR of **pentx** and **(pentx)(Hpca).** Spinning sidebands are indicated by *.

^{1}H Nuclear Magnetic Resonance Spectroscopy (NMR) of clon

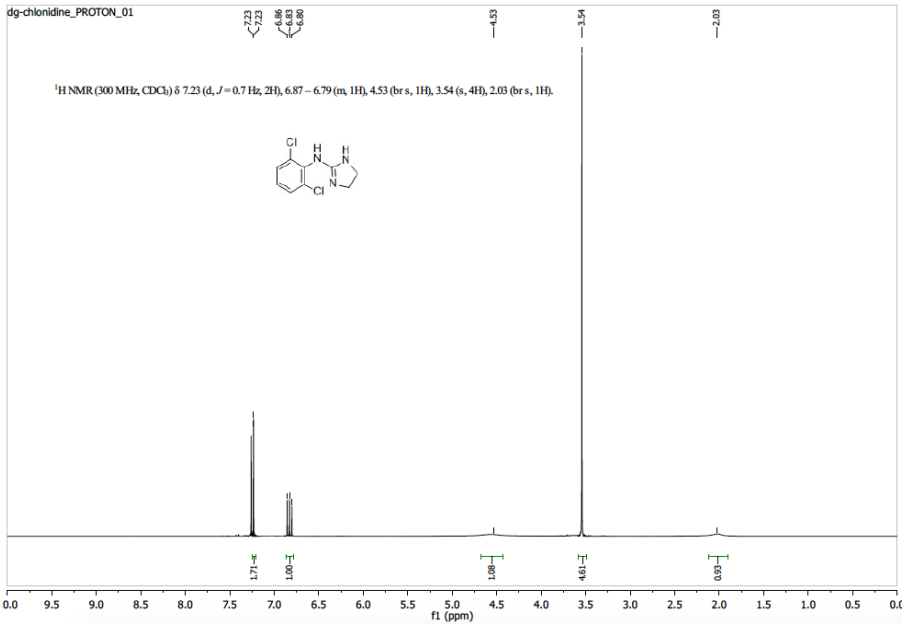

Figure S2.8 ^{1}H NMR spectrum (300 MHz) of **clon** after reaction with KOH

FTIR-ATR spectra of (H**clon**$^{+}$Cl^{-})

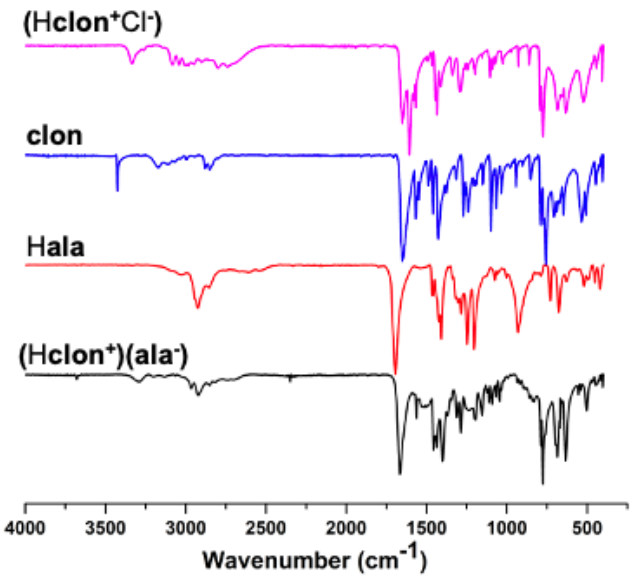

Figure S2.9 FTIR-ATR spectra for (H**clon**$^{+}$Cl^{-}), **clon**, H**ala** and (H**clon**$^{+}$)(**ala**$^{-}$)

Supporting information for Manuscript 3

A novel co-crystal of pentoxifylline & protocatechuic acid relieves allodynia in a rat model of CRPS by alleviating local tissue hypoxia

Supplementary data

Effect of topical pentx-pca on the contralateral paw of CPIP rats

To detect the contribution systemic absorption might have on the anti-allodynic effects of topical pentx-pca on CPIP rats, the drug was topically applied to the uninjured contralateral paws of the CPIP rats and resulting effects on ipsilateral PWT measured. The rats PWTs' remained low with no significant difference from values obtained post-vehicle treatment.

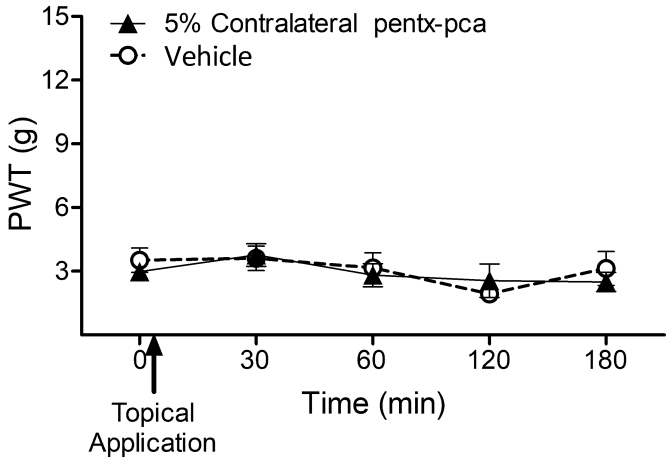

Figure S3.1 The effect of the application of topical pentx-pca (5% W/W) on the contralateral paw of CPIP rats. Ipsilateral PWT is not altered post-topical treatment of the contralateral paw indicating the absence of anti-allodynic effects due to systemic drug absorption (n = 8 rats).