CAPITAL UNIVERSITY OF SCIENCE AND TECHNOLOGY, ISLAMABAD

Pharmacological Evaluation of INCA-6 in Animal Models of Paclitaxel-induced Neuropathic Pain

by

Ibtasam Zakir

A thesis submitted in partial fulfillment for the degree of Master of Philosophy

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Department of Pharmacology

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In recognition of the perseverance, dedication, and resolve that have accompanied me throughout this challenging yet rewarding academic endeavor, I dedicate this thesis to myself with sincere humility and a profound sense of personal fulfillment. May this work serve as a gentle reminder of the importance of dedication and the pursuit of knowledge.

I am profoundly grateful to Dr. Muzaffar Abbas for his exemplary mentorship, scholarly wisdom, and unwavering support, which have been instrumental in the success of this endeavor. His exceptional capacity to provide guidance with both intellectual rigor and genuine kindness has had an indelible impact on my personal and academic development. Dr. Abbas's patience, encouragement, and thoughtful critique have motivated me to pursue excellence and approach scientific inquiry with both integrity and curiosity. It has been an immense honor to be under his guidance. The Faculty of Pharmacy and Capital University of Science and Technology provided a valuable platform to explore neuropathic pain and its management. Their supportive environment—rooted in critical thinking and innovation—greatly contributed to the depth and direction of this research. It is my sincere aspiration that this work, despite its modest scope, will contribute to the expanding body of knowledge in this field and motivate additional research for the mutual benefit of science and society.

CERTIFICATE OF APPROVAL

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Lastly, I am thankful for the opportunity to contribute to the evolving understanding of pharmacological interventions for neuropathic pain. I hope this modest effort lays the groundwork for further research in this important field.

(Ibtasam Zakir)

Abstract

Neuropathic pain is a chronic and disabling condition caused by nerve injury or malfunction. One severe variant, paclitaxel-induced neuropathic pain (PINP) frequently arises as a side effect of chemotherapy, worsening patient well-being and occasionally forcing discontinuation of treatment. The inadequate efficacy of current pain management strategies, along with their often-serious side effects, highlights the need for alternative therapeutic solutions. This study evaluates the therapeutic potential of INCA-6, a novel redox-active quinone, in addressing PIPN by targeting key neuroinflammatory and oxidative stress mechanisms involved in its development. In the experimental model, neuropathic pain was induced in mice through intraperitoneal injections of paclitaxel (2 mg/kg) on days 1, 3, 5, and 7. INCA-6 was then administered daily from days 8 to 14 at doses of 1 or 5 mg/kg (i.p.). For comparison, pregabalin (5 mg/kg, i.p.) was used as a positive control beginning on day 8. Behavioral assessments of pain sensitivity were conducted using von Frey filament, hot plate, and acetone tests. In addition, biochemical and histological analyses of spinal cord tissue were carried out to determine the levels of pro-inflammatory cytokines Interleukin - 1 beta (IL-1β), Tumor necrosis factor alpha (TNF-α) and oxidative stress markers malondialdehyde and glutathione (MDA and GSH). The findings indicated that INCA-6 significantly alleviated mechanical and cold allodynia, as well as thermal hyperalgesia, induced by paclitaxel. Moreover, INCA-6 significantly attenuated spinal cord oxidative stress and diminished elevated pro-inflammatory cytokine levels, thereby indicating potent anti-inflammatory and antioxidant activities. These findings suggest that INCA-6 targets major pathological mechanisms of PINP, presenting a promising multi-modal therapeutic approach. Its efficacy against chemotherapy-induced peripheral neuropathic pain, driven by its ability to modulate neuroinflammation and oxidative stress, positions INCA-6 as a novel and compelling candidate for the treatment of PINP.

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Abbreviations

ADMET	Absorption, Distribution, Metabolism, Excretion and Toxicity
ATP	Adenosine Triphosphate
BBB	Blood-Brain Barrier
BCA	Bicinchoninic Acid
CIPS	Chemotherapy-Induced Peripheral Sensitization
CIPN	Chemotherapy-Induced Peripheral Neuropathy
CNS	Central Nervous System
CYP1A2 Inhibitor	Cytochrome P450 1A2 Inhibitor
CYP2C19 Inhibitor	Cytochrome P450 2C19 Inhibitor
CYP2C9 Inhibitor	Cytochrome P450 2C9 Inhibitor
CYP2D6 Inhibitor	Cytochrome P450 2D6 Inhibitor
CYP3A4 Inhibitor	Cytochrome P450 3A4 Inhibitor
DMSO	Dimethyl Sulfoxide
DPN	Diabetic Peripheral Neuropathy
EAAT2	Excitatory Amino Acid Transporter 2
FLEX	Flexibility
GSH	Glutathione
H&E	Hematoxylin and Eosin
HEC	Higher Education Commission
IASP	International Association for the Study of Pain
IL-1β	Interleukin-1 Beta
IL-6	Interleukin-6
i.p.	Intraperitoneal

INSATU	Saturation
LD50	Lethal Dose 50%
LIPO	Lipophilicity
Log S (ESOL)	Logarithm of Solubility (Estimated Solubility)
LTD	Long-Term Depression
LTP	Long-Term Potentiation
MDA	Malondialdehyde
MGL-Tools	Molecular Graphics Laboratory Tools
NBF	Neutral Buffered Formalin
NFAT	Nuclear Factor of Activated T-cells
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NMDA	N-Methyl-D-Aspartate
NP	Neuropathic Pain
PDB	Protein Data Bank
Pgp Substrate	P-glycoprotein Substrate
PIPN	Paclitaxel-Induced Neuropathic Pain
PTX	Paclitaxel
ROS	Reactive Oxygen Species
SNRIs	Serotonin-Norepinephrine Reuptake Inhibitors
TCAs	Tricyclic Antidepressants
TLR4	Toll-Like Receptor 4
TNF-α	Tumor Necrosis Factor Alpha
TPSA	Total Polar Surface Area
TRPV1	Transient Receptor Potential Vanilloid 1

Chapter 1

Introduction

1.1 Background

Pain functions as a critical biological alarm that foretells possible or real damage to the body's tissues. The aspect of injury detection gives humans the ability to heal as it enables them to protect themselves from further damage. The International association for the study of pain (IASP) describes pain as "an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage" [1].

The description bears in mind the holistic balance of the experience of pain not just as an input signal but as an output which includes emotion, cognition, and complex psychological factors. The multi-dimensional nature of pain requires paradigm shifts in approaches directed towards pain management, particularly when dealing with chronic conditions

1.2 The Nature of Pain

Nociceptive pain is due to activation of nociceptors. These receptors, which detect harmful stimuli, are found in the skin, internal organs, and deeper body tissues [2].

Nociceptors respond to noxious stimuli in the form of mechanical strains like a pressure or stretching, extreme temperatures like heat or cold and chemical irritants or inflammatory mediators [3].

The activation of nociceptors will initiate the relay of nerve impulses to nervous system centers capable of receiving them, processing them, and synthesizing pain experience. Nociceptive pain is usually acute which enables it to serve as a protective mechanism informing an organism of either imminent or occurring injury to soft tissues.

1.2.1 Inflammatory Pain

Inflammatory pain follows damage to tissues or inflammation. The inflammatory process releases several different biochemical mediators: prostaglandins, bradykinin, histamine, and cytokines, which act on nociceptors and increase their sensitivity and lower their threshold.

This peripheral sensitization is responsible for hyperalgesia (increase of sensitivity to painful stimulation) and allodynia (unusual pain to stimuli which are not usually painful) which assists in tissue repair [1].

1.2.2 Neuropathic Pain

NP is a more complex form of pain that originates from damage or dysfunction within the sensory nervous system, rather than being triggered by external noxious stimuli acting on the peripheral nociceptive system [1].

1.2.3 Functional Pain

Functional pain includes pain syndromes where the etiology remains obscure, and there is no clear evidence of pathological damage to soft tissue or specific nerve lesion(s). They include fibromyalgia as well as irritable bowel syndrome [4]. The conditions are believed to have altered mechanisms of pain modulation within the central nervous system

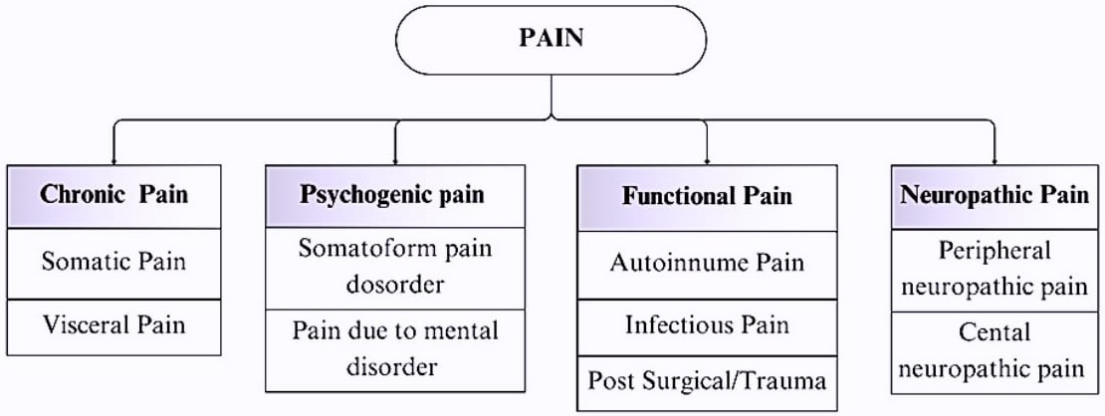


FIGURE 1.1: Types of pain

Diagram 1.1 categorizes pain into four primary types: chronic, neuropathic, functional, and psychogenic pain. Chronic pain encompasses somatic and visceral forms, frequently enduring and arising from bodily tissues or organs. Neuropathic pain, arising from nerve damage, is categorized into peripheral and central types, which can be either acute or chronic, encompassing conditions such as TMD. Functional pain occurs in the absence of evident structural damage and is linked to autoimmune, infectious, or post-surgical etiologies. Psychogenic pain is associated with psychological factors and encompasses somatoform pain disorder as well as pain resulting from mental health conditions. This classification emphasizes the varied origins and mechanisms of pain, thereby facilitating more precise therapeutic strategies (Adapted from IASP, Treede et al.,2015).

1.3 Neuropathic Pain

1.3.1 A State of Aberrant Signaling

Neuropathic pain (NP) is a distinct and chronic type of pain often resulting from injury to, or disease affecting the somatosensory nervous system. It emerges as a consequence of damage within the peripheral nervous system. Unlike nociceptive pain, which is acute in nature and resolves after tissue healing, NP is persistent and often lacks a traditional healing process, thus indicating continuous dysfunction within the nervous system. The origins of NP are diverse and include damage to peripheral

nerves, the spinal cord, and even portions of the brain; this results in intricate changes at multiple levels throughout the neurological system.

A patient's discomfort is frequently heightened due to spontaneous sensations – pain that occurs spontaneously without any external trigger which can be burning/tingling/shooting/electric shock like. Patients suffering with NP may experience allodynia which is commonly referred to as 'painful touch' wherein clothes rubbing against skin or gentle winds cause discomfort. This phenomenon reflects an underlying system that has been agitated far beyond reason or expectation. Closely related to this is hyperalgesia: an exaggerated response to mildly painful stimuli inflicts extreme agony often unbearable. These aberrant responses are quintessentially features of the neuropathic pain condition, differentiating it significantly from other pain types.

Neuropathic pain (NP) is characterized by burning, tinging, shooting and electric shock-like sensations. Stiffness and numbness, alongside an enhanced sensitivity to touch or pressure, are often seen simultaneously. NP lacking a sufficient treatment strongly decreases the patient's quality of life; it causes profound emotional distress like depression or anxiety. Patients also describe physio-emotional distress: chronic fatigue, insomnia, muscle tightness, tachycardia and heightened tonic signals all add to their suffering [3]. The multifactorial progression of neuropathic pain involves peripheral as well as central nervous system plastic changes due to a complexity of neuroplastic events.

1.3.1.1 Pain Ascending and Descending Pathways in Neuropathic Pain

To understand the syndrome of neuropathic pain one needs to grasp deeply the balance existing between ascending pathways and descending influencing systems. Sensors in the body detect harmful stimuli as well as other sensory experiences neutral or even pleasant ones, for instance on their journey to the brain through specific routes known as ascending pathways. This process begins from peripheral receptors called nociceptors which send impulses at the level of dorsal horn in spinal cord with help from peripheral nerves. The first, second order neurons begin to synapse and process signals on the contralateral side through the spinothalamic tract to the thalamus.

The projection areas of the thalamus include the somatosensory cortex, insula, and anterior cingulate cortex which are responsible for relaying information related to perception or modulation of pain.

In NP, injuries can increase excitability in ascending pathways; spontaneous nerve damage firing and synaptic reorganization in the dorsal horn led to allodynia and hyperalgesia.

Pathways originating from periaqueductal gray (PAG) and rostral ventromedial medulla (RVM) descends to spinal cord dorsal horns form descending pathways that impact both NP and PAG. These descending pathways affect with a strong inhibitory/ facilitatory control over incoming pain signals from peripheral nerves. Some important neurotransmitters include: serotonin, norepinephrine, and endogenous opioids.

Disorders associated with chronic pain such as NP experience problems maintaining these controls thus impacting overall function by lowering ability to suppress pain signals. The imbalance and hyperexcitability in ascending pathways, contributes to the intractable nature of neuropathic pain, driving both the spontaneous pain and the exaggerated responses to stimuli.

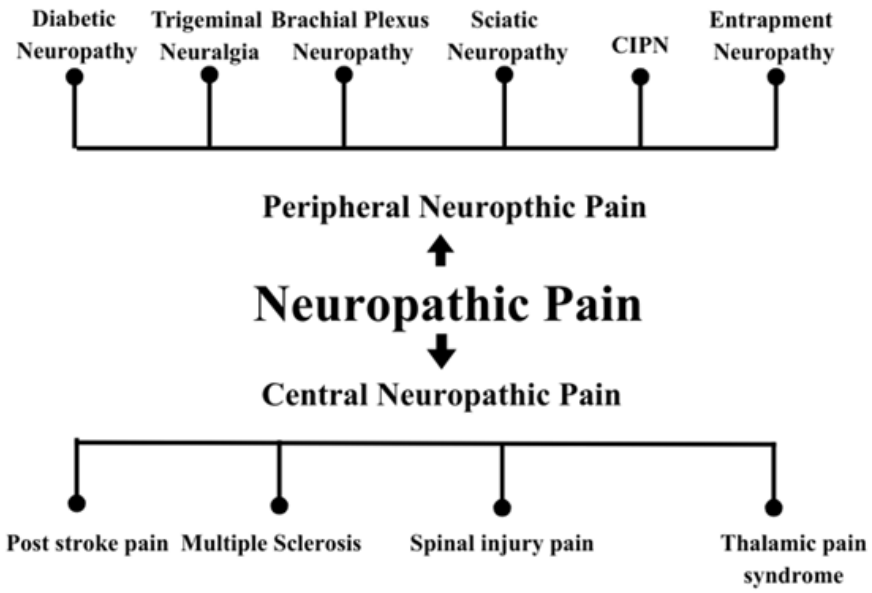


FIGURE 1.2: Neuropathic Pain

Figure 1.2 illustrates the concept of NP, which arises from injury or dysfunction within the somatosensory nervous system.

The figure explains the aberrant signaling phenomena such as spontaneous sensations, allodynic, and hyperalgesia responses which are quintessentially features of the neuropathic pain condition (Adapted from Treede *et al.*, 2008).

1.4 Types of Neuropathic Pain

Peripheral Neuropathic Pain comes from the injuries sustained to peripheral nerves outside the brain and spinal cord, while Central Neuropathic Pain arises from disorders within the brain or spinal cord. Figure 1.2 shows 11 distinct clinical types of NP. They include diabetic neuropathy, postherpetic neuralgia, trigeminal neuralgia, brachial plexus neuropathy, sciatic neuropathy, chemotherapy-induced neuropathy, entrapment neuropathies, post-stroke pain, multiple sclerosis, spinal cord injury pain and thalamic pain syndrome.

1.4.1 Peripheral Neuropathic Pain

1.4.1.1 Diabetic Neuropathy

This type of pain is often considered as one of the most common types of suffering for diabetic patients. Prolonged high blood sugar levels may lead to damage of nerve fibers known as distal symmetric polyneuropathy which predominantly affects the feet and subsequently also involves the upper limbs.

Patients may feel numbness along with burning, shooting sensations, aching pain, and more in what is described as a “stocking-glove” pattern. The underlying pathology consists of metabolic derangements alongside oxidative stress and impaired microcirculation of nerves which causes ineffective regeneration and degeneration of nerve fibers.

1.4.1.2 Postherpetic Neuralgia

Shingles or herpes zoster is associated with severe neuropathic pains localized around chest, back or face following rashes due to reactivation varicella-zoster virus responsible for chickenpox. Typically, this neuropathic pain can be classified into three

categories: sharp or throbbing or burning pain that may develop hypersensitivity to any form stimuli like touch (also termed allodynia) secondary to canonical level stimulus due to thermal burns because incisively damaged sensory neuronal pathways.

1.4.1.3 Trigeminal Neuralgia

Described as intense and shock-like electric jolts or stabbing pains that occur suddenly and in phases. These sensations often occur in the regions supplied by the trigeminal nerve, usually affecting one side of the face. The most common cause involves vascular compression of the trigeminal nerve root demyelination along with hyperexcitability of the nerve. Lastly, these excruciating episodes are triggered by a non-painful light touch, chewing, talking, or even a cool breeze.

1.4.1.4 Brachial Plexus Neuropathy

Brachial Plexus Neuropathy is also referred to Parsonage-Turner syndrome or brachial plexopathy. The condition involves inflammation alongside damage to the network of nerves known as the brachial plexus. Typically, acute onset, with severe pain located in shoulder and upper arm areas, followed by rapid development, leads to weakness along with muscle atrophy in the affected side. It is often idiopathic but can be activated by viral infections, vaccination, and an immune system response.

1.4.1.5 Sciatic Neuropathy

Sciatic Neuropathy is characterized by damage that arises from the dysfunction of the sciatic nerve, which is recognized for its pathway extending from the lower back down to the leg. The term is somewhat broad and used inconsistently, particularly in relation to radicular pain. However, true sciatic neuropathy refers specifically to an injury located directly beneath the nerve trunk, rather than in the surrounding area.

Symptoms include burning, shooting pain, numbness, tingling, and weakness along the nerve's path. It affects the buttock, posterior thigh, and calf, and can result from compression, trauma, or tumors.

1.4.1.6 Chemotherapy-Induced Neuropathy

Chemotherapy may cause neurotoxicity that damages peripheral nerves resulting in a particular form of neuropathy. It manifests as tingling, numbness, burning pain, and sharp shooting pain in a 'stocking-glove' distribution pattern. It can range from mild to severe pain such that it affects daily activities and life quality.

1.4.1.7 Entrapment Neuropathies

These syndromes arise when there is mechanical compression or "entrapment" of a peripheral nerve as it travels through an anatomical tunnel. A common example is Carpal Tunnel Syndrome where the median nerve gets compressed at the wrist leading to pain, numbness, and tingling in the thumb, index finger as well as middle finger plus half of ring finger. Other known sites are elbow ulnar nerves (cubital tunnel syndrome) and peroneal nerves at the interface with the fibula head associated with focal neuropathic symptoms.

1.4.2 Central Neuropathic Pain

1.4.2.1 Post-Stroke Pain

This is a type of chronic neuropathic pain that occurs after a person suffers infant Los Angeles or epilepsy stroke. It happens due to injury to the central nervous system structures responsible for certain perceptual tasks located mostly in the thalamus or brain stem. Patients often demonstrate persistent pain which can be described as burning, ache, 'tingle', or shoot-like and is associated with allodynia and unpleasant abnormal sensations.

1.4.2.2 Multiple Sclerosis

In Multiple Sclerosis (MS), which is an autoimmune disease characterized by demyalination and neurodegeneration changes within bones and fibers of the body;

Neuropathic Pain also manifests itself unfortunately. Depending on where the MS lesions are, patients may have different forms of neuropathic poverty such as burnings, electric-shocks like pains e.g., Lhermite's sign, facial pain like trigeminal neuralgia.

1.4.2.3 Spinal Cord Injury Pain

It is a chronic painful condition that affects almost every individual who has suffered spinal cord injury (SCI) with no easy treatment solutions. It can cause pain confined to where the injury occurred (at-level pain) or more often results in nerve injury-signal related pain occurring below where the neurological damage happened (below-level pain).

1.4.2.4 Thalamic Pain Syndrome

Sometimes grouped with post-stroke pain, Thalamic Pain Syndrome more precisely denotes intense central neuropathic pain stemming from a thalamic lesion. The thalamus functions as vital a relay and processing center of body sensory information. Its damage results in severe and often unrelenting and intensely painful suffering on the opposite side of the body - including the face—and parts of it such as skin surface and deep tissues.

It is described as a form of searing, shattering or contusive pain, which characterize by intense allodynia and dysesthesia is even more altered than normal sensation resulting from very complex disorganization within this brain focal region.

1.5 Peripheral Mechanisms

According to Vaeth and Feske (2018), in most cases, peripheral nerve damage triggers a change cascade in the injured nerves. One such example is ectopic firing, in which a damaged segment of a nerve activates on its own and is capable of generating an action potential, which is sufficient to signal pain in the absence of any provoking stimuli. This is thought to be a consequence of reorganization of ion channels at the nerve

membrane in terms of their expression and localization. In line with this, neuropathic pain involves a disruption in ion channel regulation; specifically, nerve injury alters the expression and function of voltage-gated sodium, calcium, and potassium channels.

These changes heighten the excitability of nerve fibers, making it easier for action potentials to be generated and pain signals to be transmitted—a state referred to as hyperexcitability and disinhibition of pain networks. Additionally, mitochondrial dysfunction contributes to bioenergetic failure, identifiable by diminished ATP creation and an elevation in reactive oxygen species (ROS), which are both cytotoxic and pro-inflammatory [4].

This environment fosters neuroinflammation, characterized by the mobilization and activation of immune cells like macrophages and mast cells, alongside the emission of pro-inflammatory cytokines like TNF-α and IL-1β [5]. The heightened sensitivity of pain pathways, caused by these mediators, leads to an amplified perception of pain, encompassing conditions like allodynia.

1.6 Central Mechanisms

According to Chen *et al.* (2024), injury to peripheral nerves leads to significant alterations in both the structure and function of the central nervous system. One of the most important changes is the spinal cord neuron sensitization which occurs in the dorsal horn. Persistent input from damaged peripheral nerves increases the excitability of spinal neurons, leading to an exaggerated response to sensory input that, even when it is benign or normally non-threatening, will be treated as hurtful. This phenomenon is termed as central sensitization, which is a leading cause of allodynia (pain induced by harmless stimuli) and hyperalgesia (increased pain sensitivity) [5].

In addition, glial cells that assist and protect the neurons become activated after nerve injury. Microglia and astrocytes the glial cell also produce inflammatory mediators, pro-inflammatory cytokines, chemokines, and a variety of other substances that add to neuroinflammation and hyperactivity of neurons. The descending inhibitory pathways from the brainstem that control the transmission of pain to the spinal cord also suffer

potential damage in neuropathic pain. Inhibitory control is lessened leading to greater perception of pain. Enhanced perception of pain can also be attributed to reduction in synaptic inhibition. The capacity of synapses to increase or decrease their strength: synaptic plasticity also undergoes changes.

Synaptic plasticity also undergoes changes, affecting the capacity of synapses to increase or decrease their strength. For instance, long-term potentiation (LTP), which enhances synaptic connections, may be exaggerated, while long-term depression (LTD), which entails weakening of synaptic connections, may be diminished, leading to an imbalance in these processes [4]. These fundamental developments critically impact the ever-present and long-lasting characteristic of neuropathic pain.

The pathways of concern, such as the spinothalamic tract which relays pain to the thalamus and cortex, the dorsal column-medial lemniscal pathway that primarily conveys touch and proprioception, and the trigeminal pathway that conveys facial sensory information, are also implicated in some forms of neuropathic pain [6].

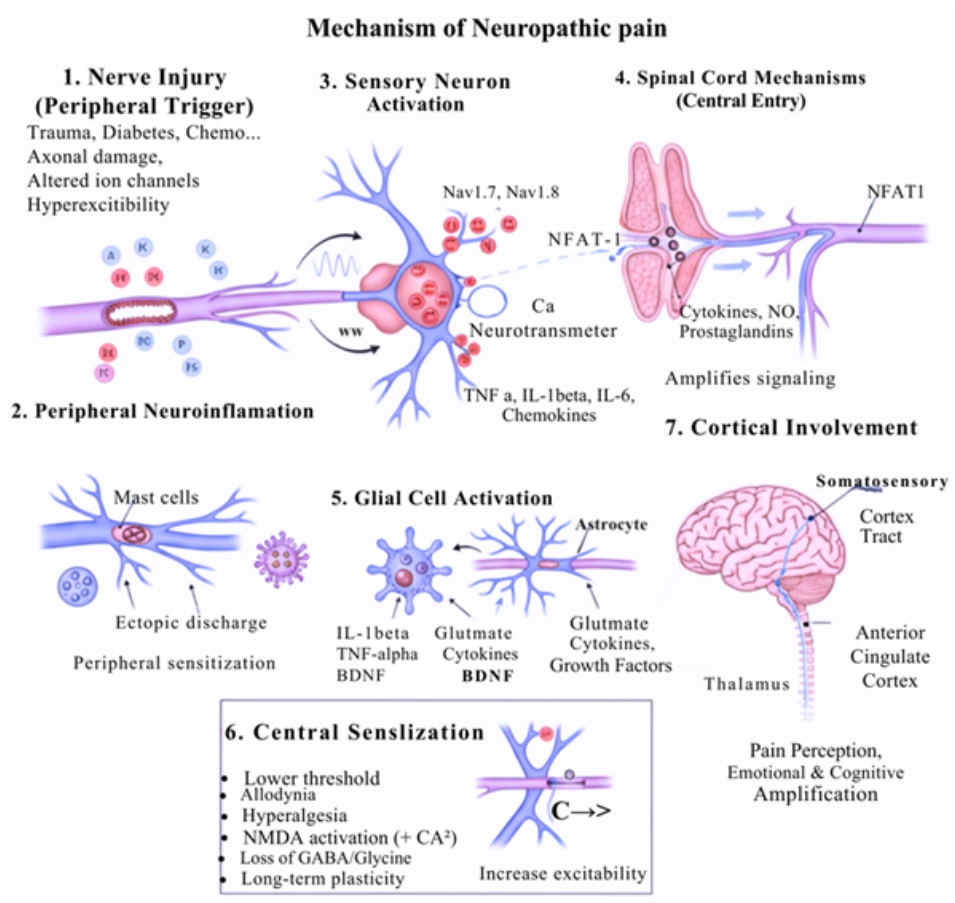


FIGURE 1.3: Neuropathic Pain Pathways.

Figure 1.3 illustrates how neuroinflammation triggers both peripheral and central pain pathways following nerve injury. This process involves the release of pro-inflammatory cytokines—such as TNF-α, IL-1β, and IL-6 from immune cells like macrophages and mast cells, which in turn activate sodium and potassium ion channels. These cytokines induce ectopic discharges and sensitize neurons by acting on sodium channels (e.g., $Na_v1.7$), thereby promoting central sensitization. In the spinal cord, cytokine signaling is augmented by transcription factors such as NF-κB and NFAT1, which are modulated by agents such as INCA-6, thereby activating microglia and astrocytes. This reinforces chronic pain pathways by resulting in enhanced pain signaling to the brain and spontaneous neuronal firing (Adapted from Roehrl *et al.*, 2004).

1.7 Prevalence and Impact of Neuropathic Pain

TABLE 1.1: Prevalence of Neuropathic Pain in different Sub-groups [7]

Subgroup prevalence	**Prevalence**
Diabetic Neuropathy	25% of diabetics
Postherpetic Neuralgia	10–20% of shingles patients
CIPN	Up to 70% in treated patients

The worldwide occurrence of neuropathic pain is also considerable, approximating at 6.9% to 10%. This accounts for several people internationally living with this painful condition. Some subgroups have even higher rates which points out the disproportionate burden of pain in certain populations. As an example, development of diabetic neuropathy as a complication of diabetes occurs in roughly 25% of the affected population [8]. Postherpetic neuralgia is another chronic pain syndrome that affects 10-20% of shingles patients. Most importantly, the patients that undergo chemotherapy are also fundamentally at a high risk, with the rate of CIPN reaching as high as 70% depending on the chemotherapeutic agent and treatment plan used [8].

Psycho-neuropathic pain may significantly influence the life of a person in its various dimensions, i.e., physical, psychological as well as social functioning. Chronic pain is one of the most significant ailments in one's life, as it disrupts sleep cycles, causing

severe fatigue and irritability. Restriction of movement and range of motions can make carrying out even the simplest tasks like dressing up or eating a challenge [6].

Chronic pains have deep emotional repercussions too. Anger, social isolation, depression and anxiety are common. Furthermore, increased healthcare costs and reduced productivity make neurologic pains a major economic issue for countries.

1.8 Paclitaxel-Induced Neuropathic Pain

1.8.1 A Chemotherapy-Related Complication

Texanes, including paclitaxel, stand out as potent neuropathic pain inducers when compared to other classes of cancer treating chemotherapeutic agents. Paclitaxel, derived from the Pacific yew tree, is recognized as highly potent chemotherapeutic agents and is widely used in treating solid tumors, including those of the breast, ovary, lung, and prostate [9].

Its anticancer efficacy is attributed to its mechanism of action, which involves binding to β-tubulin—a key component of microtubules. By stabilizing these microtubules, paclitaxel disrupts the normal dynamics required for mitotic spindle formation consequently; it brings about cell cycle arrest and fosters apoptosis within cancer cells. This inhibition of uncontrolled cell division ultimately contributes to tumor shrinkage and potential remission of the disease [10]. However, this striking mechanism that makes paclitaxel an exceptionally potent anti-cancer drug does render it neurotoxic.

Figure 1.4 illustrates the mechanism through which paclitaxel, a chemotherapeutic agent, induces chronic neuropathic pain. Paclitaxel, while stabilizing microtubules in cancerous cells, also disrupts mitochondrial function in neurons, triggering the release of ROS and cytochrome c. This cascade activates NF-κB and caspases, promoting the production of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6. These cytokines further stimulate the activation of nuclear factor of activated T-cells (NFAT) in astrocytes. NFAT activation results in the downregulation of excitatory amino acid transporter 2 (EAAT2) and promotes calcium overload, contributing to

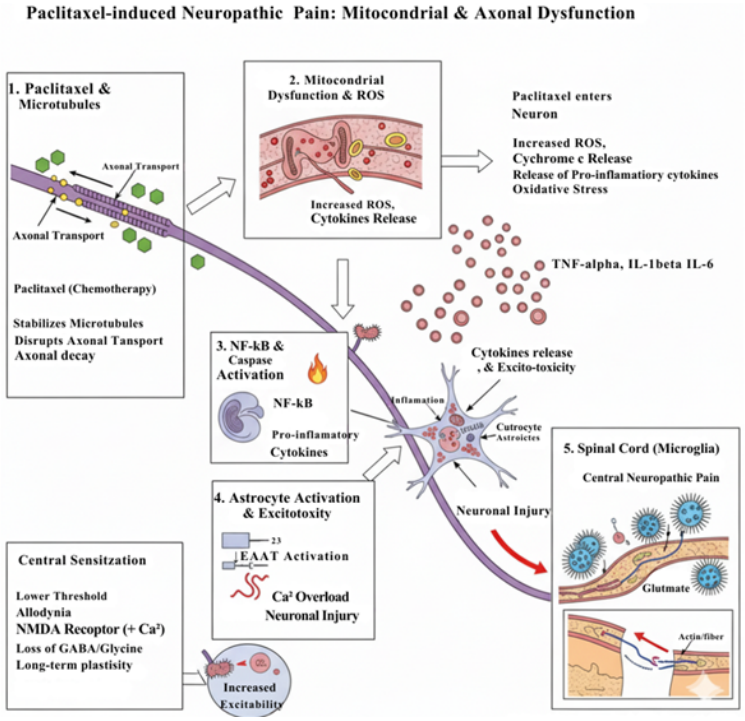


FIGURE 1.4: Paclitaxel-induced neuropathic pain via mitochondrial and astrocyte dysfunction.

excitotoxic neuronal injury. This sequence of events underlies the development of chronic neuropathic pain in the spinal cord (Adapted from Vermeer & Eijkelkamp et al., 2021).

Microtubules have a dual function: besides supporting cell division, they are instrumental in the process of axonal transport. Neurons with long axons extending to the peripheries are particularly reliant on this mechanism for their sustenance and functionality [11].

Paclitaxel's effects on microtubule dynamics and function disrupt axonal transport which sets off a chain reaction that can injure peripheral nerves and result in neuropathic pain. This becomes an issue for the long sensory neuron axons. PIPN, or paclitaxel-induced peripheral neuropathy, shows up in many patients and grows worse with higher doses; in some cases, the disability lingers for years. It usually appears as an even pattern of nerve injury that starts at the fingers and toes, creeps up the limbs, and forms the classic stocking-glove look [12].

Several interwoven cellular and molecular changes underlie the discomfort of PINP.

Chief among them is damage to microtubules, the filaments that normally support axons and ferry vital supplies. Although paclitaxel works by stabilizing these structures, that very effect robs them of the dynamic sliding needed for healthy cargo transport. When the machinery falters, materials pile up near the cell body and essential proteins dwindle at the distant tip.

Over time, the imbalance tires the neuron, causing loss of function and gradual axonal decay [11].

Paclitaxel also hurts the mitochondria, forcing cells to scrap energy. These tiny power plants slow ATP production, robbing the cell of its main fuel. Without enough ATP, everyday tasks like ion pumping and protein folding stall.

And because the damaged organelles leak excess reactive oxygen species, oxidative stress deepens [13]. That steady, high-energy demand makes neurons especially easy targets.

Calcium mishandling and NFAT turn on additional injury pathways. Paclitaxel tips the balance, letting too much calcium flood the neuron. The surplus triggers chains of signals, including the calcineurin-NFAT circuit, which fuels inflammation and pushes excitability higher [13].

Releasing NF-κB and turning on the cytokine factory matter a great deal. Paclitaxel pushes up Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB), a central pro-inflammatory messenger.

Once active, NF-κB overproduces cytokines like TNF-α and IL-1β [14]. These molecules stir up neuroinflammation, damage nerve tissue, and, in the end induces pain.

ROS buildup and cell injury also stand out in PIPN. Paclitaxel raises reactive oxygen species, which batter lipids, proteins, and DNA and lift oxidative strain. The damage weakens neurons and can set off apoptosis, or programmed cell death [13].

The ongoing activity of glial cells and their role in keeping the inflammation alive is key to the long-lasting pain seen in PIPN. After paclitaxel exposure, peripheral Schwann cells and central microglia plus astrocytes all switch on [13].

Once activated, these glial cells spill out pro-inflammatory chemicals, which worsen the nerve-immune cycle and feed the chronic pain seen in PIPN.

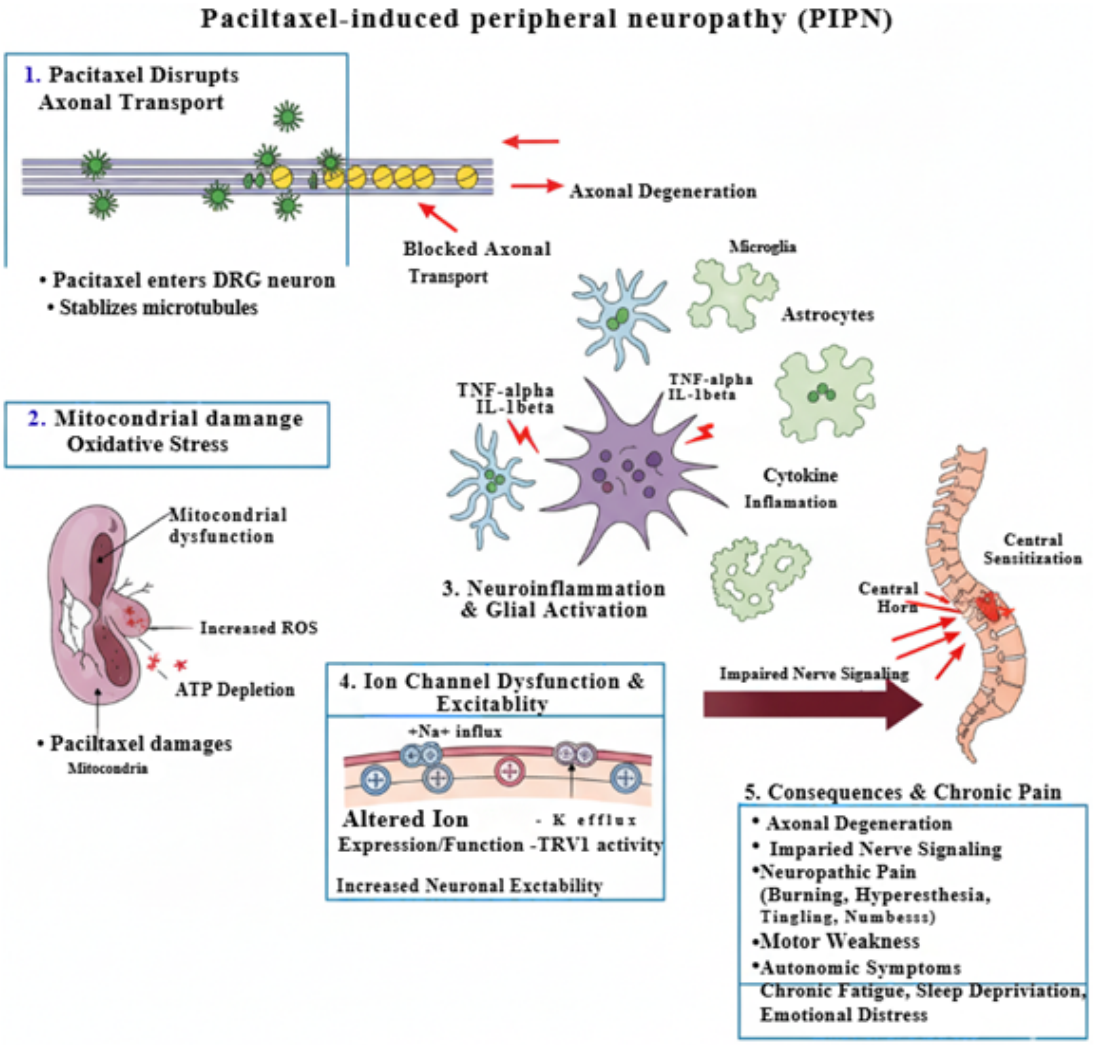


FIGURE 1.5: Cellular mechanism of PIPN.

Diagram 1.5 maps how many stressed cell types hurt sensory neurons and nearby glial neighbors in paclitaxel-induced peripheral neuropathy (PIPN). Once inside a dorsal root ganglion (DRG) neuron, paclitaxel locks microtubules in an unstable state and blocks axonal transport.

This clogging, together with damaged mitochondria, swells the organelles, boosts reactive oxygen species (ROS), and drains ATP. Sensing trouble, microglia and other glia fire back with cytokines, driving the inflammation even higher. At the same time, paclitaxel changes the expression and function of ion channels (such as Na^+, K^+, and TRPV1), which increases neuronal excitability. Axonal degeneration, impaired

nerve signaling, and neuropathic pain are the results of these effects (Adapted from Starobova & Vetter 2017).

Surgical PIPN is commonly associated with the pathological burning pain and hypersensitivity to stimuli, while blunting of neuro-sensory responses such as tingling, numbness, and paresthesis can also be hijacked by patients. Along with sensory symptoms, PIPN can also present motor symptoms like weakness of the arms and legs which makes fine motor tasks difficult, as well as gait changes. Somewhat infrequently, PIPN may have some symptoms related to autonomic functions including changes in the sweating mechanism and orthostatic hypotension [15].

According to Mehta (n.d), PIPN related pain can also severely aggravate the patient's pain boundaries. The chronic pain associated with PIPN, motor coordination sculptures with tendons, and disturbances in the limbs concludes the ability to execute basic tasks, including walking, sitting, dressing, or attending work. Additional liabilities of PIPN encompass sleep deprivation, chronic fatigue, emotional turmoil, and depressive elements. The PIPN in some cases is so severe that it requires dose cuts and at times ceases the dose of life-saving chemotherapy drugs, declining the prognosis and treatment outcomes. Also, PIPN may sustain for months, or even years after chemotherapy is completed, emerging as a persistent and most often painful complication for some survivors, emphasizing the need for clinically advanced and efficient interventions.

1.9 Limitations of Current Treatments and Need for Novel Therapies

Current pharmacological and non-pharmacological approaches like gabapentin or pregabalin to manage pain, duloxetine or amitriptyline for neuropathic pain, opioids, lidocaine patches or capsaicin cream, therapy, and acupuncture only offer transient and partial relief of symptoms. Topical therapies, physical therapy and acupuncture are not without side effects either. Severe drowsiness, dizziness, nausea, cognitive fog, and even addiction are risks associated with some treatments. These approaches

do not consider the underlying pathological mechanisms responsible for chronic pain. Additionally, current treatments for chronic pain do not incorporate changes needed to reverse nerve damage or altered signaling pathways that are linked to persistent pain states [16].

Treatments do little to repair damage and focus more on providing pain relief, which is often insufficient or high in side effects, leading patients to abandon their treatment plans. This sheds light on the need for different mechanisms aimed at treating PIPN.

1.10 INCA-6: A Potential New Therapeutic Avenue

Small, redox-active quinone containing molecules like INCA-6 (Triptycene - 1, 4 - quinone) also appears to have hope for the treatment of neuropathic pain hypersensitivity. These molecules' structural and pharmacological characteristics may be more beneficial than current treatment options. INCA-6 is more bioactive than other therapeutics because of its triptycene core which enhances its binding and effects on oxidative stress and inflammation through potent modulation of oxidative stress and inflammation [17].

INCA-6 exerts a dual mechanism of action that may be particularly relevant in the context of PIPN:

Inhibition of calcineurin and NFAT activation: Calcineurin, a calcium-dependent phosphatase that activates the Nuclear Factor of Activated T-cells (NFAT) has been shown to be inhibited by INCA-6. This means, NFAT activation leads to increased expression of proinflammatory cytokines as well as other mediators that are known to neuropathic pain. INCA-6 by inhibiting calcineurin will reduce NFAT activation, and this will reduce the inflammatory response [17].

Scavenging of reactive oxygen species (ROS): Through its structure, INCA-6 features a quinone moiety that enables the molecule to undergo redox cycling, thereby scavenging ROS. ROS is a byproduct of oxidative stress, which is an essential factor in the

development of neuropathic pain [18]. Therefore, by lessening the ROS level INCA-6 can mitigate damage to neurons and pain signaling pathways.

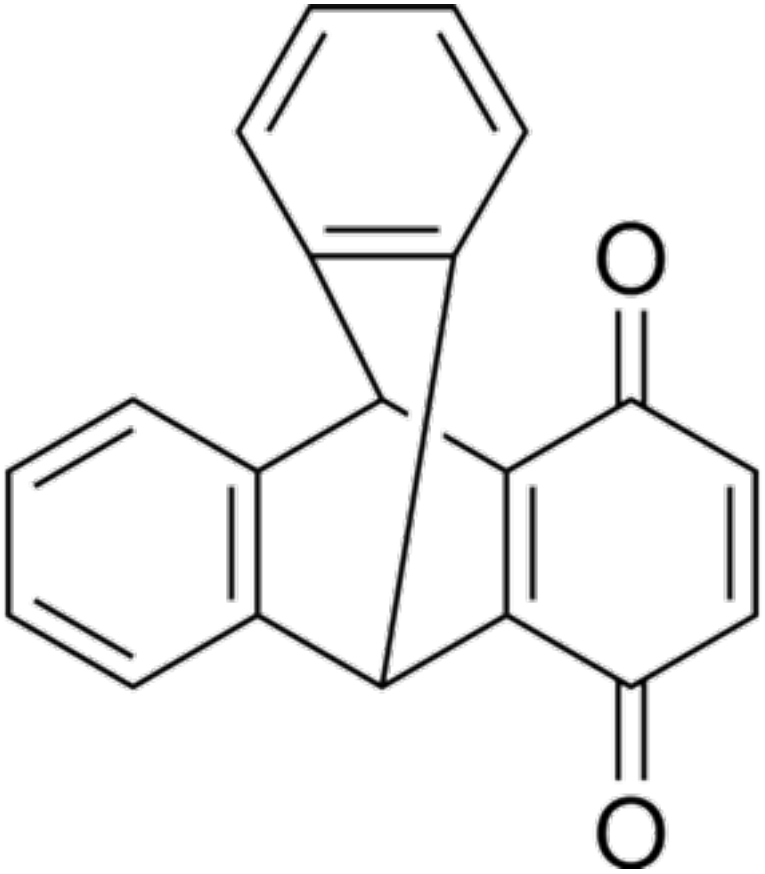


FIGURE 1.6: Chemical Structure of INCA-6.

INCA-6 is an innovative redox-active small molecule characterized by a triptycene backbone and a quinone functional group, which are pivotal to its biological efficacy. The triptycene structure offers a rigid, three-dimensional framework that improves molecular stability and interaction with biological targets.

The quinone moiety is recognized for its capacity to engage in redox cycling, enabling INCA-6 to regulate oxidative stress and neutralize reactive oxygen species. The redox properties, along with its structural characteristics, allow INCA-6 to engage with significant pro-inflammatory mediators, including NF-κB and cytokines such as IL-1β and TNF-α.

These characteristics render INCA-6 a promising multi-target agent for the treatment of inflammatory and neuropathic disorders, such as paclitaxel-induced neuropathic pain (Adapted from Kwon et al., 2018).

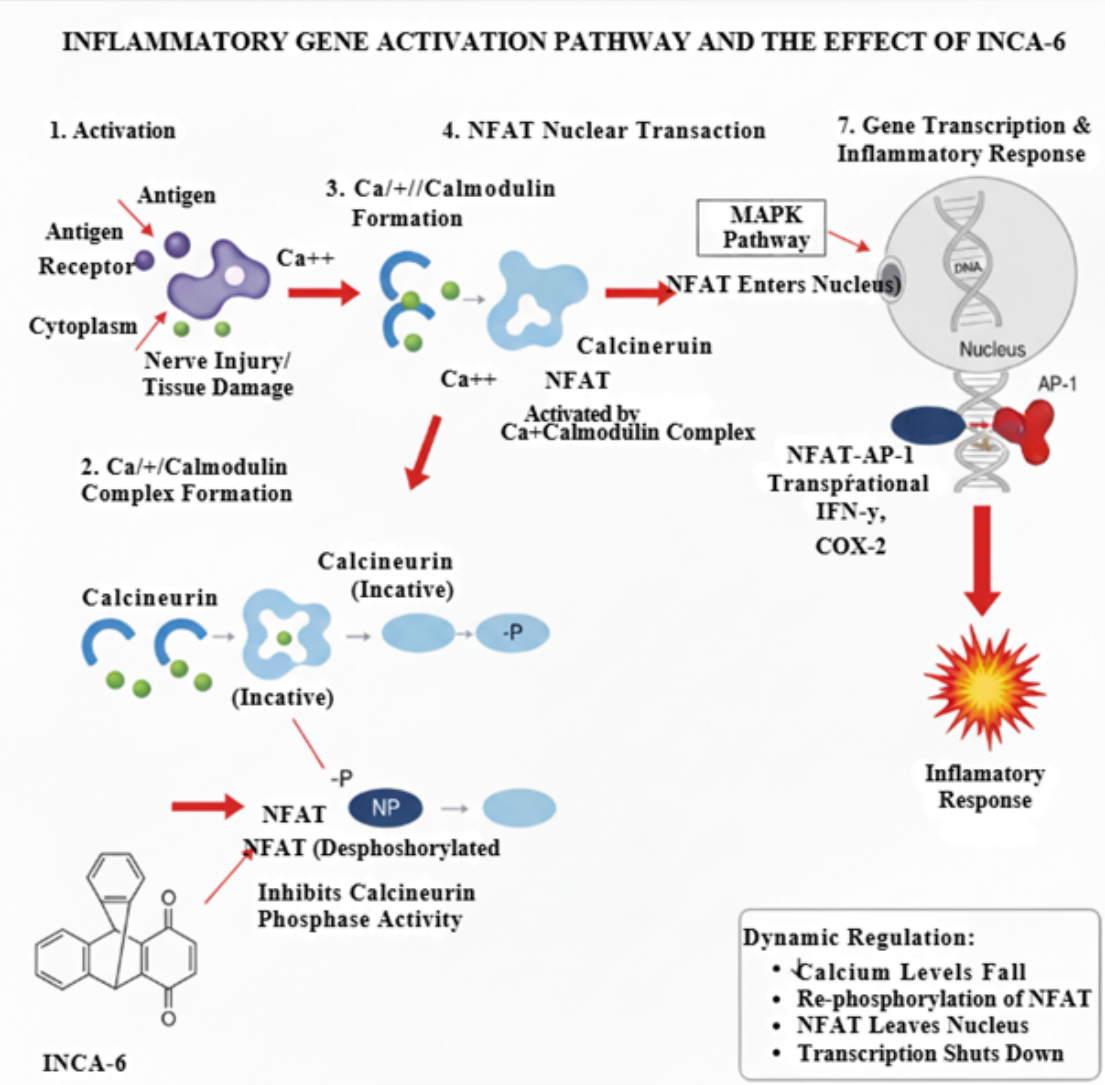


FIGURE 1.7: INCA-6 modulation of NFAT signaling pathways.

This figure delineates how INCA-6 inhibits the Calcineurin-NFAT pathway, which modulates immunity and inflammation across various cell types. By inhibiting Calcineurin, the calcium-and-calmodulin-dependent phosphatase, the drug prevents it from dephosphorylating NFAT. Consequently, NFAT is unable to translocate into the nucleus, initiate new gene expression, or revert from its phosphorylated state. This inhibitor diminishes immune activation and reduces cytokine production from T cells. INCA-6 prevents the downregulation of EAAT2 in microglia and astrocytes, thereby safeguarding against excitotoxicity and mitigating neuroinflammation. Inhibition of NFAT in endothelial cells limits the expression of cytokines and adhesion molecules, consequently reducing the migration of immune cells through the blood-brain barrier. The synergistic effects in the spinal cord and musculature contribute

to the alleviation of neuroinflammation, neuropathic pain, and muscle atrophy. The diagram demonstrates the significant neuroprotective and anti-inflammatory effects of INCA-6 through the specific interruption of the Calcineurin–NFAT signaling pathway (Adapted from Li et al., 2022).

1.11 Study Rationale and Knowledge Gap

Unlike other described calcineurin inhibitors, redox-active quinone moiety incorporated in triptycene structure of INCA-6 could uniquely address neuroinflammation caused by PIPN through NFAT's inhibition and oxidative stress at the same time. Quinone moiety allows redox activity while triptycene core serves as a rigid 3D scaffold enhancing interaction of the molecule with its biological targets. Hence, based on its two mechanisms of action INCA-6 would be more efficient than therapies targeted at one specific aspect of PIPN's complex pathophysiology. However, the precise impacts of INCA-6 on the sophisticated pathophysiology of established PIPN remain mostly undiscovered. The available literature does not provide sufficient *in-vivo* studies assessing the effect of INCA-6 on behavioral pain outcomes, level of inflammatory cytokines, and NFAT and NF-κB signaling activity in the context of paclitaxel-induced neuropathy. It is necessary to study how exactly INCA-6 is working and whether it can serve as a treatment for this condition.

1.12 Mechanistic Insight

1.12.1 Calcineurin-NFAT Pathway in Neuropathic Pain

The onset of neuropathic pain is intricately linked to cytokines and various signaling molecules. Key transcription factors in this process are NFAT (Nuclear Factor of Activated T-cells), which is pivotal in pain signaling, and NF-κB (Nuclear Factor kappa B), which primarily initiates pro-inflammatory responses. Calcineurin, a calcium-dependent phosphatase, is central to the activation of NFAT when intracellular calcium concentrations increase.

Increased calcium enhances calcineurin activity, removing phosphates from NFAT or, as posited by some, its related endonuclease, allowing dephosphorylated NFAT to translocate from the cytoplasm to the nucleus, where it activates genes responsible for the production of TNF and the interleukins IL-1 and IL-6, potent pro-inflammatory mediators [18].

Upon entering the nucleus, NFAT contributes to the surge of neuroinflammation experienced in neuropathic pain. The NF-κB pathway significantly contributes to both pain and overall inflammation along this route.

Oxidative stress, direct nerve injury, and all the cytokines that rise after tissue damage can prime or push NF-κB to awaken. While resting, NF-κB stays locked in the cytoplasm. When the signal comes, it leaves the cytoplasm, enters the nucleus, and fastens to specific gene regions [19]. There, NF-κB starts the read-out of pain-related genes and turns on new squads of pro-inflammatory cytokines, chemokines, and other mediators, which, taken together, can harm neurons.

The calcineurin-NFAT route is key in cancer pain because it shapes how neurons fire and how synapses adapt. While the same pathway has been tied to neurodegenerative diseases, studies show that blocking calcineurin or NFAT protects both central and peripheral nerves [19].

1.13 Hypothesis and Study Objectives

This thesis posits that, based on the assumption of PIPN pathology involving calcineurin and oxidative stress, administering INCA-6 to the animals will mitigate paclitaxel-induced neuropathic pain. It is hypothesized that INCA-6 will initiate the calcineurin inhibitory pathway, leading to reduced NFAT and pro-inflammatory cytokine expression, as well as diminished oxidative stress via ROS elimination. These behavioral assessments will aid in evaluating the effectiveness of INCA-6 in alleviating pain and mitigating the advancement of neuropathic symptoms. The research study examines the effect of INCA-6 on the levels of pro-inflammatory cytokines, including TNF-α and IL-1β, in the spinal cord and peripheral nerves. This analysis will

provide evidence regarding the compound's potential to modulate the inflammatory pathways responsible for PIPN. The study assesses the influence of INCA-6 on the activity of NFAT and NF-κB signaling pathways in the spinal cord and the development of peripheral nerves.

The function of INCA-6 in regulating inflammation and neuronal excitability in neuropathic pain neurons will be elucidated through its interaction with these molecular pathways. This will aid in elucidating the molecular pathways by which INCA-6 may confer therapeutic advantages.

1.14 Novelty and Significance of the Study

This thesis enhances understanding by examining the therapeutic properties of the uniquely structured dual-action molecule, INCA-6, in a preclinical context of paclitaxel-induced neuropathic pain. This study will be the inaugural investigation into the in vivo effects of INCA-6 on the integrated system governing behavior and essential molecular components (interactions: NFAT, NF-κB, and cytokine synthesis) within a PIPN model, despite previous research addressing the roles of calcineurin, NFAT, and oxidative stress in neuropathic pain.

The findings of this thesis may significantly justify further research on the molecule INCA-6 concerning its innovative therapeutic potential for PIPN and potentially other neuropathic pain conditions. Enhanced understanding of effective therapeutic agents for painful neuropathies raises the prospect of alleviating the suffering of patients afflicted by this debilitating condition.

1.15 Animal Model and Methodology

A previously established rodent model of paclitaxel-induced peripheral neuropathy (PIPN) is characterized by a core set of behavioral and pathological features of PIPN present in human patients that include distal symmetrical sensory neuropathy, allodynia and hyperalgesia to a broad range of stimuli. This model permits a more

mechanistic approach to the investigation of PIPN and the possible therapeutic solutions.

This study utilized a blend of behavioral and molecular methods to quantify and analyze pain-like behaviors. For assessing mechanical allodynia, we will utilize the von Frey test, while the Hot plate tests was used for thermal hyperalgesia and cold allodynia, respectively. The study involved multi-step approaches where spinal cord and peripheral nerve tissues were subjected to cytokine assay to evaluate the INCA-6 effect on NFAT signaling pathways activity and cytokine expression.

The widespread and debilitating effects of chronic neuropathic pain, especially paclitaxel-induced peripheral neuropathy (PIPN), highlight the urgent clinical demand for enhanced treatment options that exceed existing alternatives, which provide only minimal relief and do not address the underlying causes of the pain. This thesis posits that INCA-6, an innovative molecule featuring a redox-active quinone and triptycene core, will alleviate neuropathic pain induced by paclitaxel. Our hypothesis is based on INCA-6's two ways of working: it can block the calcineurin-NFAT pathway, which lowers the expression of pro-inflammatory cytokines, and it can also scavenge reactive oxygen species, which directly lowers oxidative stress—both of which are important factors in the development of PIPN. We know that calcineurin, NFAT, and oxidative stress play a role in neuropathic pain, but we don't know much about how INCA-6 affects the behavioral and molecular aspects of established PIPN in living organisms. This study fills in a crucial gap in our understanding by fully looking into how INCA-6 affects behavioral pain outcomes, inflammatory cytokines, and important signaling pathways (NFAT, NF-κB) in a PIPN model. The results of this study are important because they should help justify the ongoing development of INCA-6 as a new and possibly more effective treatment for PIPN and other neuropathic pain disorders. This gives patients with these difficult conditions new hope.

Chapter 2

Literature Review

2.1 Introduction

Pain is a sensory experience that elicits a fundamental response from the body's defense system, which serves as a warning of bodily tissue damage. Franjic (2022) highlights that pain is influenced by both physiological and psychological factors. Pain is a multifaceted phenomenon that is influenced by a diverse array of genetic, environmental, and psychosocial factors, resulting in a unique experience for each person. The two primary types of pain are nociceptive and neuropathic, as per the International Association of Sports Physicians (IASP) [20]. Nociceptive pain is the discomfort that individuals experience when peripheral nociceptors detect high temperatures, intense pressure, or specific chemicals [20].The distinction between chronic pain and nociceptive pain is that nociceptive pain is acutely felt but transient, dissipating with the tissue's recovery. It proactively safeguards an individual by mandating that they avoid hazards and seek medical attention when required. Neuropathic pain, as opposed to acute pain, develops due to injury within the somatosensory nervous system. Trigeminal neuralgia, a striking example of neuropathic pain is frequently characterized by the sensation of being stabbed, burned, or shocked, and it typically lasts for an extended period. Neuropathic pain may be persistent and result in severe challenges that significantly diminish an individual's quality of life [12].

It is crucial to comprehend the intrinsic characteristics of the two types of pain, as they require entirely distinct treatment approaches.

Epidemiological studies by Baskozos *et al.* (2023) indicate that 7% to 10% of the general population suffers from neuropathic pain.

The figure 2.1 represents a large number of the population, indicating a significant public health impact of this disorder.

However, the prevalence of neuropathic pain increases substantially in certain clinical populations. These populations include individuals with diabetes, where nerve damage due to high blood sugar levels can lead to diabetic neuropathy; those with shingles, where the herpes zoster virus can damage nerves, causing postherpetic neuralgia; patients with spinal cord injuries, where damage to the central nervous system can result in chronic pain; and, among patients receiving chemotherapy.

CIPN is a well-documented side effect of various antineoplastic drugs, including paclitaxel, significantly impairing patient compliance and quality of life [12].

The development of CIPN can induce dose reductions or treatment termination, thereby affecting cancer treatment outcomes.

Chronic neuropathic pain is associated with severe morbidity, contributing to a spectrum of medical, psychological, and social repercussions. The social effects include sadness, anxiety, sleep difficulties, poor physical function, and social withdrawal [21].

The neuropathic pain can sometimes be persistent and difficult-to-manage triggering deep-seated feelings of hopelessness, negatively impacting one's mental well-being.

Insomnia or other sleep problems are common and intensify the pain and fatigue. Diminished physical function can also restrict mobility and loss of independence, thereby reducing the ability to accomplish daily routines and partake in social activities.

In addition, emotional suffering and the pain itself can cause social withdrawal, contributing to isolation and reduced overall wellbeing. Lastly, healthcare expenditures, as well as lost organizational productivity and disabled individuals add enormously to the economic burden of neuropathic pain [22].

FIGURE 2.1: Global prevalence and impact of neuropathic pain.

This figure present epidemiological data on the global burden of neuropathic pain, illustrating its significant prevalence across various populations and the substantial impact it has affecting patients' quality of life in terms of their physical health, mental state, and social interactions (Adapted from Attal N et al., 2018).

2.2 The Complex Pathophysiology of Neuropathic Pain

Neuropathic pain results from intricate pathophysiological changes in both the peripheral and central nervous systems. These modifications complicate the treatment of the condition and, in most instances, present challenges in its management. The involvement of peripheral nerve pain occurs in a cascade manner, wherein cellular and molecular events are sequentially activated and enhanced following innocuous peripheral nerve injury, resulting in heightened sensitivity and convergence of pain in the affected area. The process involves the generation of numerous pro-inflammatory mediators, including certain cytokines (e.g., TNF-α and IL-1β), chemokines, bradykinin,

and nerve growth factor (NGF), which are released by damaged neurons and surrounding glial and immune cells in the affected region [22]. The mediators have an important role in starting and maintaining the pathogenic alterations that underlie neuropathic pain.

According to the mediators modulate systems that augment the primary afferent nociceptors, the neurons that sense pain, by tweaking the various ion channel in various sodium and voltage-gated ion channels (Nav1.7, Nav1.8, Nav1.9), and also TRP channels like TRPV1 and TRPA1. For the proper functioning of neurons, action potentials must be generated and propagated, which is done by the voltage-gated sodium channels.

They do have the ability to modulate neuronal excitability thru changes in expression or function. Tortuous pain response to stimuli in neuropathic pain enables excessive skin sensory response known as sensitization, and response to many factors (including chemicals, temperature and mechanical) pushing TRP channels to become more euthanized.

Along the lines of neuropathic pain supports usually spontaneous bothersome discharges of increased ion channel dysfunction provoking, along with heightened responsiveness of neurons causing irritable and ectopic discharges [21].

Ectopic firing, refers to the spontaneous generation of action potentials away from the normal initiation site. This phenomenon is a hallmark of neuropathic pain and plays a role in amplifying pain signals.

In the C.N.S., central sensitization and central hypersensitivity mechanisms occur due to the sustained input from peripherally sensitized neurons. This means that the dorsal horn of the spinal cord is capable of increasing or suppressing pain sensitivity and deep underlying rhythms of action which have a fundamental role in the sensitization processes [21].

The spinal cord is integral in modulating and amplifying pain signals. An individual may experience chronic pain due to dysfunction in the processing within the sensitization regions. Central sensitization, a fundamental mechanism, frequently results

from prolonged peripheral input and is characterized as nociception-centered pain exhibiting 'synaptic plasticity.This is the term given to the capacity of synapses to strengthen or weaken over time, thus adapting to increases or decreases in activity due to changes in the body's environment [22].

Glutamate and substance P are neurotransmitters not only involved in the generation but also in the transmission of pain and their increased releases sharpen the pain signaling. NMDA receptor is one out of the two subtypes of glutamate receptors, responsible for activities like plasticity and long-term potentiation, the Persistent nature of neuropathic pain is enhanced muscle activity due to reinforcement of strengthening connections in the nervous system [2].

Inhibitory neurotransmission, which utilizes GABA and glycine, is commonly dysregulated, allowing excitatory signals to potentiate. GABA and glycine are inhibitory neurotransmitters and, as such, serve to block the pain signals [23].

Their reduced strength, on the contrary worsens the pain. Mazzone et al. (2021) assert that the persistence of neuroinflammation is linked to the release of cytokines and neurotrophic factors by glial cells, specifically microglia and astrocytes.

The glial cells, once regarded solely as supportive components of the nervous system, are now recognised as significant modulators of pain processing. These cells are microglia, the immune cells of the central nervous system, alongside astrocytes, a subtype of glial cells that modulate neurotransmitters and cellular strength.

During neuropathic pain, the glial cells are responsive and activated to produce prominent cytokines such as TNF-α, IL-1β, and IL-6, along with the neurotrophic factor, BDNF, which exacerbates pain signaling through potential central sensitization. Impairment of descending inhibitory pain pathways from brainstem regions like the periaqueductal gray (PAG) and rostral ventromedial medulla (RVM) serves to intensify central sensitization and the chronicity of pain [24].

These areas of the brain are critical structures responsible for the modulation of incoming nociceptive signals, whose lesions upset the inhibitory mechanisms resulting in chronic pain.

2.3 Taxane Chemotherapy and Paclitaxel - Induced Neuropathic P

The Pacific yew tree (Taxus brevifolia) gives us the bark-based drug Paclitaxel which is used for chemotherapy treatments. Paclitaxel belongs to the taxane class of compounds, and it plays an integral role in treating numerous solid tumors such as breast, ovarian, lung cancer, pancreatic cancer, and even Kaposi's sarcoma [25]. It is still very popular among oncologists due to its mechanism of action and its effectiveness in halting tumor progression. Even with all these advantages, paclitaxel has a high incidence of neurotoxicity. One notable example would be paclitaxel-induced peripheral neuropathy (PIPN). PIPN not only stands out as one of the most common side effects from paclitaxil but also serves as a major contributor to long term health problems in survivors of cancer [23].

2.3.1 Chemical Structure and Mechanism of Action

The paclitaxel structure is rather complicated; it includes a tetracyclic 17-carbon taxane core which has a side chain attached to the C13 position. This attachment is crucial due to its biological activity [25]. It also has numerous modifications some of which are oxygen-bearing groups of hydroxyl, carbonyl, and acetate. These structural features make paclitaxel highly functionalized. In addition to aiding in binding with tubulin, these structures contribute to poor solubility in water making it necessary for formulation along with solvent carriers such as Cremophor EL [26].

Paclitaxel works mechanistically as a microtubule - stabilizing agent. In unaltered cells, microtubules are composed of tubulin dimers dynamically undergoing continuous polymerization and depolymerization cyclically termed "dynamic instability". This process is crucial for several cellular processes including mitosis [25].

Paclitaxel has been shown to bind specifically to β-tubulin within assembled microtubules halting their disassembly and thus reducing dynamic instability. This form of stabilization hampers the microtubule dynamics needed for mitotic spindle formation causing G2/M cell cycle arrest followed by apoptotic programmed cell death [23].

As such, paclitaxel exerts selective cytotoxicity against rapidly dividing cancer cells, making it a potent anti-neoplastic agent.

2.3.2 Clinical Applications of Paclitaxel

Paclitaxel is useful clinically for various treatments. It is used widely as a first and maintenance treatment in ovarian and breast cancer, often with other drugs like carboplatin or cisplatin. It is also indicated in non-small cell lung cancer (NSCLC), head and neck cancers, and in the advanced stage of AIDS-related Kaposi's sarcoma.

Administration is done through an IV line, and the dosage of the drug is calculated based on body surface area in a stepped fashion depending on the patient's clinical feedback [25]. The adjunctive efficacy of paclitaxel bears out for decades in trials and oncology but certainly has shortcomings.

2.3.3 Adverse Effects and the Onset of Neuropathy

With all the benefits, paclitaxel as with many medications has its adverse effects such as peripheral neuropathy which seems to be very bothersome to patients. Paclitaxel-induced peripheral neuropathy (PIPN) is a syndrome that includes dysfunction primarily involving sensory nerve fibers, although some degree of motor or autonomic nerve involvement may also occur.

This condition commonly results in abnormal sensations such as tingling or numbness, burning feelings, sharp stabbing pain mainly around hands or feet. In severe cases, patients will experience muscle weakness, gait disturbances, and impaired coordination, which can diminish quality of life and even cause discontinuation of chemotherapy [23].

2.3.4 Pathophysiological Mechanisms Leading to Neuropathic Pain

The development of paclitaxel-induced neuropathy is intimately connected to the drug's impact on neuronal microtubules, oxidative damage, and neuroinflammation.

The beneficial cancer cell-targeting effect achieved through paclitaxel's stabilization of microtubules comes with detrimental consequences as well; it interferes with axonal transport in neurons. Axonal transport is a microtubule-dependent mechanism that facilitates the movement of organelles, proteins, and even neurotransmitters over considerable distances through the axon [23].

Disruption of this transport system results in long myelinated sensory axons accumulating debris damaged organelles leading to neural injury.

Moreover, along with disruption to axonal transport, paclitaxel also causes mitochondrial dysfunction within cells. Mitochondria are critical for conserving ATP and buffering calcium levels; when they are compromised this leads to excess synthesis of reactive oxygen species or ROS. These highly reactive oxidative molecules can damage cellular DNA, proteins, and lipids increasing neuronal stress while apoptotic processes continue on a greater scale than the previous level [25].

As time goes on without hinderance agacumulative oxidative stress further underlying neuronal destruction occurs and pain pathways become increasingly sensitive.

In addition, paclitaxel initiates an acute inflammatory response in the nervous system. It triggers the activation of glial cells such as astrocytes and microglia which start releasing various pro-inflammatory cytokines like tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6). These mediators not only harm nearby neurons, but they also amplify the excitability of pain pathways in the spinal cord and brain. Furthermore, this state of neuroinflammation is worsened by the Toll like receptor 4 (TLR4) on glial cells recognizing DAMPs and activating downstream inflammatory processes.

These processes result in both central and peripheral sensitization which is characteristic features of chronic neuropathic pain. Central sensitization involves heightened responsiveness to stimuli among neurons located in the dorsal horn region of the spinal cord while peripheral sensitization refers to an increase in the "sensitivity" due to a reduction of activation thresholds in nociceptive fibers. Both mechanisms add to intensity, persistence and refractory characteristics of paclitaxel-induced neuropathic pain encountered by patients.

2.3.5 Clinical Implications and Conclusion

The emergence of neuropathic pain in relation to paclitaxel therapy poses a major clinical obstacle. It not only cap limits the dosage and duration of chemotherapy administered, which compromises its effectiveness in treating cancer, but it also leads to persistent sensory disturbances for months or years post-treatment. Current pharmacological approaches tend to rely on gabapentinoids or antidepressants. These medications do provide some relief; however, their benefits are greatly overshadowed by adverse side effects.

With emerging new therapies such as INCA-6, discussed in section 2.5, there is potential for treating the core pathophysiology of neuropathic pain by silencing oxidative stress and inflammatory mechanisms. Addressing the fundamental biological causes instead of symptom management has the potential to pave the way towards more effective treatments that will provide long-lasting improvements and recovery for patients.

2.4 Conventional Treatments for Neuropathic Pain and Their Limitations

As Franjic (2022) points out, today's pharmacies stock only a handful of drugs for neuropathic pain-antidepressants, anticonvulsants, opioids, lidocaine patches or capsaicin cream. Unfortunately these therapies rarely erase the pain and their side effects, which can be dizzying, nausea-inducing, or worse, often force patients to stop taking them at all.

The primary mechanism of action of TCAs and SNRIs is through the augmentation of descending inhibitory pathways by increasing circulating monoamines. For many years, TCAs, including amitriptyline and nortriptyline, have been prescribed to alleviate neuropathic pain. SNRIs like duloxetine and venlafaxine preferentially inhibit the reuptake of serotonin and norepinephrine [27]. Despite some achieving remarkable success, TCAs and SNRIs have many limitations.

The adverse effects that some patients suffer from, such as dry mouth, dizziness, drowsiness, and cardiovascular issues, prevent wider use. Specifically, TCAs affect the body's acetylcholine receptors, leading to side effects like dry mouth, constipation, urine retention, and even cognitive difficulties [28]. These medications can create orthostatic hypotension and cardiac arrhythmias, which limits use in the elderly or patients with heart disease. Compared with TCAs, SNRIs are considered to have a slightly better side effect profile; however, they can still create nausea, sleeplessness, and sexual dysfunction [29].

According to Sharma et al. (2023), gabapentin, which include gabapentin and pregabalin, are classified as anticonvulsants but have been increasingly prescribed for neuropathic pain. Gabapentin and pregabalin, which are structurally similar to GABA, bind to the alpha2delta subunit of voltage-gated calcium channels, thereby decreasing the release of excitatory neurotransmitters involved in pain signaling, such as glutamate.

Their effectiveness varies from person to person and type of neuropathic pain, and drowsiness, weight gain and cognitive impairment are often seen as side effects [28]. Some patients experience great pain relief due to gabapentin, while others are offered minimal pain reduction. Such side effects may reduce the ability to use them, especially for those already suffering from drowsiness or developing cognitive problems.

Morphine, oxycodone, and tramadol, which are classified as opioids, provide analgesia for acute pain in the short-term; however, their use in chronic neuropathic pain is due to long-term consequences, such as the development of tolerance, dependency, and addiction. Tolerance is described as the modifying body response to a medication due to its use which progressively increases the dose needed to achieve a specific level of pain relief. Dependence manifests when there is a reliance on a specific drug to function normally, leading to withdrawal symptoms when the drug is removed. Drug abuse refers to the use of a drug outside of medically indicated limits, which can be associated with the development of addiction. Because of these hazards, opioids are often considered as the last option for treating pain [30]. Moreover, in exceptional circumstances, prolonged use of opioids can paradoxically increase the sensitivity to pain, termed as opioid-induced hyperalgesia.

Capsaicin, a product of cayenne pepper, while usually prescribed in higher doses, topically, can be ineffective in cases of deep or generalized neuropathic pain. Lidocaine patches serve as a local anesthetic whereby it acts to numb the areas they are placed. Subsequently, they provide relief for peripheral postherpetic neuropathic pain like postherpetic neuralgia. Capsaicin cream works by depleting substance P, a pain-inducing neuropeptide, from sensory nerve endings. Nonetheless, capsaicin application can result in a burning feeling which can be intolerable [28].

Different forms of non-pharmacological therapy could help in the management of neuropathic pain, like physiotherapy, psychological support, acupuncture, and nerve stimulation. Pain physiotherapy incorporates physical exercises to improve function and relieve pain. Support from a psychologist, alongside CBT, enables patients to address the emotional and psychological burden of pain for better coping mechanisms [28].

Acupuncture may relieve some pain for some people and it involves the sharp needles being put into certain parts of the body. Direct nerve stimulation techniques, like TENS and spinal cord stimulation, assist with the modulation of pain signal transmission and reception. When these methods are used alone, patients still do not get adequate pain relief, which highlights the need for more innovative and effective treatments that go beyond simplistic understandings of neuropathic pain's complex systems [30]. Pain management requires the integration of both pharmaceutical and non-pharmacological therapy for effective relief.

TABLE 2.1: Current Neuropathic Pain Treatments and Their Limitations [31]

Class / Type	**Mechanism of Action**	**Efficacy**	**Common Limitations**
Anticonvulsants (e.g., Gabapentin, Pregabalin)	It interacts with the $\alpha 2\delta$ subunit of voltage-gated calcium channels, resulting in decreased release of excitatory neurotransmitters.	Moderate to Good for focal neuropathic pain	Include dizziness, drowsiness (somnolence), peripheral edema, and weight gain. Additionally, there is a potential risk for misuse or abuse.

Table 2.1 continued from previous page

Class/Type	**Mechanism of Action**	**Efficacy**	**Common Limitations**
Antidepressants (e.g., Duloxetine, Venlafaxine)	Inhibit reuptake of serotonin and norepinephrine, enhancing descending pain inhibition	Moderate to Good for generalized neuropathic pain (e.g., DPN)	Nausea, dry mouth, insomnia, fatigue, constipation, sexual dysfunction.
Antidepressants (e.g., Amitriptyline, Nortriptyline)	Inhibit reuptake of serotonin and norepinephrine	Moderate to Good	Include dry mouth, constipation, and difficulty urinating.
Local Anesthetics/Antiarrhythmics (e.g., Lidocaine, Mexiletine)	Block sodium channels in nerve fibers, reducing excitability	Lidocaine: Topical for postherpetic neuralgia. Mexiletine: Oral, less common.	Lidocaine: Local skin reactions. Mexiletine: Cardiac effects, nausea, dizziness.
Analgesics (e.g., Tramadol, Oxycodone, Morphine)	Activate opioid receptors within the central nervous system to modulate pain signals, reducing pain transmission	Variable; generally, less preferred for chronic neuropathic pain	Constipation, nausea, sedation, respiratory depression, addiction potential, tolerance, hyperalgesia.
Various (e.g., Capsaicin, Lidocaine)	Capsaicin: Depletes substance P. Lidocaine: Blocks sodium channels.	Capsaicin: Moderate for postherpetic neuralgia. Lidocaine: Good for localized pain.	Capsaicin: Burning sensation, skin irritation. Lidocaine: Local skin reactions.
Injections, Stimulation	Nerve blocks, epidural injections, spinal cord stimulation (SCS)	Variable; can be effective for select patients	Risk of infection, bleeding, nerve damage. High cost, need for specialized expertise.
Physical Therapy, Cognitive Behavioral Therapy (CBT), Acupuncture	Physical: Improve function, reduce pain. CBT: Coping strategies. Acupuncture: Unclear.	Physical: Moderate. CBT: Moderate. Acupuncture: Variable.	Physical: Requires patient participation. CBT: Requires trained therapist. Acupuncture: Effectiveness debated.

2.5 Comparison of INCA-6 with Current Neuropathic Pain Treatments

Tricyclic antidepressants (TCAs), serotonin-norepinephrine reuptake inhibitors (SNRIs), and gabapentinoids (e.g., gabapentin, pregabalin) are the main types of drugs used to treat neuropathic pain right now. The drugs may help with some symptoms, but they don't always work well. They only help some patients with pain, and they often come with serious side effects like sedation, dizziness, weight gain, and heart problems. Additionally, the treatments mainly work on pathways that transmit pain or change the levels of neurotransmitters. They don't directly target the underlying pathological mechanisms of neuroinflammation, oxidative stress, and neuronal damage that cause neuropathic pain to get worse [32]. People also use opioids, but they can't use them for long periods of time because of worries about addiction, tolerance, and side effects.

On the other hand, INCA-6 differs from existing treatment paradigms due to its distinct pharmacological profile. The unique pharmacological advantage of INCA-6 is its dual mechanism of action, which targets oxidative stress and the calcineurin-NFAT pathway, two important and interrelated pathways implicated in the pathophysiology of neuropathic pain. By contrast, INCA-6 offers a fresh approach; instead of acting non-specifically on individual receptors or merely masking symptoms, it inhibits calcineurin and prevents NFAT's translocation to the cell nucleus [32].

Because active NFAT cranks up many pro-inflammatory genes, blocking it could cool the neuroinflammation that feeds chronic pain. On top of that, INCA-6 contains a redox-active quinone that hunts down reactive oxygen species, the harmful leftovers of cellular metabolism, and tears them apart before they can harm nerve tissues [33].

Because INCA-6 tackles multiple pathways at once, it may bring several fresh advantages into the clinic. By cutting oxidative stress and calming neuroinflammation at the same time, the compound could offer deeper pain relief and even slow disease progression. That dual activity aims straight at the root drivers of nerve injuries, not just the discomfort on the surface. When measured against today's treatment menus-often a blend of pills, patches, and nerve blocks- the INCA-6 plan might work

faster, cover a broader range of nerve pain causes, and show milder side effects. If so, it could fill the gaps left by conventional options with a pointed, long-lasting strategy for patients.

Chapter 3

Methodology

3.1 Introduction

Neuropathic pain as a long-lasting, debilitating syndrome that emerges when lesions or disorders affect the somatosensory system; common features are allodynia, heightened sensitivity, and spontaneous pain. Despite advances in elucidating the molecular and cellular mechanisms of neuropathic pain, effective treatment options remain limited—particularly for neuropathies induced by chemotherapy. One notable example is paclitaxel, a widely used chemotherapeutic agent that frequently causes peripheral nerve damage as a side effect. This condition, known as paclitaxel-induced peripheral neuropathy (PIPN), not only restricts dosage due to its severity but also serves as a valuable experimental model for studying neuroinflammatory processes. PIPN is commonly employed in preclinical research to evaluate potential therapies aimed at relieving nerve-related pain and preventing further neural deterioration. Inflammation plays a central role in the pathogenesis of neuropathic pain as emerging evidence indicates and it manifests itself through activation of pro-inflammatory cytokines and transcription factors like NF-κB, IL-6, and NFAT1. These signals are molecular mediators; they do sensitize the pain pathways, maintaining and augmenting nociceptive messages after nerve damage. Within this paradigm, INCA-6 (Triptycene-1, 4-quinone) has been identified as a promising molecule with immunomodulatory

and anti-inflammatory properties [34]. This work combines computational and experimental methods to examine the therapeutic effectiveness of INCA-6 in treating paclitaxel-induced neuropathic pain.

Molecular docking was utilised to evaluate the interaction of INCA-6 with pivotal proteins involved in inflammation. The simulations indicated potential interactions with NF-κB, IL-6, and NFAT1, implying mechanisms that may contribute to its analgesic properties.

In-silico ADMET tools were subsequently utilised to predict absorption, distribution, metabolism, excretion, and toxicity. These analyses validated the experimental design and facilitated a more concentrated focus prior to advancing beyond in vitro studies.

Following the acquisition of consistent results from the models, INCA-6 was assessed in murine subjects exhibiting paclitaxel-induced neuropathy. Paclitaxel was administered in cycles, consistently eliciting the hypersensitivity observed in cancer patients.

Behavioural responses were evaluated utilising von Frey hairs for tactile sensitivity, a hot plate for thermal nociception, and cold acetone for cold sensitivity. These assessments produced a behavioural profile of neuropathic pain and facilitated the evaluation of the analgesic efficacy of INCA-6 under rigorously controlled conditions.

Spinal cord samples were analysed via enzyme-linked immunosorbent assay (ELISA) to quantify pro-inflammatory cytokines, namely IL-1β and TNF-α. The molecular measurements, documented in conjunction with behavioural pain scores, facilitated the correlation of inflammatory responses with neuropathic pain symptoms.

Increased cytokine levels indicated neuroinflammation, whereas decreases after INCA-6 treatment offered biochemical proof of anti-inflammatory and neuroprotective effects. The study thoroughly assessed INCA-6 as a prospective treatment for chemotherapy-induced neuropathic pain.

Through the integration of molecular docking, ADMET predictions, animal behaviour assessments, cytokine quantification, and tissue analysis, a robust translational foundation was established for progressing INCA-6 into advanced phases of preclinical and clinical research.

3.2 *In-Silico* Studies

A virtual study looked at how well INCA-6 connects with proteins linked to nerve-pain signaling, naming NF-κB, IL-6, NFAT1, IL-1β, and TNF-α.

Because these molecules sit at the heart of pain and inflammation, docking studies tried to measure how tightly INCA-6 can bind to each one. Making the model, we assigned non-polar atoms, set realistic charges, and aligned hydrogen bonds for both ligand and target.

AutoDock Vina 1.1.2 ran the scans, placing the search grid around critical residues that control the proteins activity.

To round out the picture, Swiss ADME and ProTox 3.0 estimated INCA-6 drug-like traits and flagged any possible toxicity. Together these steps painted a clearer map of the compound's absorption, fat solubility, water ease, and safety, giving earlier clues to its use in pain care.

3.3 ADMET Prediction

ADMET profiles were generated using Swiss ADME (http://www.swissadme.ch), a web tool hosted by the Swiss SIB (http://www.sib.swiss), plus ProTox 3.0 to estimate toxicity for each ligand. Molecules were submitted as single-line SMILES strings, entered one at a time, and results were returned in tabulated form for every compound [35].

The built-in bioavailability radar quickly visualizes drug-likeness by blending six core physicochemical traits-lipophilicity (LIPO), size, polarity, solubility, saturation (INSATU), and flexibility (FLEX).

The value of Lipophilicity is evaluated by XLOGP3 and should ideally range between -0.7 and +5.0, the Molecular size is based on weight range between 150 to 500 grams per mole The acceptable solubility is determined by a logarithmic S value over six, while polarity is quantified by the total polar surface area (TPSA) and must fall

within the range of 20 to 130 Å^2. Regarding the saturation criterion, it is required that more than 25% of the carbon insults should be sp3 hybridized and clinically, FLEX is capped at nine rotatable bonds.

3.4 Molecular Docking

3.4.1 Ligand Preparation

The 3D structure of INCA-6 was retrieved from the PubChem database (ID: 230748). Prior to molecular docking, hydrogen bonds were assigned to the ligand, followed by non-polar atom merging and Kollman and Gasteiger charge assignment. The rotatable bonds were assigned, and the ligand was converted to pdbqt format with MGL-Tools 1.5.7.

3.4.2 Preparation of Receptors and Grids Configuration

The Protein Data Bank (PDB) was used to get three-dimensional crystal structures of the following proteins: 3GUT (NF-κB), 1P9M (IL-6), 1PZU (NFAT1), 2AZ5 (TNF-α), and 1ITB (IL-β). Protein preparation was carried out using MGLTools version 1.5.7. The method involved removing water molecules and heteroatoms, incorporating polar hydrogen atoms, combining non-polar hydrogens, and allocating Kollman and Gasteiger charges. The changed proteins were then stored in the pdbqt format required for molecular docking research.

A grid box was created for each protein to delineate the docking search space, centred on residues essential to the protein's functional activity. The grid box size was chosen to guarantee full coverage of the binding site while preserving computational efficiency. The grid parameters were delineated as follows:

The grid box for NF-κB (PDB ID: 3GUT) was centered at coordinates (38.45, -22.94, 55.37) with dimensions of 15 $\times$ 15 $\times$ 15 Å^3. The grid included essential residues such as Tyr357, Cys359, Glu360, Lys444, Lys445, and Lys541.

The grid center for IL-6 (PDB ID: 1P9M) was established at coordinates (-36.75, 185.66, 29.52) with dimensions of 55 × 55 × 55 Å^3. The targeted residues comprised Leu57, Ala58, Glu59, Asn60, Leu62, Trp157, and Leu158.

The grid for NFAT1 (PDB ID: 1PZU) was centered at coordinates (27.31, -3.15, 141.79) with size of 15 × 15 × 15 Å^3. The residues chosen for docking comprised Arg421, Tyr424, Thr426, Glu427, Arg430, Lys434, Lys520, Arg522, Asn523, Arg537, Lys538, Gln571, Lys664, and Arg665. The residues were identified as contact hot spots by DNA-bound crystal structure analysis utilizing the Protein-Ligand contact Profiler (PLIP) service.

The docking grid for TNF-α (PDB ID: 2AZ5) was established at coordinates (-20.798, 70.950, 37.699) with dimensions of 53 × 46 × 41 Å^3. The critical residues implicated were Leu57, Tyr59, Ser60, Gln61, Tyr119, Leu120, Gly121, Gly122, and Tyr151.

The grid box for IL-1β (PDB ID: 1ITB) was centered at coordinates (30.100, 2.678, 15.942) and measured 71 × 78 × 75 Å^3. The docking site included residues Arg11, Ser13, Gln14, Gln15, Met20, Gly22, Lys27, Leu29, His30, Leu31, Gln32, Gly33, Gln34, Asp35, Met36, Gln38, Gln126, Thr127, Lys128, Gly129, Met130, Pro131, Thr147, and Gln149.

These setups guaranteed that docking simulations concentrated on biologically pertinent binding sites, hence augmenting the reliability and interpretability of the molecular interaction outcomes.

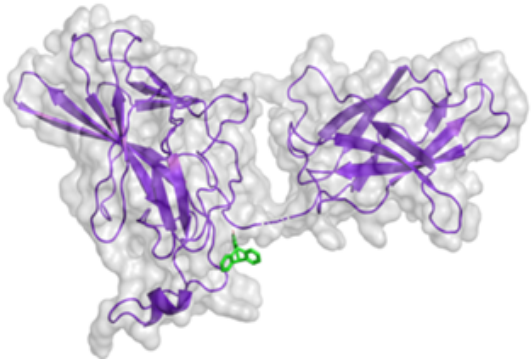


FIGURE 3.1: Molecular Docking.

Figure 3.1 shows visual outputs from molecular docking simulations, demonstrating the predicted binding interactions and affinities of INCA-6 with key inflammatory

signaling proteins such as NF-κB, IL-6, and NFAT1. These visualizations provide insight into the potential molecular basis for INCA-6's therapeutic actions.

3.4.3 Chemical and Reagents

Table 3.1: Chemical and reagents

Category	**Chemical / Material**	**Source**
Solvents	Dimethyl sulfoxide (DMSO), Ethanol, Methanol, Xylene	Sigma, USA
Cytokine Analysis	ELISA inflammatory cytokines analyzing kit	Nanjing Pars Bio-chem CO. Ltd
Biological Materials	Fetal bovine serum (FBS)	Commercially available
Test Compound	INCA-6 (Triptycene-1, 4-quinone)	Commercially sourced
Buffer Solutions	Phosphate Buffered Saline (PBS)	Sigma, USA
Injectable Solutions	Normal Saline	Amson Pharmaceuticals, Islamabad

3.4.4 Animals

A neuropathic pain model was established using male BALB/c mice weighing between 20 grams and 30 grams , procured from the Faculty of Pharmacy. The research was performed at Capital University of Science and Technology in Islamabad, Pakistan. The animals were accommodated in hygienic stainless-steel enclosures with suitable bedding, under regulated conditions: temperature sustained at 24 $\pm$ 0.5 ^{o}C , relative humidity ranging from 55–60%, and a 12-hour light/dark cycle. They possessed unimpeded access to conventional sustenance and hydration. All experimental protocols were evaluated and sanctioned by the University Research and Animal Ethics Committee (Approval No: REC/FoP/F2024/07). The animals underwent a one-week acclimatization prior to the commencement of any procedures

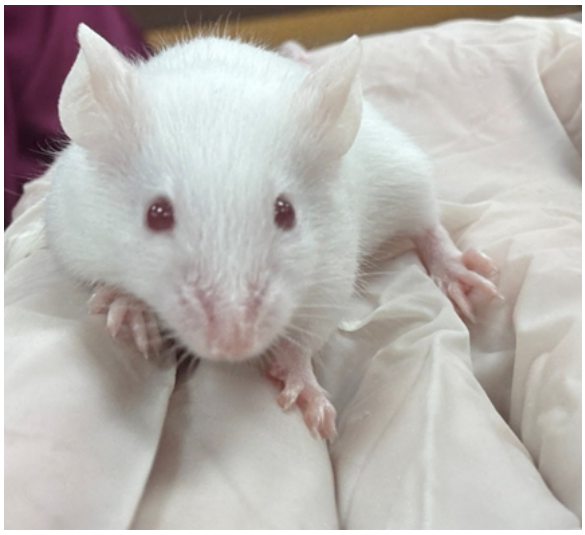


FIGURE 3.2: Animal handling.

This figure illustrates the standardized and controlled housing conditions of the mice used in the study. The image emphasizes the adherence to ethical research standards and proper animal care protocols, ensuring reliable experimental conditions (Photograph taken at Faculty of Pharmacy CUST).

3.4.5 Animal Care and Ethical Statement

The researchers did the 3Rs-Replacement, Reduction, and Refinement-to restrict the number of animals and reduce their pain and suffering during all phases. Mice were housed within a controlled facility in which the temperature was held at a constant condition, and the housing had constant humidity, 12 hours/light, 12 hours/dark among other conditions like ad libitum food and water.

3.5 Justification of Animal Group Sizes

Drawing on power calculations and earlier studies that looked at similar nerve-pain models and drug treatments, we settled on eight mice per group. This number is common in preclinical work because it keeps animal use low and still gives enough statistical power to spot real differences between therapies. The approach therefore balances animal welfare with the need for reliable results.

3.6 Paclitaxel-induced Neuropathic Pain Model

In the current experiment, nerve damage and pain were triggered in mice by giving them paclitaxel via an intraperitoneal injection. The drug was mixed in normal saline at 2 mg/kg and delivered every other day-on days 1, 3, 5, and 7-making four total doses. On the same day paclitaxel was first given [11] tests were already run to record baseline behavior.

3.7 Study Design

Initially, eight mice were allocated to each experimental group. However, during the course of the study, the final number of mice analyzed for behavioral assessments was reduced to seven per group (n=7) due to unforeseen animal attrition. For the subsequent biochemical analyses (cytokine levels and oxidative stress markers) in spinal cord tissue, a representative subset of three mice (n=3) from each group was utilized due to the destructive nature of the assay and to optimize resource utilization. To evaluate pain, the mice were randomly divided into five groups of seven so treatment effects could be compared fairly across the board.

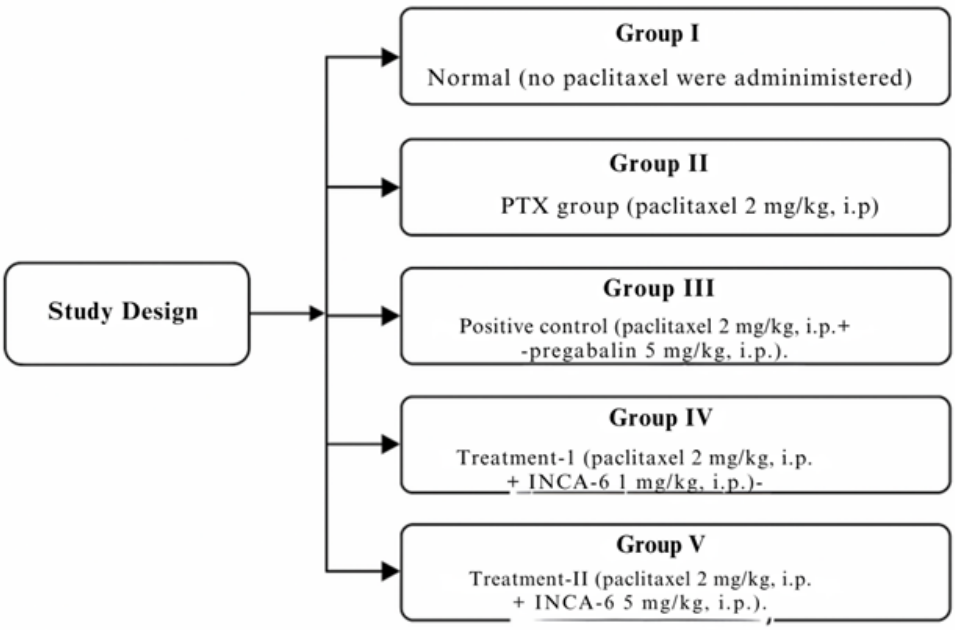


FIGURE 3.3: Study design

Table 3.2: Dose Calculation for PTX and INCA-6 during *in-vivo* studies

Group	**Compound**	**Dose (mg/kg)**	**Avg. Mouse Weight (kg)**	**Dose per Mouse (mg)**	**Total Mice**	**Frequency (Days)**	**Total Dose for Group (mg)**
Group I	PTX	2 mg/kg	0.03	0.06	8	4 days (Alt.)	1.92
Group I	INCA-6	1 mg/kg	0.03	0.03	8	7 days	1.68
Group I	Pregabalin	5 mg/kg	0.03	0.15	8	7 days	8.4
Group II	PTX	2 mg/kg	0.03	0.06	8	4 days (Alt.)	1.92
Group II	INCA-6	1 mg/kg	0.03	0.03	8	7 days	1.68
Group III	PTX	2 mg/kg	0.03	0.06	8	4 days (Alt.)	1.92
Group III	INCA-6	5 mg/kg	0.03	0.15	8	7 days	8.4
Group IV	PTX	2 mg/kg	0.03	0.06	8	4 days (Alt.)	1.92
Group IV	INCA-6	Vehicle (2% DMSO + Sal- ine)	0.03	-	8	7 days	-
Group V	PTX	2 mg/kg	0.03	0.06	8	4 days (Alt.)	1.92
Group V	INCA-6	Vehicle (2% DMSO + Sal- ine)	0.03	-	8	7 days	-

3.8 Dose Calculation Table for PTX and INCA-6

The table 3.2 below summarizes the calculated dosages for Paclitaxel (PTX), INCA-6, and Pregabalin based on an average mouse weight of 30 grams, along with the total amount required for each group during the 7-day experimental period.

3.9 Solution Preparation and Drug Administration

3.9.1 Drug Administration

Stability and drug dosage accuracy were ensured by carefully formulating every drug solution before distributing them. All drug substances were injected (10 mL/kg by intraperitoneal) in the same way. The dose of individual animal was carefully adjusted based on its daily weight. Paclitaxel was administered in Paclitaxel was administered in normal saline (i.p.) at a dose of 2 mg/kg.

All the groups other than the negative control were PTX treated on alternating days (1, 3, 5 and 7). This was aimed at inducing neuropathic pain by reproducing the effects of chemotherapy neuropathy on repeated systemic exposure.

INCA-6 Management: INCA-6 was dissolved in DMSO 2% and then mixed up with normal saline before administration via intraperitoneal injection. Intraperitoneal injections of 1 mg/kg/d and 5 mg/kg/d were administered to the animals once daily over 7days. Variability during the circadian rhythms was minimized by the daily therapy. Analgesic efficacy INCA-6 was compared to pregabalin, in a neuropathic pain paradigm.

All drugs were delivered intraperitoneally via sterile syringes and needles. Mice were restrained to minimize stress and ensure accurate solution administration. Under standardized conditions, control groups were administered a same quantity of suitable solutions.

The image below displays mice post-treatment with INCA-6 or control substances, visually representing the experimental handling and the various treatment protocols applied during the study.

FIGURE 3.4: Drug Administration in Mice.

The administration of Intra-Peritoneal drugs entails the restraint of mice and the intraperitoneal injection of a sterile syringe and needle. The needle was inserted into the lower right quadrant of the abdomen at a 30–45-degree angle, carefully avoiding major organs, and the predetermined drug volume (10 ml/kg body weight) was delivered gradually. Because of its rapid systemic absorption and less stressful nature than intravenous injection, this route was appropriate for repeated dosing in experimental studies. Animals were safeguarded and complications are prevented by maintaining aseptic conditions and restraint during the procedure (Photograph taken at Faculty of Pharmacy CUST)

3.10 Pain Hypersensitivity Test

3.10.1 Assessment of Mechanical Allodynia

Mechanical allodynia was assessed using the von Frey test, following established methods described in earlier studies [36]. Mechanical sensitivity of the mice's right hind

paw was assessed using calibrated von Frey filaments. Before testing began, each mouse was placed in a clean beaker on a von Frey mesh platform and given 30 minutes to acclimate to the environment. The assessment involved applying a series of filaments with progressively increasing force to the plantar surface of the right hind paw. Each filament was applied five times, and a withdrawal reflex observed in at least three of the five applications was considered a positive response.

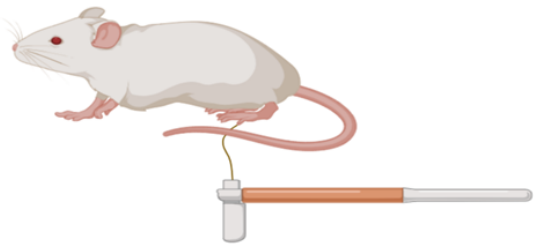


FIGURE 3.5: Mechanical Allodynia von Frey hair filament test.

Mice were tested for mechanical allodynia using von Frey filaments weighing 0.5g, 1g, 2g, 3g, and 4g. The animals acclimatize in chambers on an elevated mesh platform for 15-30 minutes until exploratory behavior stops. Apply each filament perpendicularly to the plantar surface of the hind paw in ascending order of force, starting at 0.5g and progressing through 1g, 2g, 3g, and 4g, five times at 5-second intervals, to determine the threshold force that elicits withdrawal responses in at least three out of After development time and experimental treatment, injury, or inflammatory agent administration, the von Frey testing protocol is repeated at predetermined intervals using the same filament series to assess mechanical sensitivity. A withdrawal threshold that is significantly reduced (Adapted from Mogil *et al.*, 2017).

3.10.2 Assessment of Thermal Hyperalgesia

The hot plate test was utilized to evaluate the inhibitory effect of INCA-6 on paclitaxel-induced neuropathic pain by measuring paw withdrawal thresholds. The hot plate test was performed at a constant temperature of 54^{o}C to evaluate thermal nociception. Mice were placed individually on a heated surface, and the duration until they

displayed pain-related behaviors, such as paw withdrawal or licking, was recorded as the thermal withdrawal threshold. A strict cutoff time of 30 seconds was implemented to prevent tissue injury. Evaluations were conducted at multiple intervals both before and after paclitaxel administration.

Test latency: The duration (seconds) from the moment the animal is positioned on the hot plate (54 ± 0.1 54^{o}C) until it exhibits licking or flicking of the hind limb or jumping following the administration of the test compound.

Control latency: The duration (seconds) from the placement of the animal on a hot plate (54 ± 0.1 ^{o}C) until the animal exhibits licking, hind limb flicking, or jumping following saline administration.

Cut-off time: It is the temporal point at which the antinociceptive effect is deemed to be at its maximum efficacy of 100%.

FIGURE 3.6: The hot plate test process.

Illustrated in the figure 3.6, involves the evaluation of thermal hyperalgesia in mice. The animals were first allowed to acclimatize to the testing environment for 30 minutes. Subsequently, baseline response latencies are obtained by placing each mouse on a heated surface set to 52-55^{o}C and timing until the first pain behavior (paw licking, jumping, or vocalization) occurs, which typically takes 8-12 seconds in healthy

mice. The experimental treatment was administered and allowed to develop over the designated time period after these baseline measurements are recorded. A decrease in response latencies compared to the baseline indicates the development of thermal hyperalgesia or heightened pain sensitivity. Subsequently, the mice are retested on the hot plate at specific time intervals. Mice are promptly removed upon exhibiting pain responses, and a cutoff time of 20-30 seconds is maintained throughout the procedure to prevent tissue damage (Adapted from Mogil *et al.*, 2017).

3.10.3 Evaluation of Cold Allodynia

Cold allodynia was performed to investigate the effect of INCA on paclitaxel-induced cold hypersensitivity as reported [37]. Briefly, 200 μl of acetone were sprayed on the planter surface of mice right hind paw. Immediately, the mice were observed for 30 second and the responses were noted. The scoring of mice in response to acetone were measured as follow. The figure 3.7 below shows the experimental setup for the cold allodynia test, which is utilized to assess cold hypersensitivity in mice. It shows how acetone application is used to trigger responses in neuropathic models, quantifying the degree of allodynia based on paw withdrawal reflexes.

FIGURE 3.7: Acetone induced cold allodynia.

0 = no response

1 = brisk withdrawal of paw

2 = repeated paw withdrawal

3 = paw withdrawal and flickering

4 = paw licking, paw withdrawal, and flickering

The readings were observed at different time interval (day 0, 3, 7, 8, 10, & 13)(Adapted from Mogil *et al.*, 2017).

3.11 Biochemical Parameters

3.11.1 Protein Extraction and Quantification Using the Bicinchoninic Acid Assay

Tissue samples were collected from mice following institutional ethical guidelines. After dissection, tissues were rinsed with ice-cold PBS to remove blood and debris, gently dried, and weighed. Protein was extracted by homogenizing tissues in RIPA buffer on ice using a mechanical homogenizer. The homogenates were centrifuged at 8,000 $\times$ g for 30 minutes at 4^{o}C to obtain the soluble protein fraction, which was stored at -20^{o}C. A total of 30 samples were processed. Protein concentration was determined using the BCA assay. Working reagent was prepared by mixing Reagents A and B in a 50:1 ratio per manufacturer instructions. BSA standards (25–2000 μg/mL) were used to generate a standard curve. For each sample or standard, 25 μL was added to a 96-well plate, followed by 200 μL of BCA reagent. After mixing, plates were incubated at 37^{o}C for 30 minutes, and absorbance was measured at 562 nm with a microplate reader.

3.12 ELISA

Pro-inflammatory cytokines in mouse spinal cord tissues were measured using ELISA kits (TNF-α: PRS-20505Mo), (IL-1β : PRS-20295Mo). After behavioral tests, mice

were humanely euthanized, and spinal cords were carefully collected. Tissues were homogenized and centrifuged at room temperature for 20 minutes to isolate soluble proteins.

Levels of IL-1β and TNF-α were assessed in all groups using ELISA kits, following manufacturer protocols with slight modifications. For sample preparation, spinal cords were lysed in ice-cold RIPA buffer using a homogenizer. Lysates were centrifuged at 10,000 rpm for 20 minutes to remove debris.

Supernatants containing target cytokines were collected, and total protein concentrations were determined using the BCA assay to ensure accuracy in cytokine quantification.

The ELISA reagent comprises a pre-coated 96-well microplate for analysis. Immunoglobulins directed against IL-1β and TNF-α were administered into the wells. The plate was rinsed three times with wash buffer to eliminate residual chemicals.

Subsequently, experimental samples and standards were introduced into the wells. Subsequently, 100 μL of spinal cord tissue supernatant and 100 μL of standard solutions were introduced into the designated wells.

Each sample underwent two analyses. The plates were gently agitated at ambient temperature for 1 to 2 hours to facilitate antigen-antibody binding. Subsequent to incubation, the plate was rinsed once more with wash buffer before the application of the detecting antibody.

Subsequently, 100 μL of biotinylated detection antibody specific to the cytokine was introduced to each well. The dishes were stirred periodically following one hour of incubation at room temperature.

The reaction was ended by using the stop solution. The microplate reader was then used to measure the absorbance at 450 nm.

The quantities of TNF-α and IL-1β in the samples were assessed using a standard curve built from the kit's recombinant cytokine standards (Crowther 2009; Lequin 2005).

FIGURE 3.8: Protein Concentration (Photograph taken at Faculty of Pharmacy CUST).

3.13 Statistical Analysis

Quantitative data from behavioral (e.g., mechanical allodynia, thermal hyperalgesia), biochemical (e.g., cytokine levels, oxidative stress markers), and molecular (e.g., protein expression) assays was presented as mean ± SEM. Data distribution was assessed using normality tests such as the Shapiro-Wilk test. Statistical analysis was performed using Python. For two-group comparisons, unpaired Student's t-tests was used. Multi-group, time-point data was analyzed via two-way ANOVA, followed by Tukey post-hoc tests significant differences were detected. A p-value < 0.05 was considered statistically significant.

Chapter 4

Results

4.1 Evaluation of INCA-6 ADMET Properties

4.1.1 Physicochemical Properties

INCA-6 exhibits favorable drug-like characteristics, including a molecular weight of 284.31 Da (under 500 Da), 0 rotatable bonds (fewer than 10), 2 hydrogen bond acceptors (no more than 10), and 0 hydrogen bond donors (maximum 5). Its topological polar surface area (TPSA) is 34.14 $Å^2$ (below 140 $Å^2$), supporting favorable absorption and permeability. These parameters collectively indicate good oral bioavailability and membrane penetration, as excessively large molecular weights, high hydrogen bonding, or high lipophilicity can impede a compound's ability to traverse biological membranes. Similarly, a higher number of rotatable bonds and a larger TPSA value can negatively affect bioavailability by compromising membrane permeability.

4.2 Lipophilicity

Regarding lipophilicity, the log-P value, specifically XLOGP3 (which ranges from 1 to 4), is indicative of good absorption for oral drug candidates. Lower log-P values generally improve solubility in aqueous environments, enhancing dissolution, while

higher values improve membrane permeability, facilitating better absorption across lipid membranes. INCA-6 demonstrates an acceptable XLOGP3 value (2.96) as shown in table 4, placing it within this favorable range.

TABLE 4.1: Physicochemical properties of INCA-6

Physico-chemical Properties		**Values**
	Compound	INCA-6
	MW (g/mol)	284.31
	Rotatable bonds	0
	H - bond acceptor	2
	H - bond donor	0
	TPSA	34.14
Lipophilicity	LogP (o/w) (XLOGP3)	2.96
Drug-Likeness	Lipinski Violation	NO
BBB permeability	Permeability	YES
Bioavailability Score	Score	0.55

4.3 Solubility

In terms of solubility, the Log S value was predicted using the ESOL model. A Log S value of less than -4 indicates poor solubility, which can significantly affect bioavailability due to inadequate amounts of the drug being available in systemic circulation. INCA-6 demonstrates good Log S solubility value, which is -3.87. The different parameters are listed in table 4.1.

4.4 Blood-Brain Barrier Penetration

The ability of a compound to cross the blood-brain barrier (BBB) is closely related to its Log-P value, with moderate values favoring permeability [38]. INCA-6 displays properties that support BBB penetration, as shown by the computational prediction in Figure 4.1. The model's findings indicate that INCA-6 is likely to cross the BBB, supporting its potential for central nervous system activity and underscoring its therapeutic relevance in treating centrally mediated pain.

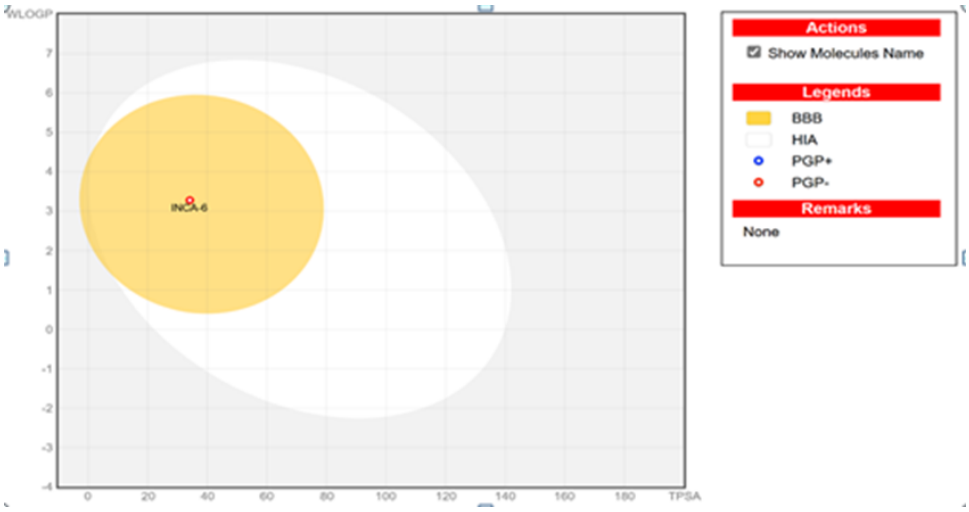


FIGURE 4.1: The BOILED-egg model indicating BBB permeability of INCA-6.

This figure employs the BOILED-Egg model to forecast the pharmacokinetic properties of INCA-6, predicated on its lipophilicity (WLOGP) and polarity (TPSA). The location of INCA-6 in the yellow region signifies a substantial likelihood of blood-brain barrier (BBB) permeability, indicating its potential for central nervous system (CNS) engagement. This underscores its appropriateness for addressing centrally mediated conditions such as paclitaxel-induced neuropathic pain. The model emphasizes advantageous molecular characteristics, bolstering INCA-6's therapeutic potential in addressing neuroinflammatory pathways within the CNS.

4.5 Pharmacokinetics

From a pharmacokinetic standpoint, INCA-6 was predicted to inhibit CYP1A2, CYP2C19, and CYP2D6 (table 4.2). These enzymes are essential for the metabolism of a wide variety of drugs, and their inhibition can lead to significant alterations in pharmacokinetics, impacting both efficacy and safety profiles of therapeutic agents.

4.6 Toxicity Assessment

The toxicity of INCA-6 was assessed, revealing an LD_{50} value of 1520 mg/kg as shown below

TABLE 4.2: Water solubility, pharmacokinetics, and toxicity of INCA-6

Categories	**Properties**	**Values**
Water Solubility	Compounds	INCA-6
	Log S (ESOL)	-3.87
Pharmacokinetics	GI Absorption	High
	Pgp Substrate	NO
	CYP1A2 Inhibitor	YES
	CYP2C19 Inhibitor	YES
	CYP2C9 Inhibitor	NO
	CYP2D6 Inhibitor	YES
	CYP3A4 Inhibitor	NO
Toxicity (ProTox-III Server)	Predicted LD50 (mg/kg)	1520mg/kg
	Toxicity Class	4

4.7 Molecular Docking Study of INCA-6 Against Various Protein Targets

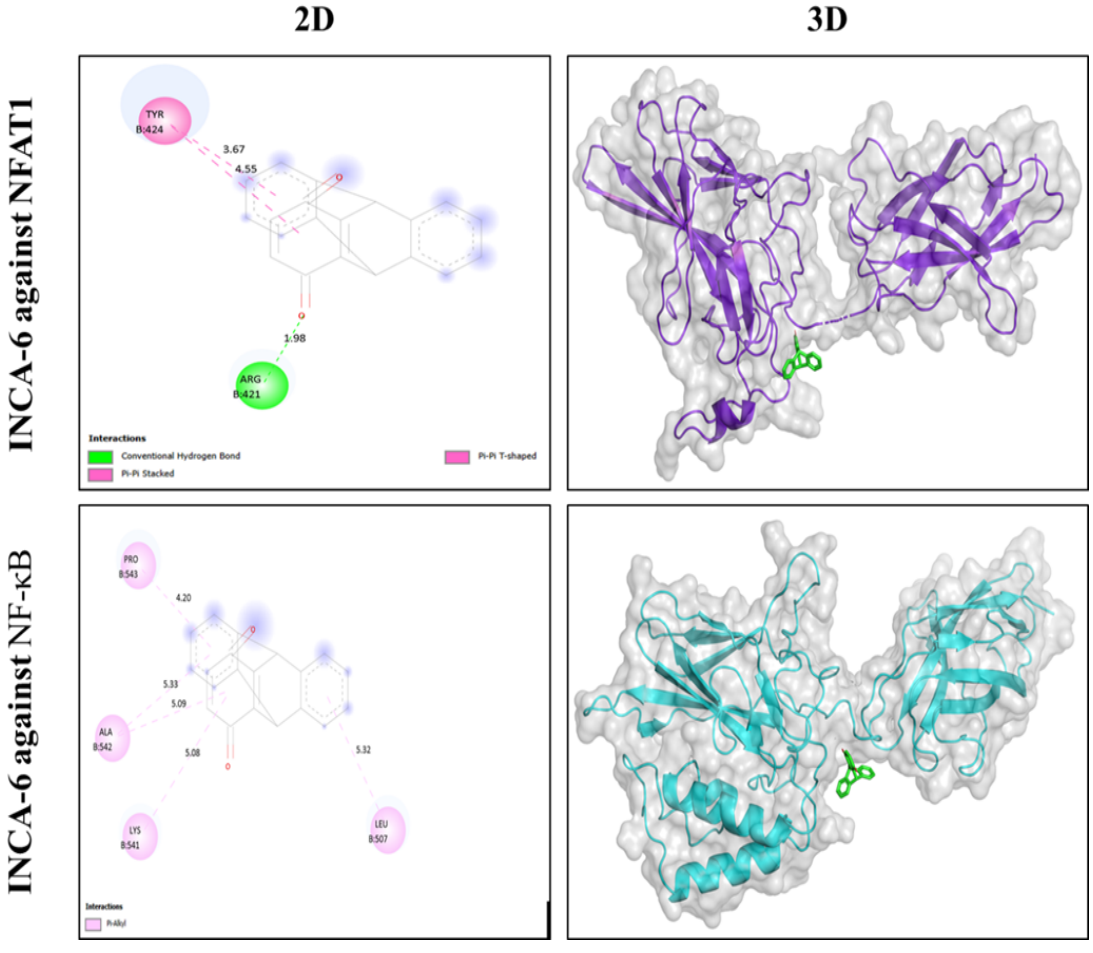
The binding interaction and affinity of INCA-6 were evaluated in the current study through molecular docking. It was observed that INCA-6 exhibits a moderate binding affinity against NF-κB, with no hydrogen bond formation (only hydrophobic interactions were identified). Similarly, promising binding affinities were observed between INCA-6 and the remaining protein targets, which include NFAT1, IL-1β, TNF-α, and IL-6.

Additionally, the presence of hydrophobic interactions and hydrogen bond formation indicated that these proteins exhibited strong binding affinities. Table 4.3 displays the binding affinities of INCA-6.

Additionally, Figure 4.2 illustrate the binding interactions (2D & 3D) of INCA-6 with a variety of protein targets.

TABLE 4.3: Molecular docking study of INCA-6 against various protein targets

Ligand - Protien	**PDB ID / Protein**	**Molecular Docking Score (kcal/-mol)**	**Hydrophobic Interaction**	**Hydrogen interaction**
Ligand-NF-κB	3GUT (NF-κB)	-4.9	LEU507, LYS541, ALA542, PRO543	
Ligand-IL-6	1P9M (IL-6)	-8.0	ARG B:104	THR B:43, LYS B:46
Ligand-NFAT1	1PZU (NFAT1)	-7.0	TYR B:424	ARG B:421
Ligand-TNFα	2AZ5 (TNF-α)	-6.8	TYR A:115, LEU A:63, PRO A:117	TYR A;119
Ligand-IL-1β	1ITB (IL-β)	-7.0	PRO A:91	TYR A:68



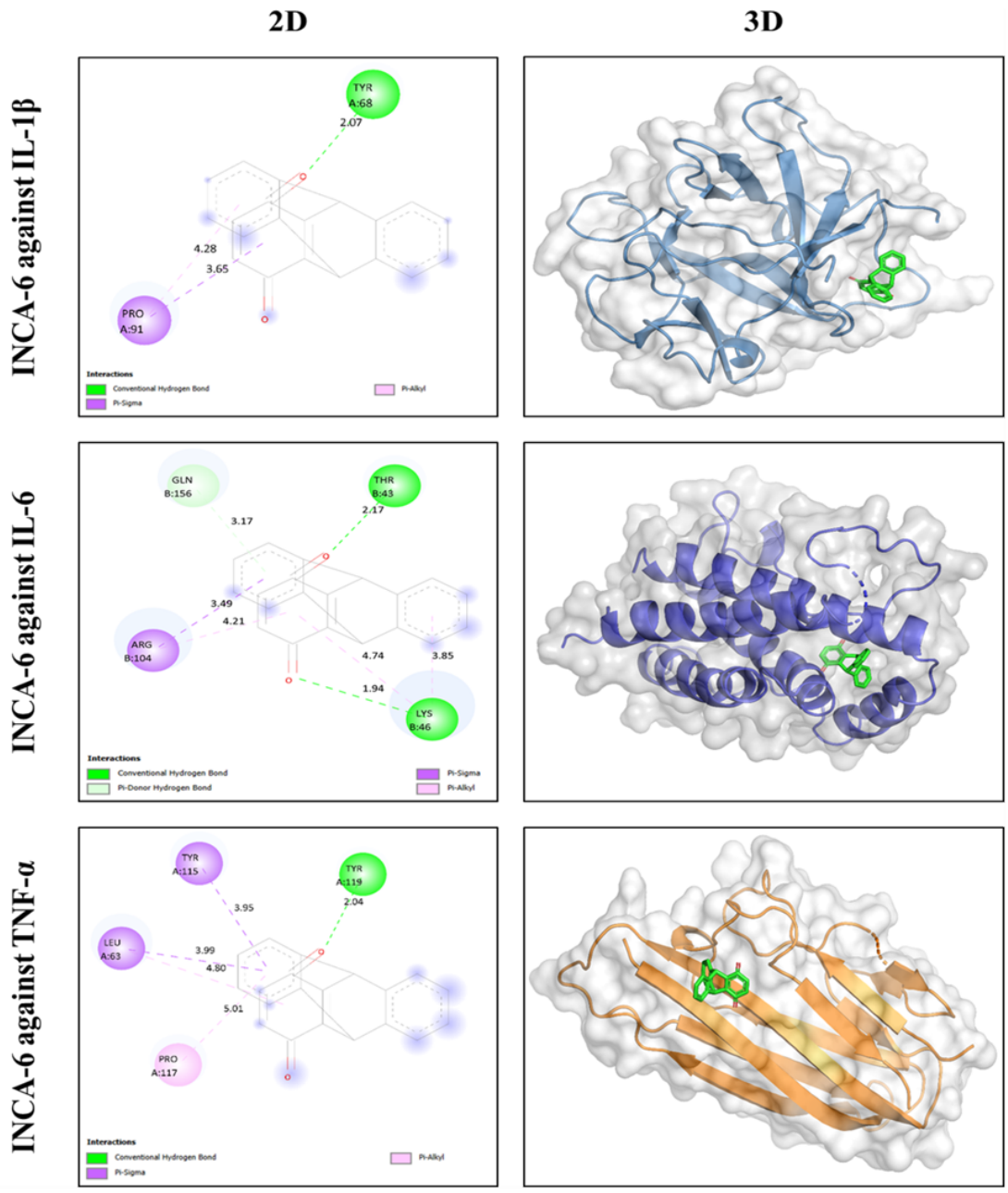


FIGURE 4.2: Binding interaction of INCA-6 against TNF- α, IL-1β and IL-6

The molecular docking results demonstrate that the ligand exhibits the strongest binding affinity with IL-6 (PDB ID: 1P9M) at –8.0 kcal/mol, driven by key hydrogen bonds with THR B:43 and LYS B:46 and hydrophobic interaction with ARG B:104, indicating a potentially strong inhibitory effect. NFAT1 (1PZU) and IL-1β (1ITB) also show favorable binding affinities (–7.0 kcal/mol), stabilized by both hydrogen bonds (ARG B:421 and TYR A:68, respectively) and hydrophobic interactions, particularly aromatic (π-π) and alkyl contacts.

TNF-α (2AZ5) shows moderate binding (–6.8 kcal/mol) through hydrogen bonding with TYR A:119 and hydrophobic interactions involving TYR A:115, LEU A:63, and PRO A:117. In contrast, NF-κB (3GUT) displays the weakest binding score (–4.9 kcal/mol), lacking hydrogen bonding and relying solely on hydrophobic interactions with residues like LEU507 and LYS541. Overall, the ligand demonstrates promising multi-target binding potential, especially with IL-6, suggesting a possible role in modulating inflammatory responses.

4.8 Effect of INCA-6 on Paclitaxel-induced Mechanical Allodynia

INCA-6 (1 and 5 mg/kg, i.p) was tested with respect to the ability to reduce mechanical allodynia caused by paclitaxel through used Von Frey filaments. Mechanical allodynia was evident in the paclitaxel group (2 mg/kg, i.p.) from day 2 onwards, with measurements also taken on days 1, 3, 5, and 7. Following paclitaxel administration, a significant reduction in pain threshold was observed in this group ($p < 0.001$). INCA-6 treatment led to a statistically significant increase in withdrawal thresholds and a great decrease in mechanical allodynia ($p < 0.001$), as it can be seen in Figure 4.3. The lipophilic antiepileptic pregabalin (5 mg/kg, i.p.), used as a positive control, also showed a significant pain reduction.

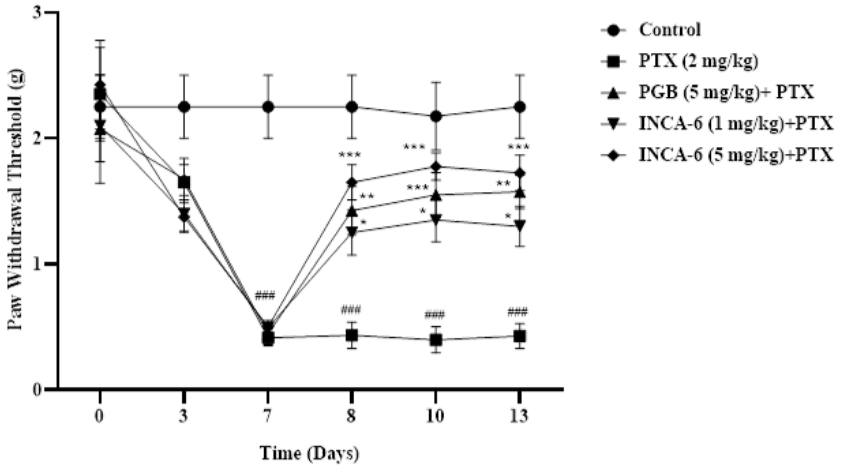


FIGURE 4.3: Effect of INCA-6 and PGB on PTX-Induced Mechanical allodynia

This graph shows dose effect of INCA-6 on mechanical allodynia of paclitaxel (PTX) injected mice. INCA-6 as well as pregabalin (PGB) exhibited potent effects in relieving mechanical hypersensitivity caused by PTX.

In order to elicit neuropathic pain, paclitaxel (2 mg/kg, i.p.) was administered on days 1, 3, 5 and 7. INCA-6 was then administered intraperitoneally at 1mg/kg and 5mg/kg at the same rate of seven days a row whereas, as a reference drug, pregabalin was tested intravenously at a rate of 5mg/kg.

Tukey post-hoc test was conducted as a statistical evaluation of two-way ANOVA. The INCA-6+treated group differed significantly with the PTX-Treatment only group (***P < 0.001, *P < 0.01), whereas there were significant differences (###) between the control and PTX group. Results are expressed as a means ± SEM and each of the groups was performed with seven animals (n = 7).

4.9 Effect of INCA-6 on Paclitaxel-induced Thermal Hyperalgesia

In order to evaluate the effects of INCA-6 on paclitaxel-induced thermal hypersensitivity, hot plate testing was used.

Altogether, mice were treated non-consecutively with paclitaxel (2 mg/kg) every other day during week-long periods by intraperitoneal administration and demonstrated a significant decrease in paw withdrawal latency following thermal stimulation ($p < 0.001$), equivalent to a greater sensitivity to a painful stimulus.

INCA-6 (1 and 5 mg/kg, i.p) produced significant prolongation of withdrawal times and this indicated that heat hypersensitivity was diminished in comparison with paclitaxel-only group.

Thermal sensitivity also demonstrated a significant reduction in the presence of Pregabalin (5 mg/kg, i.p.), thus further objectifying the effectiveness of the treatment option.

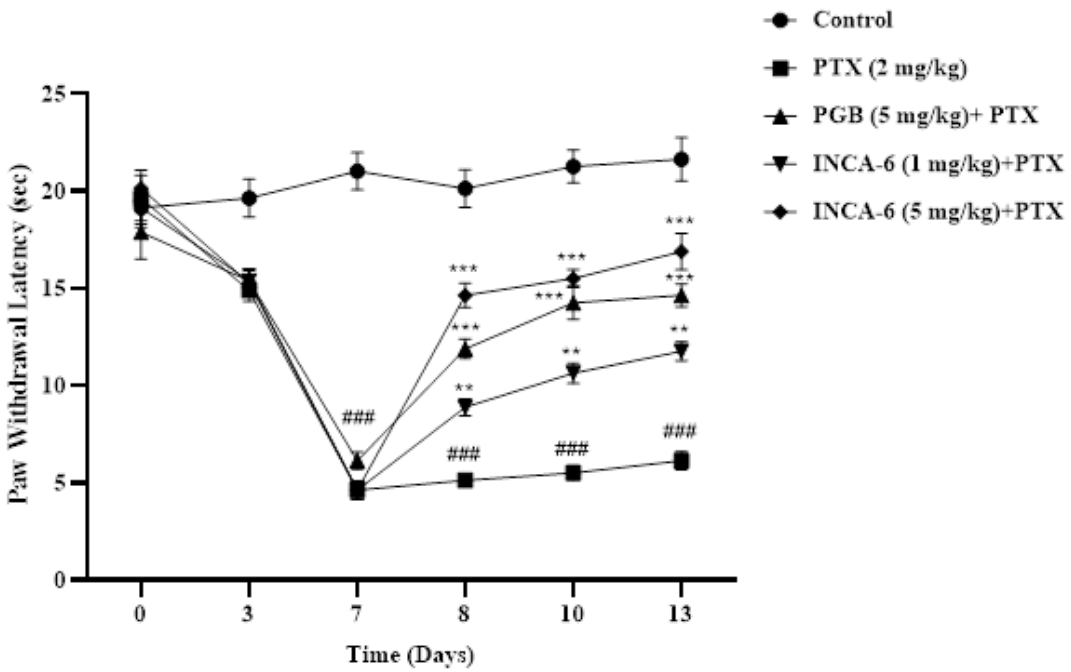


FIGURE 4.4: Effect of INCA-6 and Pregabalin on thermal Hyperalgesia in PTX-Induced Neuropathy

This graph offers insights regarding the effectiveness of INCA-6 in alleviating thermal hyperalgesia. It also shows that INCA-6 aids in reducing heat sensitivity in PTX neuropathic mice. The graph corroborates the previously discussed qualitative effects of pregabalin and INCA-6 on heat pain hypersensitivity quantifiably.

Warmth induced hyperalgesia due to PTX neuropathic pain was reduced by INCA-6 in a dose dependent manner (1 and 5 mg/kg, i.p.) in rats. Neuropathic pain was established by administering paclitaxel 2 mg/kg i.p. on alternate days—injections were given on days 1, 3, 5 and 7.

Subsequently, daily treatments with INCA-6 for 7 consecutive days were given. Pregabalin was administered at 5 mg/kg i.p. as a reference. Two-way ANOVA with Tukey's post hoc test was used for the data evaluation. Differences between treatment and PTX were significant (**P<0.01, ***P<0.001), while (###) denotes significant difference from control to PTX.

Results are reported as mean ± SEM for seven-mouse groups (n = 7). Evaluation of INCA-6 effect on paclitaxel-induced cold allodynia.

4.10 Effect of INCA-6 on paclitaxel-induced Cold Allodynia

This study employed the acetone test to assess the effect of INCA-6 (1 and 5 mg/kg, i.p.) on paclitaxel-induced cold hypersensitivity. Compared to the control group, the repeated administration of paclitaxel (2 mg/kg, i.p.) on alternate days for one week led to a significant increase ($p < 0.001$) in behaviors such as paw withdrawal, flicking, or biting, indicative of hypersensitivity to typically non-painful stimuli, particularly cold. The INCA-6 treatment markedly diminished cold-induced pain responses ($p < 0.001$), evidenced by a reduction in paw withdrawal and flicking occurrences relative to the paclitaxel group Figure 4.5. Pregabalin (5 mg/kg, i.p.) significantly diminished sensitivity to cold stimuli relative to the PTX-treated group.

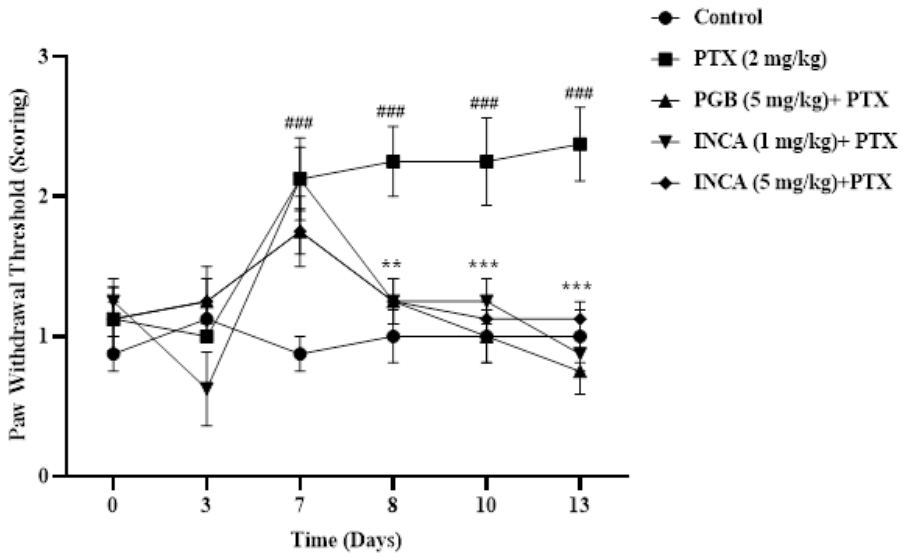


FIGURE 4.5: Effect of Treatments on Paclitaxel-induced cold allodynia signs and symptoms

This graph shows the results of the cold allodynia test performed in a mouse model of neuropathic pain induced by paclitaxel (PTX). On day 1,3,5, and 7, paclitaxel (2 mg/kg, i.p.) was given to mice and neuropathic symptoms were induced. Then, INCA-6 was administered intraperitoneally at dosage of 1 or 5 mg/kg daily (every one day) during 7 days. As a reference compound, 5 mg/kg of Pregabalin (i.p.) was used. The statistical analysis was done on two-way ANOVA with Tukey post hoc. Following

the procedure of Chaplan *et al.* (1994), statistically significant changes were found between the PTX-only and the treatment groups (**P<0.01, ***P<0.001) as well as in the comparison between the control and the PTX groups (###). The data were presented as mean ±SEM and each group in the tests had seven mice (n = 7).

4.11 Effect of INCA-6 on Paclitaxel-induced Proinflammatory Cytokines

This experiment was used to determine the anti-inflammatory effects of INCA-6 in lessening the degree of spinal cords cytokines that are related to neuroinflammation inflicted by paclitaxel. Paclitaxel treatment (2 mg/kg, i.p.) resulted in the pronounced increase in the IL-1β and TNF-α pro-inflammatory cytokine expression (p<0.001). Nevertheless, INCA-6-treated mice had decreased significantly these levels of cytokines in comparison with the PTX-alone group ($p < 0.001$) indicating that INCA-6 could be counteracting neuroinflammation occurring during chemotherapy treatment (see Figures 4.6 and 4.7). The drop was comparable to the after pregabalin treatment and that makes it a positive control.

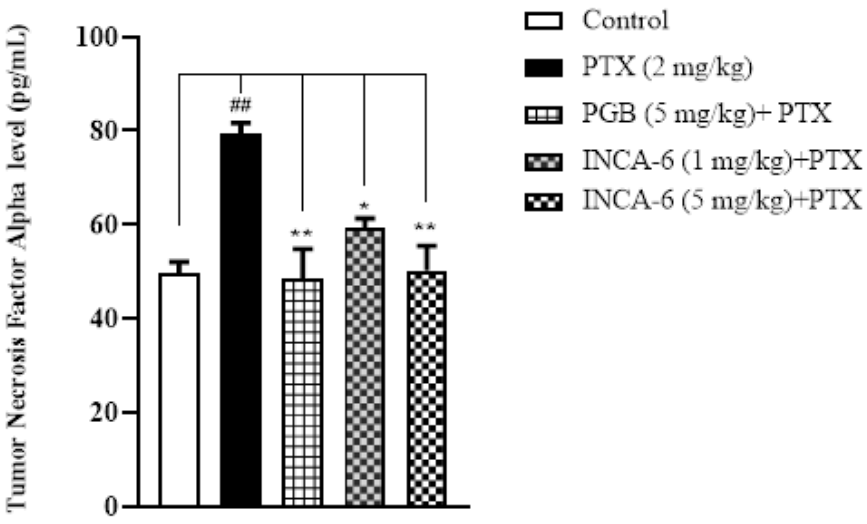


FIGURE 4.6: Tumor Necrosis Factor Alpha (TNF-α) levels.

This bar graph shows quantified concentrations of Tumor Necrosis Factor Alpha (TNF-a) in the spinal cord tissue where they reveal a considerable reduction in this

aggravator of the inflammatory agent after treatment using INCA-6. Such decrease indicates that INCA-6 possesses significant anti-inflammatory properties in paclitaxel (PTX)-induced neuropathic pain model. Neuropathy was induced by intraperitoneal (i.p.) administration of paclitaxel (PTX) at a dose of 2 mg/kg on days 1, 3, 5, and 7. Following the induction phase, INCA-6 was administered daily for seven days at doses of either 1 mg/kg or 5 mg/kg (i.p.). Pregabalin (5 mg/kg, i.p.) was used as a reference treatment. Data analysis was conducted using one-way ANOVA, followed by Tukey's post hoc test to determine specific group differences. Statistical significance was established at *P $<$ 0.05 and **P $<$ 0.01. The symbol (###) indicated substantial differences between the control group and the PTX-only group. Results are reported as mean ± SEM, with each experimental group consisting of three mice (n = 3).

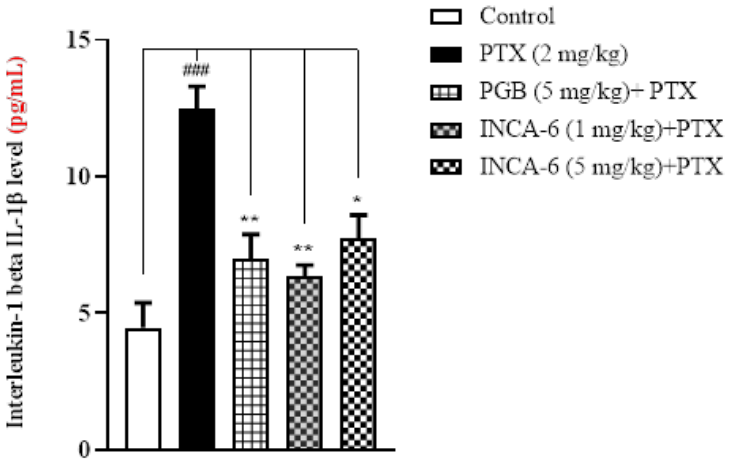


FIGURE 4.7: Effect of INCA-6 on IL-1β Levels in PTX-Induced Neuropathy.

This graph shows the level of an important pro-inflammatory cytokine called Interleukin-1 beta (IL-1β. There was a remarkable reduction in the IL-1β levels with INCA-6 treatment than with paclitaxel alone, which further confirms the potential application of INCA-6 to reduce neuroinflammation in paclitaxel (PTX) induced neuropathy. This was done in the same mouse model, wherein neuropathic pain was elicited by the intraperitoneal injection of PTX (2 mg/kg), which was repeated on the fourth, sixth, and eighth days. After receiving PTX, INCA-6 was administered daily during seven days. A previous comparator was a dose of Pregabalin (5 mg/kg, i.p.). One-way ANOVA with Tukey's post-hoc was used to analyze data where significance was

reported as *P $<$ 0.05 and **P $<$ 0.01. The symbol (###) implies that there is a statistically significant difference between the control and the PTX groups. Means and standard error of the mean ±SEM are reported using three animals per group (n = 3).

4.12 Effect of INCA-6 on Paclitaxel-induced Oxidative Stress Markers

This part of the research was devoted to the antioxidants potential of INCA-6 by performing tests on the level of malondialdehyde (MDA), which is one of the biomarkers of oxidative stress and lipid peroxidation in the spinal cord. IV administration of paclitaxel (2 m g/kg, i.p.) also had a significant effect on MDA level (p $<$ 0.001) showing the presence of high oxidative stress. INCA-6 1 and 5 mg/kg, i.p. treatment continued on seven consecutive days significantly reduced MDA concentration (p¡0.01) compared to the PTX group showing that INCA-6 can be potentially used to overcome oxidative damage. The significant decrease in the level of the MDA (p $<$ 0.001) after the administration of pregnabalin (5 mg/kg, i.p.) illustrates its antioxidative properties.

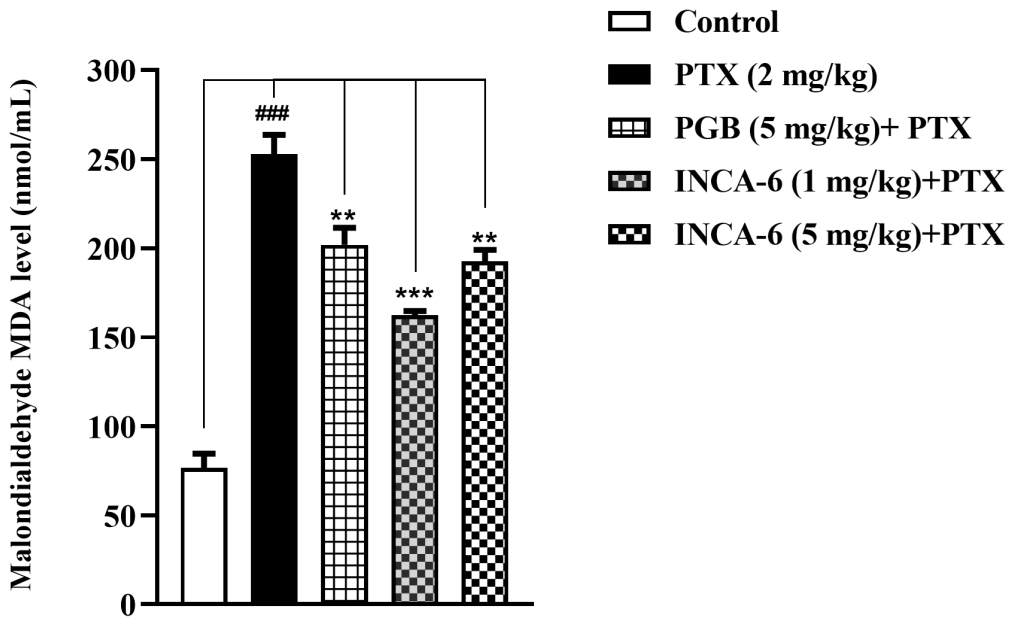


FIGURE 4.8: Effect of INCA-6 on MDA Levels in PTX-induced neuropathic pain.

The graph demonstrates a significant decrease in malondialdehyde (MDA) levels following INCA-6 treatment.

This reduction indicates a clear mitigation of lipid peroxidation and oxidative stress, which are critical pathological components of paclitaxel-induced neuropathic pain.

We looked at how two doses of INCA-6, given by injection (1 or 5 mg/kg), changed the amount of malondialdehyde-MDA-a marker of oxidative stress-in the spinal cords of mice with paclitaxel-linked nerve pain.

The injury was created by injecting paclitaxel (2 mg/kg) on days 1, 3, 5 and 7, then the chosen dose of INCA-6 was given once a day for seven days. Pregabalin (5 mg/kg, also by injection) served as a standard treatment.

Results were compared using one-way ANOVA and Tukey's test, with significance marked as *P $<$ 0.05, **P $<$ 0.01 or ***P $<$ 0.001; the symbol ### shows that the control and paclitaxel groups differed.

Values are reported as mean ± SEM and each experimental group included three mice (n = 3).

4.13 Effect of INCA-6 on Paclitaxel-induced Changes In Glutathione Levels

The team measured spinal-cord glutathione (GSH) in paclitaxel (PTX)-induced mice to see whether INCA-6 helps the bodys own antioxidant shield. When given PTX (2 mg/kg, i.p.), mice showed a sharp drop in GSH ($p < 0.001$ versus controls), an obvious sign of oxidative damage.

By contrast, INCA-6 (1 or 5 mg/kg, i.p.) lifted spinal GSH to much higher levels than PTX alone ($p < 0.01$, see Figure 4.9).

The antioxidant potential of INCA-6 under PTX-induced oxidative stress was further supported by a notable ($p < 0.05$) elevation in GSH levels when compared with pregabalin (5 mg/kg, i.p.), which served as the standard comparator.

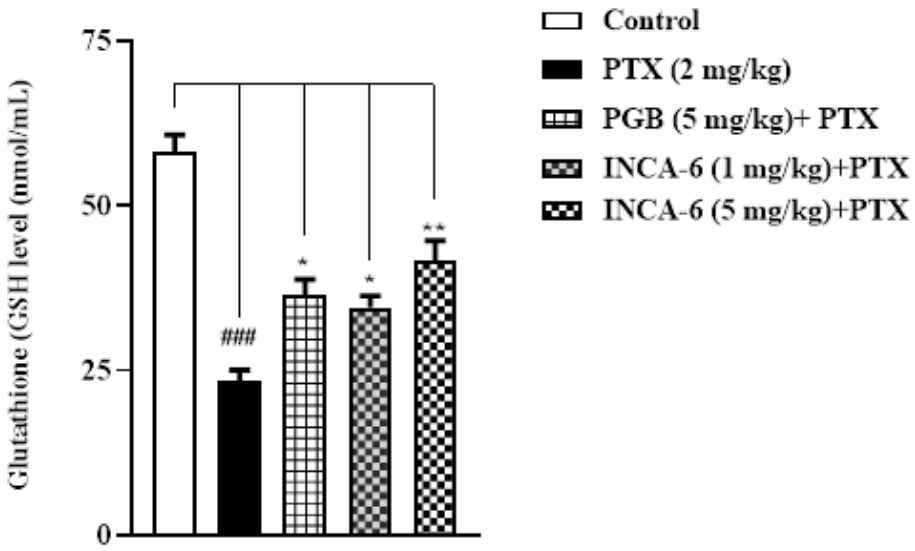


FIGURE 4.9: Effect of INCA-6 on GSH Levels in PTX-Induced Neuropathy.

the figure shows how INCA-6 affects glutathione (GSH) levels, offering a clear picture of its antioxidant boost. That trend highlights INCA-6s power to refill the cells fading GSH pool after paclitaxel wipes it out, thus protecting nerves from ongoing oxidative harm. We measured spinal-cord GSH in mice given paclitaxel and then treated with either 1 or 5 mg/kg of INCA-6 by intraperitoneal injection. Paclitaxel was delivered at 2 mg/kg by the same route on days 1, 3, 5, and 7 to trigger neuropathic pain. After the last drug, mice received INCA-6 every day for seven days. A group also received pregabalin (5 mg/kg, i.p.) as a reference drug. Results passed through one-way ANOVA plus Tukey's test, setting significance at *P < 0.05 and **P < 0.01. The symbol ### marks comparisons between control and the paclitaxel group. Values appear as means SEM with three mice per group (n = 3).

Chapter 5

Discussion

5.1 Introduction

A comprehensive pharmacological investigation of INCA-6, a novel redox-active quinone, was conducted to evaluate its efficacy in alleviating paclitaxel-induced neuropathic pain in rodents. Due to the significant clinical challenges posed by paclitaxel-induced pain (PIPN), physicians often deem conventional analgesics insufficient, necessitating dose reductions or cessation of chemotherapy [39]. Our goal was to test whether INCA-6 could calm PIPN symptoms while also probing its effects on spinal cord inflammation and oxidative stress at the molecular level. The results show that INCA-6 sharply eased mechanical allodynia, thermal hyperalgesia and cold allodynia linked to paclitaxel treatment. It also lowered spinal TNF-a and IL-1b levels, pointing to reduced neuroinflammation, and cut MDA while boosting glutathione, indicating less oxidative damage. Together, these data suggest that INCA-6 acts as a potent analgesic, anti-inflammatory and anti-oxidant agent for patients suffering PIPN.

5.2 Interpretation of Results

The behavioral analyses of our study illustrate utilizing an INCA-6 compound as having analgesic effects. It was previously reported that the administration of paclitaxel

led to severe medical conditions such as mechanical allodynia, thermal hyperalgesia, and cold allodynia, which is in sync with the sensory hyper-responsiveness seen in human PIPN patients [12].

In our studies, the subjects treated with 1 mg/kg and 5 mg/kg doses of INCA-6 showed a strong reversal of these pain-like activities, restoring the withdrawal thresholds and latencies towards normal levels. This level of analgesia is almost indistinguishable from that of pregabalin (a commonly used medication for neuropathic pain), suggesting that there is promise from INCA-6 in countering the varied sensory disturbancestypical of neuropathic pain. The effects of INCA-6 on mechanical allodynia (pain from light touch), thermal hyperalgesia (increased pain from heat), and cold allodynia (pain from cold stimuli) demonstrate possible modulation of disturbed pain signaling pathways in the peripheral and central nervous systems.

Besides providing symptomatic relief, our biochemical studies focus on the INCA-6's efficacy further exploring deeper mechanisms of action. Paclitaxel treatment markedly increased the levels of pro-inflammatory cytokines, namely TNF-α and IL-1β in the spinal cord. Cytokines are well-known factors enhancing neuroinflammation and causing central sensitization, which leads to the development of neuropathic pain [40]. The substantial decrease of both TNF-α and IL-1β suggests that their reduction by INCA-6 treatment was due to strong anti-inflammatory activity. This reduction of dominant inflammatory mediators is critical because chronic neuroinflammation leads to hyperexcitability of neurons, which is one reason for the development of neuropathic pain that can hardly be left untreated [41]. The anti-inflammatory effect of INCA-6 is a part of therapeutic potential, targeting one of the core processes in PIPN pathophysiology.

In addition, oxidative stress markers reinforce mechanistic understanding, completing the puzzle. Paclitaxel is known to cause mitochondrial damage and raise oxidative stress and ROS overproduction, which results in stress and harm to the cellular structure of neurons [13]. In this work, increased levels of MDA, which signifies lipid peroxidation and oxidative injury, were observed to be elevated in the presence of paclitaxel while GSH, an important endogenous antioxidant, was shown to be depleted. INCA-6 treatment more than counteracted those changes by reducing MDA

and restoring GSH to significant levels. This illustrates INCA-6's strong antioxidant activity which is important for safeguarding the integrity and function of neurons from oxidative injury caused by paclitaxel. INCA-6's protective scavenging of ROS and enhancement of antioxidant defenses removes one of the major pathological processes underlying PIPN, thus explaining the neuroprotective impact and overall pain attenuation effect of INCA-6.

The in-silico molecular docking studies provided preliminary rationale concerning the interaction of INCA-6 with inflammatory and pain-related protein targets. INCA-6's notable binding affinities with NFAT1, IL-1β and TNF-α are indicative of direct molecular interactions which would account for the compound's anti-inflammatory properties. Along with NF-κB showing moderate binding affinity, the overall profile still supports multi-target potential activities of INCA-6.

The ADMET predictions of INCA-6 also highlighted clear pharmacokinetic concerns, where the compound's desirably balanced physicochemical parameters alongside good solubility and predicted BBB access, implies favorable chances for systemic circulation and central nervous system target engagement [35].

These insights bolster the rationale regarding the biological activity of INCA-6 and assist in guiding subsequent experimental steps.

5.3 Mechanistic Implications

The observed therapeutic effects of INCA-6 in PIPN can be mechanistically linked to its dual action as an NFAT inhibitor and a redox-active quinone, targeting the interconnected pathways of neuroinflammation and oxidative stress.

5.4 NFAT Pathway Modulation

The erroneous neuronal functioning and neuroinflammation causing neuropathic pain is notogenetic in nature, depicting the importance of the calcineurin-NFAT pathway

[19]. The calmodulin-dependent phosphatase calcineurin perpetuates NFAT signaling by dephosphorylation, enabling the nuclear translocation of NFAT, which then activates the transcription of pro-inflammatory genes like TNF-α and IL-1β [17]. Dysregulated calcium from paclitaxel is known to stimulate this pathway [13].

That INCA-6 reduces significantly TNF-α and IL-1β concentrations so strongly supports the thought of calcineurin-NFAT mechanisms inhibition. This convoluted pathway of signalling is important because INCA-6 diminishes and alters the inflammatory profile that leads to neuroplastic changes in sensitization of pain and its chronicity whole directly targeting the peripheral aspects of inflammation.

INCA-6's differences highlight gaps in an expanse of analgesics whose action is devoid of the upper tier inflammatory pathways.

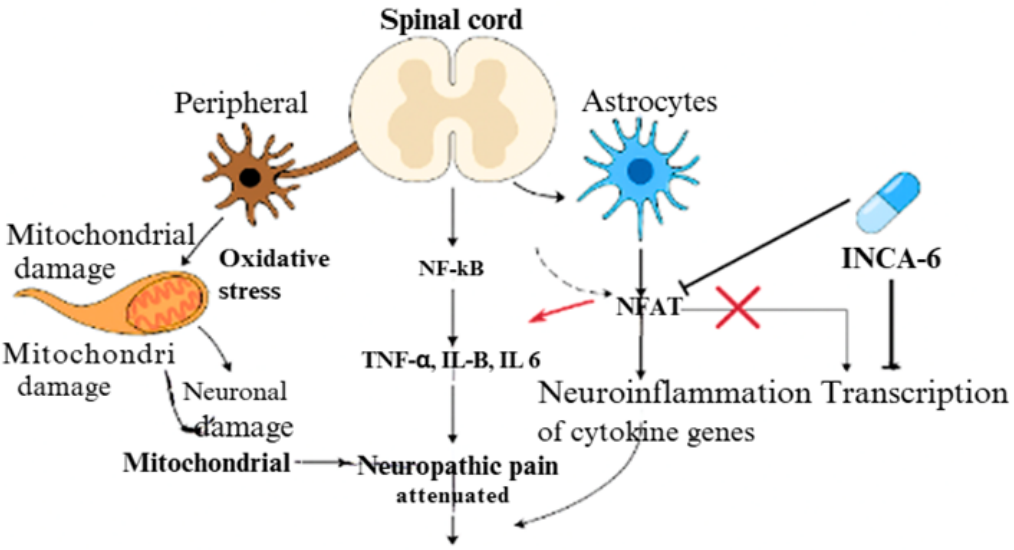


FIGURE 5.1: Effect of INCA-6.

The pathophysiology of PTX-induced neuropathic pain and the role of INCA-6 in reducing neuroinflammation are depicted in the diagram. It demonstrates how PTX induces neuronal injury and mitochondrial damage, resulting in the activation of the NF-κB and NFAT pathways in astrocytes and microglia. This process is responsible for the production of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6). INCA-6 reduces neuropathic pain by inhibiting NFAT, which in turn prevents the transcription of cytokine genes [42, 43].

5.5 Redox Activity and Oxidative Stress Mitigation

The triptycene-1, 4-quinone structure of INCA-6 provides its redox-active properties, enabling the compound to scavenge reactive oxygen species (ROS) [17]. Production of reactive oxygen species is greatly elevated in paclitaxel-induced peripheral neuropathy and causes oxidative damage to lipids, proteins, and DNA of the neuron [13]. Restoration of GSH levels and reduction of MDA by INCA-6 in our study suggests its strong antioxidant capacity. By neutralizing detrimental ROS, INCA-6 preserves neuronal cells from oxidative damage, INCA-6 preserves neuronal cells from oxidative damage, and therefore, cellular integrity and function.

This is critical for the long sensory neuron axons that are extremely susceptible to paclitaxel's impact on the microtubules and the mitochondria [11]. The relationship between oxidative stress and inflammation is notoriously known; inflammation can induce more ROS and in return, ROS can activate inflammatory pathways [26]. Because of these reasons, addressing both simultaneously falls into the powerful therapeutic advantage INCA-6 has.

The molecular docking results further reinforce these mechanistic insights. The strong binding affinities of INCA-6 with NFAT1, IL-1β and TNF-α suggest that INCA-6 may directly interact with and modulate the activity of these proteins, thereby contributing to the observed anti-inflammatory effects. While the exact nature of these interactions (e.g., competitive inhibition, allosteric modulation) requires further elucidation, the computational data provides a plausible molecular basis for INCA-6's biological actions.

5.6 Consistency with Previous Research

As with previous studies, our results also emphasize the prominent role neuroinflammation and oxidative stress play in the development of neuropathic pain, particularly PIPN. Pro-inflammatory cytokines such as TNF-α and IL-1β are known to be elevated

in the PIPN animal models and even in human patients [44]. The reduction of these cytokines by INCA-6 clearly underlines the growing belief that targeting neuroinflammation could provide relief from neuropathic pain. Tshe role of oxidative stress in paclitaxel-induced neurotoxicity where there is an increase in MDA and decrease in GSH. Confirming the antioxidant effects of INCA-6 aligns with the growing belief on the applicability of other antioxidants in treating neuropathic pain [45].

The efficacy of INCA-6 in ameliorating behavioral pain hypersensitivity is comparable to that of pregabalin, a widely used gabapentin. While pregabalin's primary mechanism involves modulating voltage-gated calcium channels to reduce excitatory neurotransmitter release [27], it does not directly target the inflammatory and oxidative stress pathways as comprehensively as INCA-6 appears to. This suggests that INCA-6's multi-modal action may provide a more robust and sustained therapeutic benefit by addressing upstream pathological events.

Moreover, the approach taken in our work corresponds to the most recent progress made in PIPN treatment research that focuses on modulation of neuroinflammation and specific pathways. Studies involving the S1PR1 antagonist FTY720, as well as compounds such as CN016 and artesunate, emphasize the therapeutic potential of anti-inflammatory and neuroprotective strategies in managing paclitaxel-induced peripheral neuropathy (PIPN). These findings suggest that targeting neuroinflammatory pathways and promoting neuronal protection can play a critical role in both the prevention and treatment of PIPN [46].The peculiar NFAT suppression and redox behavior in INCA-6 provides a distinct mechanism that complements these approaches and as such broadens the therapeutic profile. Also, works proposing that Keap1/Nrf2, a major cellular antioxidant defense signal, is activated by other quinones [47] supports broader hypothesis about INCA-6's cytoprotective ability.

5.7 Novelty and Significance

This study makes several significant contributions to the field of neuropathic pain research and the development of novel therapeutics for PIPN.

This is the first comprehensive investigation involving the *in-vivo* study of the therapeutic effects of INCA-6, a uniquely structured dual-action molecule, in the context of paclitaxel-induced neuropathic pain. Calcineurin, NFAT, and oxidative stress have all been studied in isolation within the context of their role on neuropathic pain, but assessing the integrated impact of INCA-6 at the behaviorally relevant pain outcome level on the NFAT, NF-κ B, cytokine production within a single model of PIPN was unique at the time of our study.

Such an analysis serves to underscore in a single PIPN model integrate all relevant constituents to derive a holistic understanding of vasculogenic PIPN's INCA-6's effects on peripheral and central components of complex path. This exposes glob therapeutic potential of INCA-6.

Second, an important step forward in therapeutics is the dual mechanism of action of INCA-6, a combination of direct inhibition of NFAT and redox active character. Treatment options offer relief solely within the specified target areas, unless accompanied by various adverse effects [48].

These findings underline PIPN's proxies targeting brain-inflammation and oxidative damage, incidentally INCA-6, as a more aggressive and perhaps effective approach to managing the condition. Multi-targeted treatment appears to be vital, considering the complex and integrated nature of underlying mechanisms like neuropathic pain, which involve interplay of multiple pathways responsible for its onset and perpetuation [49].

If these core pathological processes are targeted simultaneously, there is likely to be much greater efficacy, broader scope of action, and possibly even an improved side-effect profile compared to standard approaches.

The optimistic outcomes of our in silico ADMET predictions, along with the molecular docking studies, provides a strong rationale for further development of INCA-6. The predicted drug-like characteristics and favorable binding affinities with relevant protein targets enhances confidence in the therapeutic potential of INCA-6 and suggests validation tests should be performed experimentally at the molecular level [50, 51].

In this context, we proposed a new promising therapeutic model for PIPN in the form of INCA-6 which, due to its dual anti-inflammatory and antioxidant action, is

unique. This approach may be particularly useful in filling the existing gap in controlled clinically-useful chemotherapy-induced neuropathic pain treatments, thereby enhancing cancer patients' life quality.

5.8 Limitations

Despite the compelling findings, this study has several limitations that warrant consideration for future research. Firstly, the current study employed an acute PIPN model with a treatment duration of 7 days which is relatively short.

This model successfully induced neuropathic pain and facilitated evaluation of INCA-6's acute impacts, but chronic neuropathic pain is typically alleviated through long-term approaches. Future works should focus on determining the long-term efficacy and safety of INCA-6 within chronic PIPN paradigms to evaluate its enduring therapeutic effects alongside potential cumulative side effects.

Secondly, in addition to monitoring changes in spinal cord cytokine levels and oxidative stress markers, a more complete understanding of the action of INCA-6 would come from studying specific neuronal populations like dorsal root ganglia neurons and specific glial cell types such as microglial and astrocytic cells in the peripheral and central nervous system. That would enable the determination of precise cellular targets of INCA-6.

Thirdly, although molecular docking gave insights into prospective protein interactions, this study did not experimentally test the controlling action of INCA-6 on the NFAT and NF-κB signaling pathways.

Methods such as Western blotting for nuclear translocation of NFAT or NF-κB subunits, or reporter gene assays, could provide these proofs. Such experiments would provide stronger evidence for the proposed mechanistic implications.

Finally, while the ADMET predictions were favorable, comprehensive *in-vivo* pharmacokinetic and pharmacodynamic studies are needed to fully characterize INCA-6's absorption, distribution, metabolism, and excretion in animal models, as well as its

dose-response relationship in greater detail. This would inform optimal dosing strategies for future translational studies.

5.9 Future Directions

In light of the encouraging findings of this study and its recognized limitations, multiple directions for future research are suggested to further investigate the potential of INCA-6. A more comprehensive explanation of molecular mechanisms is essential. This entails conducting comprehensive molecular analyses utilizing techniques such as Western blotting, immunohistochemistry, and immunofluorescence [52].

The objective is to confirm that INCA-6 modifies the modulation of NFAT and NF-κB pathways, particularly by assessing NFAT nuclear translocation and NF-κB phosphorylation, subsequently followed by nuclear translocation. Furthermore, subsequent research should examine the effects of INCA-6 on additional pertinent ion channels and distinct markers of glial cell activation, including GFAP and Iba1, in muscle, spinal cord, and peripheral nerve tissues.

A thorough evaluation of long-term effectiveness and safety is essential. This entails conducting and implementing research in chronic neuropathic pain models to assess the prolonged efficacy of INCA-6 over extended treatment periods. It will be essential to document any long-term adverse effects to facilitate further developmental enhancement and ensure future clinical applicability.

Third, thorough pharmacokinetic and pharmacodynamic assessments should be performed. Comprehensive *in-vivo* pharmacokinetic investigations of INCA-6 are required to elucidate its absorption, distribution, metabolism, and excretion in animal models. Complementary pharmacodynamic investigations will reinforce the established correlation between dose response and the therapeutic window of the compound.

Fourth, looking at drug combinations opens a promising path. Future studies should test INCA-6 alongside standard chemotherapy drugs or common pain relievers given to cancer patients. Blending therapies could lower the dose of each medicine, cut

unpleasant side effects, and lift overall benefit-especially for people battling tumor-related pain.

Fifth, research must spread into other models of nerve pain. Evaluating INCA-6 in diabetes-related neuropathy, post-herpetic neuralgia, and surgical nerve lesions will show whether the compound works similarly or differently across these varied disorders. In the end, translational work will move INCA-6 from bench to bedside. First, larger animals must undergo standard toxicity tests to build a clear safety record. With those results in hand, scientists can wisely decide whether to push forward into human trials for cancer pain and other complex nerve syndromes.

Chapter 6

Conclusion and Recommendations

In tests on mice, the work showed that INCA-6-a new redox-active quinone-eases pain caused by paclitaxel, hinting at real clinical promise. Behaviorally, INCA-6 cut back mechanical allodynia, thermal hyperalgesia, cold allodynia, and other signs of heightened pain sensitivity. Changes in the spinal cord matched those gains: TNF-α and IL-1β dropped, while markers of oxidative stress-MDA-fell and GSH rose. Taken together, these results credit INCA-6s strong pain relief and nerve protection to its twin powers of blocking inflammation and mopping up free radicals, framing it as a hopeful option for chemotherapy-related neuropathy.

6.1 Recommendations

Several clear directions emerge for future work if INCA-6 is to move beyond the bench. First, studies should dig deeper into the exact molecules that INCA-6 acts on. That effort can include standard techniques like Western blotting and immunohistochemistry to track whether INCA-6 really tunes NFAT and NF-kB up or down. Researchers should also look at how INCA-6 influences other key targets, such as ion channels and markers of glial cell activation, in both central and peripheral nervous tissues [53, 54]. Doing so will paint a fuller picture of the drug's therapeutic action.

Another vital topic for future work is the drugs long-term safety and benefit. Studies ought to track the lingering results of INCA-6 in models of chronic neuropathic pain, taking into account both extended dosing and recovery phases. Such experiments will clarify how well the compound holds up over time and reveal any delayed side effects that could shape its use in clinics.

In addition, broad dose-response and pharmacokinetic studies are essential. This work should start with careful stepwise dosing to pinpoint the narrow range where INCA-6 works best. At the same time, *in-vivo* tests of how the drug is absorbed, spreads through tissues, transformed by the body, and excreted will give a realistic picture of its fate within living animals. Together, these data will guide dose selection and delivery routes in later trials.

Research into combination treatments also looks very encouraging. Future trials need to test INCA-6 alongside current cancer drugs and standard neuropathic pain meds. The goal is to spot any additive action that would let doctors use smaller doses of each drug, cut side effects, and give patients a bigger overall benefit.

It is equally vital to test INCA-6 in other pain models. That effort means studying it in diabetic neuropathy and nerve-ligation setups to see if the compound works across different causes of nerve injury. Solid data from diverse tests would widen the medicine's target population.

Strong translational work will be the next step toward human use. Early safety tests in larger animals must be run to shape a solid risk profile for INCA-6. Positive results will guide clinicians as they consider the compound for the first formal studies in people.

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