CHAPTER 2: Rationale, Objectives and Plan of Work

1.1 Rationale

Chemotherapy-induced neuropathic pain (CINP) is a prominent and severe adverse effect caused by antineoplastic agents. The prevalence of neuropathic pain in patients undergoing chemotherapy varies widely, ranging from 18% to approximately 88%, contingent upon the specific treatment regimen employed. The frequency of CINP tends to be lower in individuals undergoing treatment with a single agent, ranging from 3% to 7%. However, this incidence can elevate significantly, reaching up to 68.1%, in patients subjected to chemotherapy regimens. Despite sustained and comprehensive research efforts over recent decades, the development of effective therapeutics for the treatment of CINP remains markedly constrained, primarily due to the intricate and multifaceted nature of the underlying pathophysiology and the absence of well-defined molecular targets. Notably, the current therapeutic landscape lacks US-FDA approved interventions and effective treatment of CINP in patients. The current pharmacotherapeutics is inadequate, further raising the concern of researchers toward this unmet medical need. The pathophysiology of CINP involves impaired nociceptive signaling and the generation of uncontrolled and inappropriate responses. It involves nociceptor activation, peripheral sensitization, and neuronal hyperexcitability. Chemotherapy-induced damage initiates a cascade of events, including the generation of free radicals, dysregulation of ion channels, and activation of glial cells. This process is further intensified by the infiltration of leukocytes, resulting in inflammation in both the dorsal root ganglions and the spinal cord. Ultimately, these intricate molecular and cellular changes contribute to the development of peripheral neuropathy. Transient

receptor potential (TRP) channels, including TRPA1, TRPV1, and TRPM8 play a crucial role in both the initiation and perpetuation of neuropathy. Activation of TRP channels leads to an upsurge in the release of calcitonin gene-related peptide (CGRP) and substance P, triggering inflammatory responses. In the context of chemotherapy-induced peripheral neuropathy (CINP), N-methyl-D-aspartate receptors (NMDARs), particularly those containing NR2B subunits, contribute significantly to the process of central sensitization, influencing the development and persistence of CINP. The cumulative impact of chronic pain conditions exacerbates these effects, leading to an increased production and delivery of receptors, ultimately heightening sensitivity and contributing to the complex dynamics of neuropathic pain in affected individuals.

Despite numerous attempts in drug development for CINP, the rate of successfully translating these drugs into clinical settings remains notably low, mainly due to several underlying factors. One such factor is the lack of reliable animal model which can clinically mimic the CINP and correlate the outcome to bridge the translational gap. In response to this challenge, our study aimed to address this limitation by hypothesizing the development of a new clinically mimicable animal model for CINP, correlate the outcome to bridge the translational gap. This rodent model utilized a combination of three commonly used chemotherapeutic drugs (paclitaxel, cisplatin, and vincristine), referred to as the combined chemotherapy model. We compared this new model with the conventional (paclitaxel) monotherapy model in rats. The purpose of developing a combination chemotherapy animal model was to establish an alternative experimental framework designed to facilitate the development or screening of novel pharmacotherapeutics targeted at treating CINP. Subsequently, we explored the potential of Bergenin, a natural phytochemical, in alleviating

neuropathic pain induced by the newly developed combined chemotherapy model. Our investigation extended to unraveling the cellular and molecular mechanisms underlying the anti-nociceptive effects of Bergenin, assessing its effect on both evoked and spontaneous ongoing pain. Moreover, we conducted an evaluation of Bergenin treatment on base pain responses in healthy naive animals, using the tail flick and tail clip assays to assess acute pain responses, as compared the effect of morphine. Considering the central nervous system (CNS) side effects pose a significant challenge with current analgesics in clinics, thus we have also investigated Bergenin effect on motor in-coordination and locomotion activity.

Recognizing the clinical significance of transient receptor potential ankyrin 1 (TRPA1) in pain patients, we observed elevated levels of TRPA1 in rats treated with chemotherapy or even in the CCI model. Given the imperative to minimize off-target effects commonly associated with antagonists, there is a pressing need for a highly specific gene silencing approach. In response to this challenge, we have engineered a liposomal formulation that encapsulates small interfering RNA (siRNA) targeting TRPA1 gene. This liposomal formulation aims to specifically silence the TRPA1 gene, offering a precise and effective strategy to alleviating this debilitating condition. This multifaceted approach aims to not only enhance our understanding of the underlying mechanisms of CINP but also explore potential therapeutic interventions, addressing the current translational gap in drug development for this challenging condition.

2.2 Objectives

The primary objective of the study was to establish a clinically mimicable animal model for CINP through the implementation of a combination chemotherapy

approach. The model's fidelity was systematically validated in comparison to conventional monotherapy models, with a specific focus on exploring the analgesic properties of the natural phytochemical Bergenin and elucidating the associated cellular and molecular mechanisms. Additionally, the study sought to propose an innovative therapeutic avenue by developing a nano-formulation loaded with TRPA1-siRNA, intending to address the translational gap in CINP drug development. Below are the key objectives of this study:

Aim 1: Establishment and Validation of Animal Model for Chemotherapy-Induced Neuropathic Pain

Aim 1A: Development and Validation of a Clinically Relevant Animal Model for Chemotherapy-Induced Neuropathic Pain

Aim 1B: Mechanistic Validation of Animal Model for CINP Using Molecular Assays

Aim 2: Preclinical Investigation of Bergenin in the Animal Model of Combination Chemotherapy-Induced Neuropathic Pain

Aim 2A: Investigating the Effects Bergenin on Combination Chemotherapy Model Using a Battery of Pain Behavioral Assays

Aim 2B: Elucidation of the Cellular and Molecular Mechanisms Underlying Bergenin Attenuation of Neuropathic Pain in a Combination Chemotherapy Model

Aim 2C: Investigation of the effect of CINP and its Modulation by Bergenin on Spontaneous Ongoing Pain and Central Nervous System Toxicity

Aim 3: Development, Characterization, and Evaluation of TRPA1-siRNA Loaded Liposomal Formulation in the Animal Model of CINP

Aim 3A: Development of Liposomal TRPA1-siRNA Nanomedicine Using State-of-the-Art Facilities

Aim 3B: Performance of Characterization Studies for the Lipid-Based siRNA Nanomedicine

Aim 3C: Determination of the Efficacy and Potency of Liposomal TRPA1-siRNA Nanomedicine in Combination Chemotherapy Model and Elucidates Downstream/Upstream Signaling

2.3 Plan of work

2.3.1 Study I

In our initial series of experiments, we utilized both behavioral and pharmacological methodologies to establish and validate a novel model for peripheral neuropathy, focusing on a combination-based chemotherapeutic approach. This involved the integration of three commonly used chemotherapeutic drugs paclitaxel, cisplatin and vincristine termed the combined chemotherapy model. We compared this model with the conventional paclitaxel monotherapy model in rats.

The first study was divided into two parts; in the first half (Table 2.1), we have performed the time courses of pain responses due to chemotherapeutic agents. In the second half of the study (Table 2.2), we have conducted the pharmacological validation of our novel model with conventional paclitaxel model.

Table 2.1 Animal grouping for time course study of animal model

S. No	Group	Number of animals (Male Sprague Dawley rats)
1.	Vehicle	8
2.	Paclitaxel (2 mg/kg, i.p.)	8
3.	Combination chemotherapy (Paclitaxel 2 mg/kg, Cisplatin 2 mg/kg, Vincristine 0.5 mg/kg, i.p.)	8

In the second half of the study, we have examined the predictive validity (gabapentin) of our novel animal model. The grouping for this set of experiment was mentioned below.

Table 2.2 Animal grouping for pharmacological validation of animal model

S. No	Group	Number of animals (Male Sprague Dawley rats)
1.	Vehicle	8
2.	Paclitaxel (2 mg/kg, i.p.)	8
3.	Combination chemotherapy (Paclitaxel 2 mg/kg, Cisplatin 2 mg/kg, Vincristine 0.5 mg/kg, i.p.)	8
4.	Paclitaxel + Gabapentin 60mg/kg i.p.	8
5.	Combination Chemotherapy + Gabapentin 60mg/kg i.p.	8

2.3.2 Study II

In this study, we have investigated the effect of Bergenin in our newly developed animal model of chemotherapy-induced neuropathic pain. Behavioral responsiveness assays were systematically conducted in rats both before and after the induction of CINP with subsequent assessments at various time intervals following Bergenin treatment. The study comprehensively examines the effects of Bergenin on

both evoked and spontaneous pain responses. Moreover, a detailed mechanistic exploration was undertaken to elucidate the mechanism of action of Bergenin on modulation of TRP channels mediated NR2B signaling in the dorsal root ganglia (DRG) and spinal cord of neuropathic rats. Finally, *in-silico* studies were performed to validate Bergenin binding affinity with different TRP channels. The grouping for the study was as follows:

Table 2.3 Animal grouping to investigate the effect of Bergenin on chemotherapy-induced neuropathic pain in rats

S. No	Group	Number of animals (Male Sprague Dawley rats)
1.	Naive	8
2.	Combination chemotherapy+ Vehicle	8
3.	Combination chemotherapy+ Bergenin 25 mg/kg i.p.	8
4.	Combination chemotherapy+ Bergenin 50 mg/kg i.p.	8
5.	Combination chemotherapy+ Bergenin 100 mg/kg i.p.	8
6.	Combination chemotherapy + Gabapentin 60 mg/kg i.p.	8

In the subsequent experimental phase, we evaluated the effect of Bergenin on blood-spinal cord barrier integrity through the implementation of the Evans blue assay. Additionally, we quantified the mRNA expressions of tight junction proteins including occludin and claudin-5, in the spinal cord of neuropathic rats. The study systematically examined the influence of Bergenin on the expression levels of these tight junction proteins to gain insights into its effects on blood-spinal cord barrier function. The grouping for this set of the study was as follows:

Table 2.4 Animal grouping to study the effect of Bergenin on blood-spinal cord barrier integrity in animal model of CINP in rats

S. No	Group	Number of animals (Male Sprague Dawley rats)
1.	Naive	8
2.	Combination chemotherapy+ Vehicle	8
3.	Combination chemotherapy+ Bergenin 25 mg/kg i.p.	8
4.	Combination chemotherapy+ Bergenin 50 mg/kg i.p.	8
5.	Combination chemotherapy+ Bergenin 100 mg/kg i.p.	8
6.	Combination chemotherapy + Gabapentin 60 mg/kg i.p.	8

Moreover, we investigated the effect of Bergenin on normal pain thresholds in naïve rats by using tail-flick and tail clip tests, with morphine employed as a positive control. These tests were specifically designed to assess hypersensitivity responses to noxious thermal and mechanical stimuli, respectively. The grouping for this set of the study was as follows:

Table 2.5 Animal grouping to study the effect of Bergenin on basal pain response in rats

S. No	Group	Number of animals (Male Sprague Dawley rats)
1.	Naive	8
2.	Naïve+ Bergenin 25 mg/kg i.p.	8
3.	Naïve+ Bergenin 50 mg/kg i.p.	8
4.	Naïve+ Bergenin 100 mg/kg i.p.	8
5.	Naïve+ Gabapentin 60 mg/kg i.p.	8
6.	Naïve+ Morphine 10 mg/kg i.p.	8

2.3.3 Study III

In this study, we intrathecally administered TRPA1 siRNA (at the lumbar L4-L5 vertebra level) to evaluate the efficacy of gene knockdown. Subsequently, we conducted molecular assays, including western blotting and real-time polymerase chain reaction (RT-PCR), to quantify the expression levels of TRPA1 mRNA and protein. The grouping for this set of the study was as follows:

Table 2.6 Animal grouping for the validation of TRPA1-siRNA knockdown efficacy in rats

S. No	Group	Number of animals (Male Sprague Dawley rats)
1.	Naïve +Scramble siRNA(i.t.)	8
2.	Naïve +TRPA1 siRNA (i.t.)	8

Following the successful *in-vivo* validation of TRPA1 siRNA, we proceeded to formulate and develop a liposomal-based nano-formulation. This nano-formulation, encapsulated within liposomes synthetic carriers characterized by a lipid bilayer resembling cellular membranes was designed for the efficient delivery of TRPA1-siRNA, specifically targeting the treatment of chemotherapy-induced neuropathic pain. Following the successful development of the TRPA1 siRNA nano-formulation, we systematically assessed its therapeutic efficacy through both intrathecal and intravenous administration in neuropathic rats. Our evaluation included comprehensive pain behavioral assays, specifically focusing on mechanical allodynia and cold allodynia in neuropathic rats. Furthermore, we conducted central nervous system (CNS) toxicity tests, such as assessments of locomotor and exploratory activities, to ensure the safety and efficacy of the formulated treatment. The grouping for this set of the study was as follows:

Table 2.7 Animal grouping to study the efficacy and safety of TRPA1-siRNA liposomal formulation in neuropathic rats

S. No	Group	Number of animals (Male Sprague Dawley rats)
1.	Naïve	8
2.	CINP + Scramble siRNA (i.t.)	8
3.	CINP+ Plain siRNA (i.v.)	8
4.	CINP+ Blank formulation (i.v.)	8
5.	CINP+ Blank formulation (i.t.)	8
6.	CINP+ Plain siRNA (i.t.)	8
7.	CINP+ Liposome formulation (i.t.)	8
8.	CINP+ Liposome formulation (i.v.)	8