CHAPTER 7: Summary & Conclusions

7.1 Summary

Chemotherapy-induced neuropathic pain is a major side effect associated with antineoplastic agents, impacting a substantial percentage of patients, ranging from 19% to over 85%. This condition arises due to damage to structures within the somatosensory nervous system, resulting in diverse neuropathies. Classical symptoms including numbness, tingling, altered touch sensation, and various forms of pain. The pathophysiology of CINP involves the stimulation of nociceptors, leading to changes in electrical activity within the peripheral and central nervous systems. Chemotherapy-induced damage triggers cascades of reactive oxygen species, mitochondrial dysfunction, inflammation, and activation of ion channels, collectively contributing to the development of painful neuropathy. Activation of glial cells and infiltration of leukocytes into dorsal root ganglions exacerbate inflammation and promoting peripheral sensitization. Transient receptor potential (TRP) channels, particularly TRPA1, TRPV1, and TRPM8, play a significant role in progression and transmission of pain processing. Their activation releases neuropeptides such as calcitonin gene-related peptide (CGRP) and substance P, leading to neuro-inflammation and T cell recruitment. N-methyl-D-aspartate receptors (NMDARs), especially those with the NR2B subunit, contribute to CINP development and maintenance by facilitating central sensitization.

In spite extensive efforts to develop new therapeutics for treating CINP, the translation of these treatments into clinical practice faces significant challenges,

resulting in limited success rates. One prominent obstacle is the lack of a robust animal model that faithfully replicates the complexities of CINP during preclinical evaluation. The commonly used mono-chemotherapy model, focusing on drugs like paclitaxel, cisplatin etc. has been the standard for decades in pain research. However, in clinics, antineoplastic agents are prescribing in a combination of two to three drugs such as paclitaxel/platinum/vincristine, which has shown significant success in phase III clinical trials for various advanced carcinomas.

To address this disparity, we hypothesized and established a new animal model, utilizing a combination of three frequently prescribed chemotherapeutic drugs (paclitaxel, cisplatin, and vincristine). This novel animal model was compared systematically with the conventional paclitaxel monotherapy model in rats. The combination chemotherapy model exhibited marked behavioral and molecular changes, closely resembling the signs and symptoms observed in patients with CINP. Behavioral studies revealed that rats treated with combination chemotherapeutic displayed prolonged and heightened pain responsiveness to thermal and mechanical stimuli for up to the 8^{th} week post-last injection. In contrast, rats treated with paclitaxel alone showed pain hypersensitivity for only up to the 5^{th} week post-last injection. Molecular analysis revealed upregulation of TRP channels, pro-inflammatory cytokines, and neuropeptides in both models. Notably, the combined chemotherapy model showed increased expression of NR2B in the dorsal root ganglia and spinal cord compared to paclitaxel alone. Gabapentin treatment effectively mitigated pain hypersensitivity in both groups, reversing cellular and molecular changes. The combined chemotherapy model, with heightened and prolonged hypersensitivity, mimics clinical biomarkers and provides a potential platform for screening analgesic drugs targeting CINP. Our results

suggest that this newly developed combined chemotherapy model successfully recapitulates clinical features associated with CINP, exhibiting characteristic markers relevant to CINP including TRP channels, the NR2B subunit of NMDA receptors, neuropeptides, neuro-inflammatory markers, and reactive oxygen species. This model holds promise for evaluating potential CINP treatments and advancing our understanding of the underlying neurobiological mechanisms, providing a more effective platform for drug discovery in the field of CINP.

Unfortunately, to date there are no US-FDA approved drugs or effective treatments available and currently available therapeutics in the clinic do not produce effective analgesia and are coupled with several severe side effects. Therefore, there is an unmet need for the discovery of novel analgesics for the treatment of CINP devoid of classical side effects associated with opioids and other non-opioid drugs. Members of the TRP family are implicated in CINP, with chemotherapy treatment increasing TRPV1, TRPA1, and TRPM8 mRNA expression in cultured DRG neurons. Bergenin, known for its anti-nociceptive, anti-inflammatory properties and neuroprotective potential. Therefore, in the in the second study designed with an aim to investigate the effect of Bergenin on CINP and dissect the underlying cellular and molecular mechanisms. We have found that Bergenin demonstrated significant and dose-dependent attenuation of mechanical and thermal pain-like behavior in chemotherapy-treated rats. It also alleviated cold allodynia but showed no significant effect on cold hyperalgesia. Bergenin did not interfere acute pain responses in healthy rats, suggesting potential application in CINP. Importantly, Bergenin and gabapentin elicited place preference behavior in neuropathic rats. However, no such preference behavior was observed in healthy rats, suggesting a lack of addictive potential for these analgesic

agents. However, morphine treatment leads to the development of significant place preference behavior in rats irrespective of their CINP state confirming its analgesia coupled with addictive potential.

Molecular findings revealed Bergenin's selective modulation of TRP channels and downregulation of NR2B expression in DRG and spinal cord tissues. Bergenin maintained blood-spinal cord barrier integrity, preventing immune cell infiltration and cytokine-mediated damage. Biochemical assays showed Bergenin's effectiveness in lowering oxidative stress markers and restoring GSH levels in sciatic nerves of neuropathic rats. *In-silico* investigations suggested Bergenin's robust binding affinity with TRPA1 and TRPV1, indicating its potential as a natural compound for CINP treatment. However, further research and clinical trials are essential to comprehensively evaluate Bergenin's safety and efficacy in addressing chemotherapy-induced neuropathic pain.

The clinical significance of TRPA1 in pain patients is well recognized Interestingly, our study corroborated the elevated levels of TRPA1 following the administration of combined chemotherapy in rats. Recently, small interfering siRNA emerges as a powerful tool to silence the gene expression by neutralizing targeted mRNA molecules and it has become more popular in the application of targeted interference for pain regulation. In 2018, the FDA granted approval for RNAi-based therapeutics in clinical applications, significantly shaping research and market perspectives towards the exploration of RNAi-based gene therapy for clinical utility. The effective delivery of siRNA poses a considerable challenge due to the risk of degradation and the potential for triggering undesired immunogenic responses, factors that could compromise the overall efficacy of siRNA. Liposomes, synthetic carriers

with a lipid bilayer resembling cellular membranes, provide an effective means for delivering encapsulated therapeutics while protecting them from premature degradation.

In this study, we propose the design and evaluation of a nano-formulation incorporating TRPA1-siRNA for the purpose of treating chemotherapy-induced neuropathic pain (CINP). Initially, we validated the efficacy of TRPA1 siRNA in rats, employing mRNA and protein expression analyses. Results indicated a successful knockdown of TRPA1 in the spinal cord subsequent to intrathecal administration of TRPA1-siRNA. To ensure the specificity of the injection, we meticulously assessed the expressions of trpa1 miRNA and protein in the dorsal root ganglion (DRG). Notably, we observed no significant changes of TRPA1 gene expressions, affirming the specificity of the administered siRNA.

Subsequently, we developed a liposomal nano-formulation of TRPA1-siRNA, subjecting it to comprehensive characterization through various techniques, including dynamic light scattering (DLS), atomic force microscopy (AFM), scanning electron microscopy (SEM), transmission electron microscopy (TEM), and other advanced analytical methods. Addressing a critical concern associated with the administration route of plain siRNA, which conventionally involves intrathecal injection, we pioneered the utilization of an intravenous route for the delivery of the liposomal TRPA1-siRNA formulation for CINP. This novel approach was chosen to assess the therapeutic effect of the developed formulation on alleviating CINP symptoms while also elucidating the underlying cellular and molecular mechanisms. Behavioral studies conducted revealed a noteworthy reduction in mechanical and cold hypersensitivity in neuropathic rats treated with TRPA1-siRNA-loaded nanoparticles, administered both

intrathecal and intravenously. Molecular analyses further unveiled that our formulated treatment mitigates neuropathic pain through TRPA1-mediated PERK inhibition and subsequently suppression of microglial activation and neuroinflammation in the DRG and spinal cord of the rats. In summary, our findings highlight the promising potential of the liposomal TRPA1-siRNA formulation, delivered intravenously, in ameliorating CINP symptoms. The observed behavioral improvements are supported by a detailed understanding of the associated molecular and cellular mechanisms, shedding light on a novel therapeutic avenue for managing chemotherapy-induced neuropathic pain. The outcomes of this study hold promise for the development of innovative therapeutics targeting chemotherapy-induced neuropathic pain, with the potential to mitigate side effects and toxicities. This knowledge forms a foundational basis for future investigations into the application of siRNA-nanomedicine targeting TRP channels for the treatment of chronic pain and other neurodegenerative disorders.

7.2 Conclusion

In summary, our studies introduce a novel, comprehensive approach to addressing CINP. The development of a combined chemotherapy model, utilizing paclitaxel, cisplatin, and vincristine, faithfully recapitulates behavioral and molecular changes akin to the clinical manifestations of CINP. Notably, the model encompasses key indicators of chronic pain, including TRP channels and the NR2B subunit of NMDA receptors, thereby providing a robust platform for evaluating potential therapeutic interventions and deepening our understanding of the neurobiological underpinnings of CINP. In the pursuit of novel analgesics for CINP, Bergenin, identified as a promising phytochemical, demonstrated significant and dose-dependent mitigation of pain-like behavior in chemotherapy-treated rats. Bergenin's non-addictive

analgesic potential is significant, suggesting its suitability for application in CINP management without precipitating severe side effects. Expanding the therapeutic paradigm, this study delves into RNA interference (RNAi) as a potent tool for targeted gene therapy in CINP. Through the utilization of a liposomal nanoformulation loaded with TRPA1-siRNA, the study successfully achieved TRPA1 knockdown, resulting in a marked alleviation of mechanical and cold hypersensitivity in neuropathic rats. This innovative approach holds promise as a viable avenue for the development of novel therapeutics for CINP, addressing existing limitations associated with current treatment modalities. In overarching terms, the multifaceted strategy encompassing the establishment of a reliable animal model, exploration of natural compounds such as Bergenin, and the application of RNAi for targeted gene therapy emerges as a pivotal advancement in the treatment landscape for CINP.

7.3 Limitations and outlook for future work

The present study furnishes crucial preliminary data and insightful observations that pave the way for future investigations in the development of innovative therapeutics for CINP. The findings from this study form a foundational basis for subsequent research endeavors delving into novel TRP channels implicated in the progression of pain. This knowledge is integral for the development of pharmacotherapies targeting CINP, aiming to mitigate pain without inducing significant side effects. In the context of CINP related research, numerous avenues beckon future exploration. Firstly, the refinement and diversification of animal models are paramount. While the combined chemotherapy model shows promise and could be useful to screening a new molecule for the treatment of the CINP but tumor injections could heighten its specificity and relevance to human CINP experiences. Given the challenging nature of treating established CIPN, prevention is of high clinical

significance, especially considering the scheduled nature of chemotherapy. Future studies could explore whether pre-treatment or co-treatment with specific drugs could prevent the onset of CIPN in this new model.

Our research framework proposes the exploration of Bergenin for its therapeutic potential at low doses, specifically targeting TRP channel-mediated regulation of nociceptors. Notably, the single-dose administration of Bergenin in our present work revealed no acute toxicity, central nervous system (CNS) toxicity, or addictive potential. However, comprehensive safety profiling, particularly with repeated dosing in preclinical settings, is imperative before advancing Bergenin towards drug development.

Addressing a major limitation observed in preclinical and clinical setups involving TRPA1 antagonists and their potential side effects, our study introduces a specific gene silencing formulation as an alternative therapeutic approach. Future investigations should elucidate the detailed interaction of this formulation with cellular systems, potentially employing intricate functional assays for Bergenin to enhance our understanding of its molecular intricacies with TRP channels. Simultaneously, the promising avenue of RNA interference (RNAi) for targeted gene therapy in CINP warrants focused efforts on refining siRNA delivery systems, addressing challenges related to stability, efficacy, and potential immunogenic responses. The exploration of alternative delivery methods and optimization of formulations stands to enhance the sustained efficacy of siRNA in clinical applications. Crucially, long-term safety studies for Bergenin and potential siRNA-based therapeutics are imperative prerequisites for clinical translation. This multifaceted approach holds significant promise to advance our understanding of CINP and facilitate the development of targeted, effective therapeutic interventions for individuals grappling with CINP.