Chapter-5

Modulation of KIF-17/NR2B Crosstalk by Tozasertib in Inflammatory Pain Rat Model

5.1 Introduction

Chronic pain may consist of both neuropathic and inflammatory components, involving complex mechanisms such as excitatory synaptic transmission, microglial and macrophage activation, altered action potential in nociceptive fibers, and central as well as peripheral sensitization [145,182]. Release of various inflammatory mediators from the tissue injury site or activated immune system occurs in chronic pain condition that simulates the nociceptors and manipulate the central projections involved in pain processing. The complex pathophysiological mechanisms and lack of potential druggable targets are the key factors that put a substantial barrier to analgesic drug development [183]. Moreover, currently available analgesics fail to treat chronic pain adequately and produce several side effects that lead to treatment withdrawal and poor quality of life [85,184]. Opioids are one of the most commonly prescribed medicines for the treatment of chronic pain but they carry potential side effects including drug addiction, respiratory depression, hypotension, sleep apnea and constipation [85,182]. Non-steroidal anti-inflammatory drugs are the first line of therapy for the treatment of chronic inflammatory pain but their contraindications and adverse effects along with drug-drug interaction are the key limiting factors putting them on the back-foot [4,185].

Recent studies from our and other labs suggest the role of kinesins in nociceptive response and trafficking of the cargo loaded with receptors that are involved in pain pathophysiology [53,125,145,186]. A member of the kinesin

superfamily, KIF17, is localized in somata and dendrites of neurons and plays an important role in the trafficking of the NR2B subunit of the N-methyl-D-aspartate receptor (NMDAR) system [145]. This NR2B subunit is crucial for the functionalization of NMDARs on the synaptic membrane and is observed to be upregulated in chronic pain conditions [101]. The activation of NMDARs causes central sensitization in the spinal cord whereas in DRG they initiate various downstream signaling cascades that promote the pathophysiology of chronic pain [126,187–189]. Recent report suggest that glutamate is present in the DRG leading to the retrograde sensitization in DRG itself [190]. Further, it has been observed that glutamate released in DRG activates NMDARs present on glial cells [189]. Thus, DRG residing NR2B subunit also plays critical role in development of peripheral hyperalgesia. NMDAR induced excitotoxicity is sensed by the microglia which further causes the release of inflammatory cytokines which is one of the key mechanisms involved in pain hypersensitivities [191,192]. KIF17 comprises the tail domain that binds to the PDX domain of scaffolding protein mLin-10 which attaches to the large scaffolding cargo complex consisting of NR2B. Finally, the whole complex binds to the microtubule tracks and the cargo are delivered to the surface of neurons. Inflammation is known to induce the NR2B subunit expression in the central nervous system (CNS) and alter the NMDARs mediated behavioral sensitization [193,194]. Findings by Liu *et al.* (2015) suggest that KIF17-NR2B expressions were significantly increased during bone cancer pain and drive behavioral hypersensitivities. A recent report has demonstrated that downregulation of KIF17 by using shRNA targeting activating transcription factor 4 has led to the regulation of nociceptive response [145]. Aurora kinase is a novel serine/threonine class of enzymes that regulate the distribution

of kinesin nanomotors across the microtubule track [195]. In our previous studies we found that inhibition of pan aurora kinase attenuates the nerve injury- induced chronic pain in animal model of neuropathic pain [140].

Therefore, we hypothesized that chronic inflammatory pain may lead to the activation of KIF17-NR2B signaling which will cause the release of pro-inflammatory cytokines along with activation of macrophage and microglia in DRG and spinal cord (respectively) of rats followed by central sensitization. In the present study, we investigated the effect of tozasertib, a pan aurora kinase inhibitor on pain hypersensitivity in animal models of acute and chronic inflammatory pain along with dissecting the cellular and molecular mechanisms involved in the same.

5.2 Experimental design

To investigate the effect of tozasertib on acute inflammatory pain condition we used formalin model in rats. Rats were divided into five groups with eight animals each; first group consisted of naïve of healthy rats, second, third and fourth groups consisted formalin + tozasertib (10, 20 and 40mg/kg *i.p.* respectively) and fifth group was formalin + diclofenac (10mg/kg *i.p.*). The details of formalin model are already discussed under chapter 3. In the acute inflammatory pain model animals were injected with formalin by the intraplantar route and behavioral testing was performed 30 min after the drug administration.

Next, we examined effect of tozasertib on chronic inflammatory pain model, total forty-eight animals were randomized into different groups namely: naïve, inflammatory pain, treatment groups (tozasertib 10, 20 and 40 mg/kg *i.p.* and diclofenac 10mg/kg *i.p.*). The experiment commenced with habituation in the laboratory room

followed by acclimatization in elevated von-Frey mesh and Hargreaves apparatus. Baselines for evoked pain behavior in rats were measured before complete Freund's adjuvant (CFA) administration. The drug treatment was given day 4 post intraplantar CFA injection and behavioral testing were performed at 0.5, 1, 2, 4, and 24 hrs after the drug and vehicle treatment. After behavioral testing rats were sacrificed and tissues were harvested including sciatic nerve, DRG and spinal cord. Samples were stored in cryobox in -80°C until further processing for biochemical and mRNA and protein expression studies.

5.3 Results and discussion

5.3.1 Tozasertib attenuates only the second phase of formalin-induced inflammatory pain

The inflammatory response to formalin and CFA are well-established models in pain research for testing analgesics and identifying the novel pathways associated with chronic inflammatory pain [142,143]. The outcome measure in the formalin test is marked flinching behavior in rats that increases post-formalin administration [142]. Formalin-induced acute inflammation is characterized by two phases namely phase I and phase II. The first phase is characterized by the direct activation of nociceptors whereas activation of the downstream singling is the classical feature of the second phase. There was a significant effect across the groups on flinching per minute observed in two-way ANOVA model followed by the Bonferroni's multiple comparison ($p<0.0001$; $F(4, 35) = 60.5$) (figure 5.1). Tozasertib did not affect the first phase of the acute inflammation however the standard drug diclofenac (10mg/kg *i.p.*) significantly ($p<0.0001$) attenuated the ipsilateral paw flinching post formalin administration.

Further, we have observed a significant decrease in paw flinching during second phase of acute inflammation post tozasertib treatment (10, 20 and 40mg/kg *i.p.*) ($p<0.0001$). Diclofenac (10mg/kg *i.p.*) has also rescued the acute inflammation in second phase and decreased the ipsilateral paw flinching in rats ($p<0.0001$). Our results are in line with the reports suggesting a marked increase in flinching response in the ipsilateral paw of formalin injected rats which was significantly attenuated in both phase 1 and phase 2 after diclofenac administration [196]. Interestingly, we found that pan aurora kinase inhibition by tozasertib selectively attenuates only phase 2 of formalin-induced acute pain in rats. These findings suggest that tozasertib may not have any direct effect on nociceptors but might interfere with secondary mediators associated with downstream of post-formalin pain.

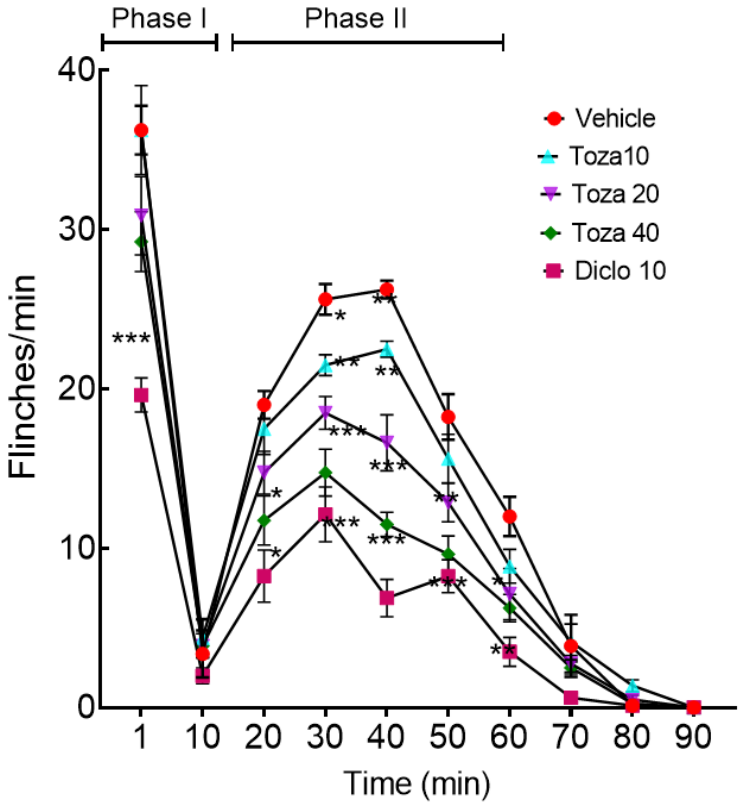

Figure 5.1 Effect of tozasertib on formalin induced acute inflammatory pain. Tozasertib (10, 20 and 40mg/kg *i.p.*) treatment significantly attenuated formalin-

induced flinching behavior in rats during secondary phase of acute inflammatory pain. (n=8/group). Data were presented as mean ± SEM. A two-way ANOVA followed by Bonferroni's multiple comparison test was used. $^{###}P<0.001$ indicates statistical significance as compared to the Naïve rats. *p<0.05, **p<0.01, and ***p<0.001 indicates statistical significance as compared to the nerve injured rats. P<0.05 was considered statistically significant. Doses: Toza10, Tozasertib 10mg/kg; Toza 20, Tozasertib 20 mg/kg; Toza40, Tozasertib 40 mg/kg; Diclo 10, Diclofenac 10mg/kg; CFA, Complete Freund's Adjuvant.

5.3.2 Effect of tozasertib on thermal, mechanical and cold pain hypersensitivities in CFA injected rats

5.3.2.1 Pan aurora kinase inhibition decreases heat hyperalgesia in CFA injected rats

Complete Freund's adjuvant-induced pain was used as a model of chronic inflammatory pain for investigating the effect of pan aurora kinase inhibition on the same. The intraplantar injection of CFA lead to the significant and time-dependent development of hyperalgesia and allodynia in rats [143]. To test the effect of tozasertib in chronic inflammatory pain we have studied nociceptive response to a variety of stimuli in CFA injected rats. Increased pain from the thermal stimulus that normally provokes pain is known as hyperalgesia which is the primary feature of chronic pain. We assessed the heat hyperalgesia in rats using Hargreaves apparatus and paw withdrawal responses were recorded as the outcome measure. There was a significant effect of CFA injection on heat-induced hyperalgesia as evident in two-way ANOVA test followed by Bonferroni's multiple comparisons on ipsilateral paw withdrawal ($p<0.0001$; $F(5, 42) = 134$). CFA injection decreased the withdrawal latency in ipsilateral paw of rats as compared to their pre-CFA baseline, contralateral recordings and naïve group rats ($p<0.0001$) (Figure 5.2A). Treatment with tozasertib (10, 20 and 40 mg/kg *i.p.*) significantly attenuates the heat hyperalgesia in ipsilateral paw of rats at

1 hr (p=0.0353, p<0.0001 and p<0.0001 respectively), 2 hr (p=0.0046, p=0.0007 and p<0.0001 respectively) and 4 hr (p=0.0316, p=0.0022 and p=0.0075 respectively) as compared to the pre-drug baselines and CFA injected rats. Standard drug diclofenac (10mg/kg *i.p.*) also attenuated thermal hyperalgesia in CFA injected rats at 0.5 hr (p=0.0013), 1 hr (p<0.0001), 2 hr (p<0.0001) and 4 hr (p=0.0089) post administration as compared to the CFA injected rats. Contralateral paw did not show any significant difference in paw withdrawal latency before and after drug treatment (Figure 5.2B).

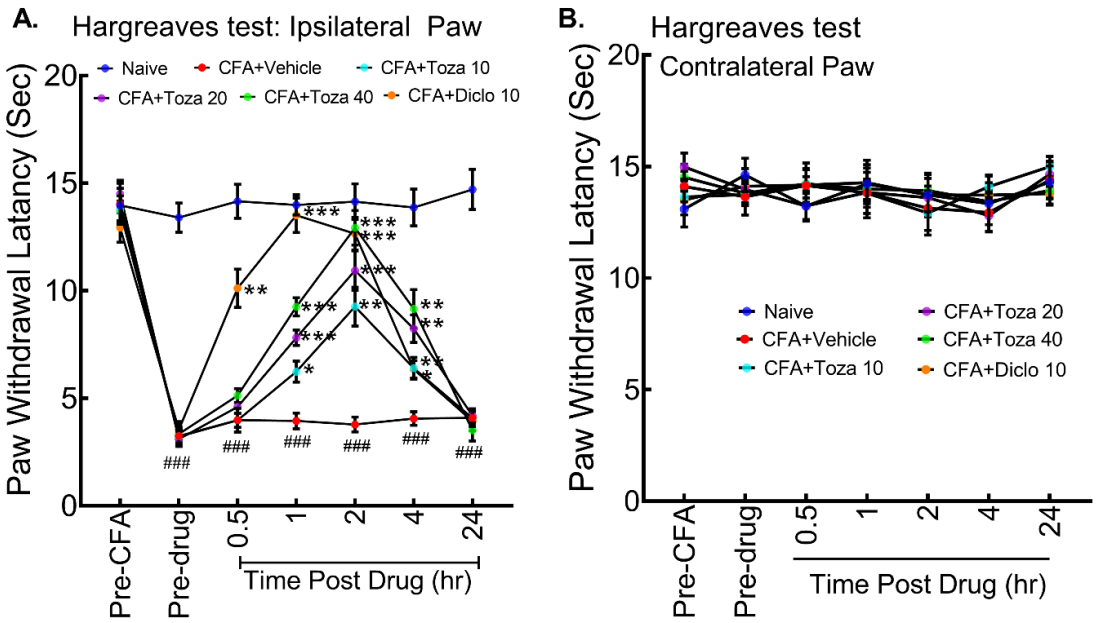

Figure 5.2 Effect of pan aurora kinase inhibition on CFA-induced thermal hyperalgesia in rats. Hargreaves test: (A) Tozasertib (10, 20 and 40 mg/kg *i.p*) and diclofenac (10mg/kg *i.p*) administration resulted in a significant increase in PWL of CFA injected rats as compared to their pre-drug baseline. **(B)** Contralateral PWL showed no significant differences between the groups. A two-way ANOVA followed by Bonferroni's multiple comparison test was used. Data were presented as mean ± SEM. ###P<0.001 indicates statistical significance as compared to the Naïve rats. *p<0.05, **p<0.01, and ***p<0.001 indicates statistical significance as compared to the nerve injured rats. P<0.05 was considered statistically significant. Doses: Toza10, Tozasertib 10mg/kg; Toza 20, Tozasertib 20 mg/kg; Toza40, Tozasertib 40 mg/kg; Diclo 10, Diclofenac 10mg/kg; CFA, Complete Freund's Adjuvant.

5.3.2.2 Tozasertib attenuates cold-hyperalgesia in CFA injected rats

Hyperalgesia to cold stimulus is well evident under various chronic pain conditions including inflammatory pain. Two-way ANOVA followed by Bonferroni's multiple comparison test demonstrated a significant ($p<0.0001$) effect across the groups on number of ipsilateral paw lifts (Figure 5.3A). CFA injected rats exhibited cold stimuli induced hyperalgesia in ipsilateral paws as compared to the respective contralateral recordings and naïve rats. Pan aurora kinase inhibition by tozasertib (10, 20 and 40 mg/kg *i.p.*) significantly reduces number of paw lifts in CFA injected-

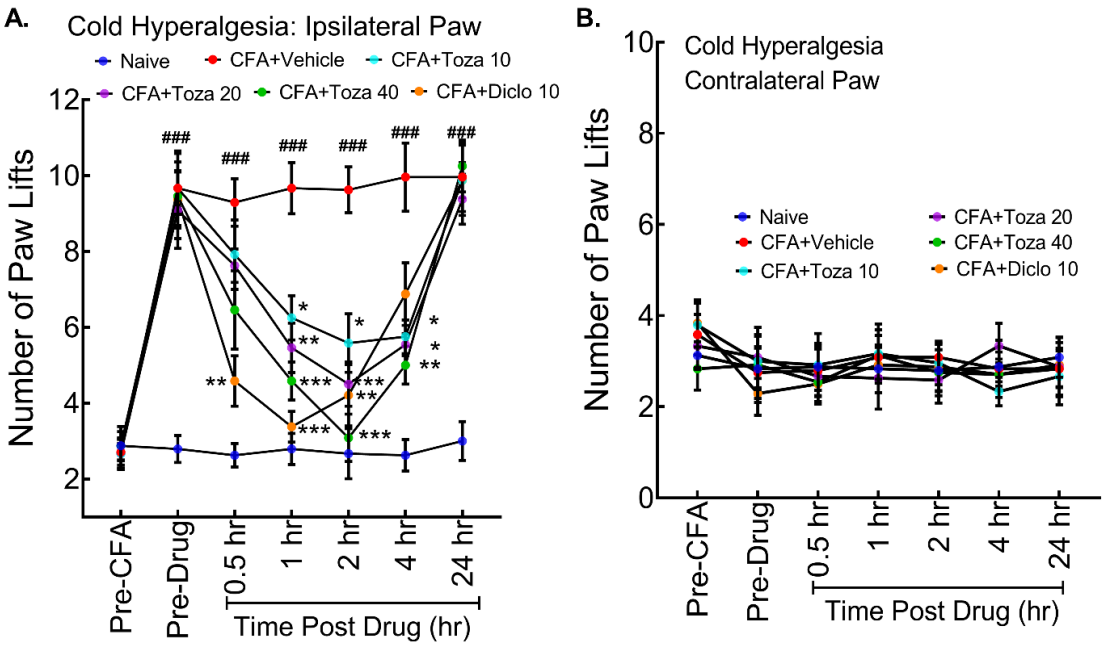

Figure 5.3 Effect of pan aurora kinase inhibition on CFA-induced cold hyperalgesia in rats. Cold hyperalgesia test: (E) CFA induced cold hyperalgesia was significantly decreased by tozasertib (10, 20, and 40 mg/kg *i.p*) and diclofenac (10mg/kg *i.p*). **(F)** Contralateral paw has no significant effect on cold induced hyperalgesia. (n=8/group). A two-way ANOVA followed by Bonferroni's multiple comparison test was used. Data were presented as mean ± SEM. $^{###}P<0.001$ indicates statistical significance as compared to the Naïve rats. *p<0.05, **p<0.01, and ***p<0.001 indicates statistical significance as compared to the nerve injured rats. P<0.05 was considered statistically significant. Doses: Toza10, Tozasertib 10mg/kg; Toza 20, Tozasertib 20 mg/kg; Toza40, Tozasertib 40 mg/kg; Diclo 10, Diclofenac 10mg/kg; CFA, Complete Freund's Adjuvant.

-rats as compared to the vehicle treated rats post 1 hr ($p=0.0287$, $p=0.0077$ and $p=0.0006$ respectively), 2 hr ($p=0.0171$, $p=0.0004$ and $p<0.0001$ respectively) and 4 hr ($p=0.0266$, $p=0.0210$ and $p=0.0083$ respectively). These results suggest the anti-hyperalgesic effect of pan aurora kinase inhibition in chronic inflammatory pain rat model. Moreover, standard drug diclofenac (10mg/kg i.p.) also leads to significant decrease in cold hyperalgesia at 0.5 hr ($p=0.0022$), 1 hr ($p<0.0001$) and 2 hr ($p<0.0020$) post drug administration.

5.3.2.3 Pan aurora kinase inhibition reduced CFA induced mechanical hyperalgesia in rats

Hyperalgesia due to the noxious mechanical stimuli is another important symptom of chronic pain, thus we examined the same in rats using pin prick test. We observed a significant ($p<0.0001$; $F(5, 42) = 91.1$) effect across the groups on paw withdrawal frequency as analysed by two-way ANOVA test followed by Bonferroni's multiple comparison test (Figure 5.4A). Rats administered with intra planter CFA showed significant ($p<0.0001$) increase in ipsilateral paw withdrawal frequency as compared to their pre-CFA baselines, respective contralateral paw recordings, and control group rats (Figure 5.4A). these results indicates that, the intraplantar injection of CFA lead to the development of mechanical hypersensitivity in rats.

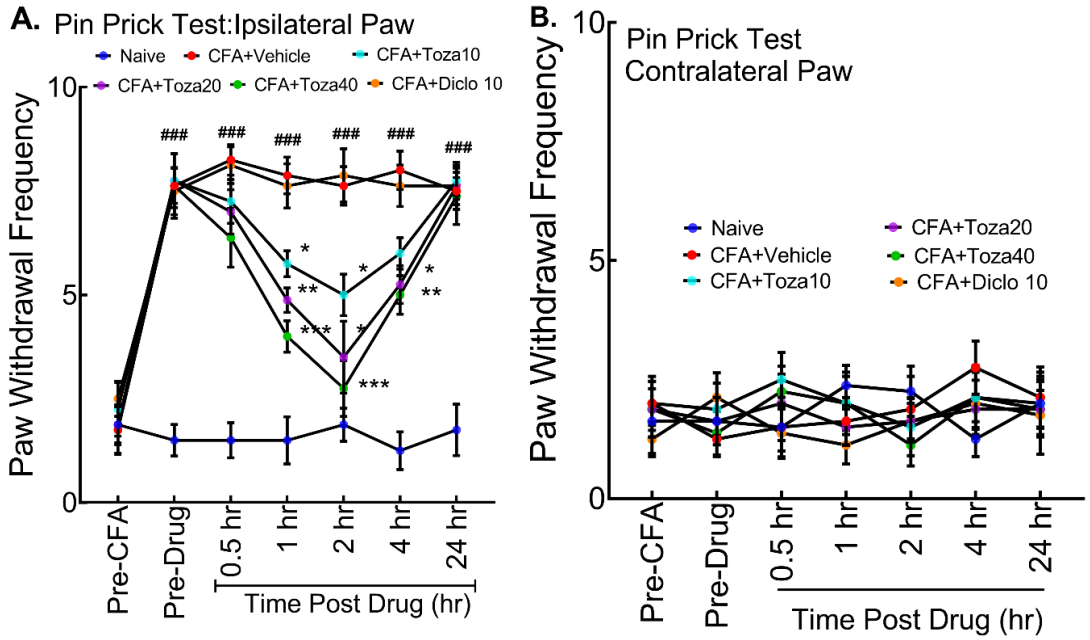

Figure 5.4 Effect of pan aurora kinase inhibition on CFA-induced mechanical hyperalgesia in rats. Pinprick test: (C) CFA-injection resulted in increased paw withdrawal frequency (PWF) to noxious mechanical stimuli in rats. Tozasertib (10, 20, and 40 mg/kg *i.p*) treatment significantly decreased the mechanical hyperalgesia in CFA injected rats. **(D)** Contralateral paw withdrawal frequency was not significantly altered in different groups.

Treatment with tozasertib (10, 20 and 40mg/kg i.p.) significantly attenuated the mechanical hyperalgesia in chronic inflammatory rat model as evident by significant decrease in paw withdrawal frequency at 1 hr (p=0.0273, p=0.0015 and p=0.0002 respectively), 2 hr (p=0.0262, P=0.0236 and p=0.0009) and 4 hr (non-significant, p=0.0122 and p=0.0064 respectively) post drug administration. However, diclofenac treatment showed no significant effect on mechanical hyperalgesia in CFA injected rats. Contralateral paw response was not significantly altered in any of the groups (Figure 5.4B).

5.3.2.4 Tozasertib attenuates mechanical allodynia in CFA injected rats

Chronic pain is accompanied by the lower pain threshold to the innocuous stimuli, hence we performed evaluation of allodynia using mechanical and thermal stimulus. Two-way ANOVA followed by Bonferroni's multiple comparisons-

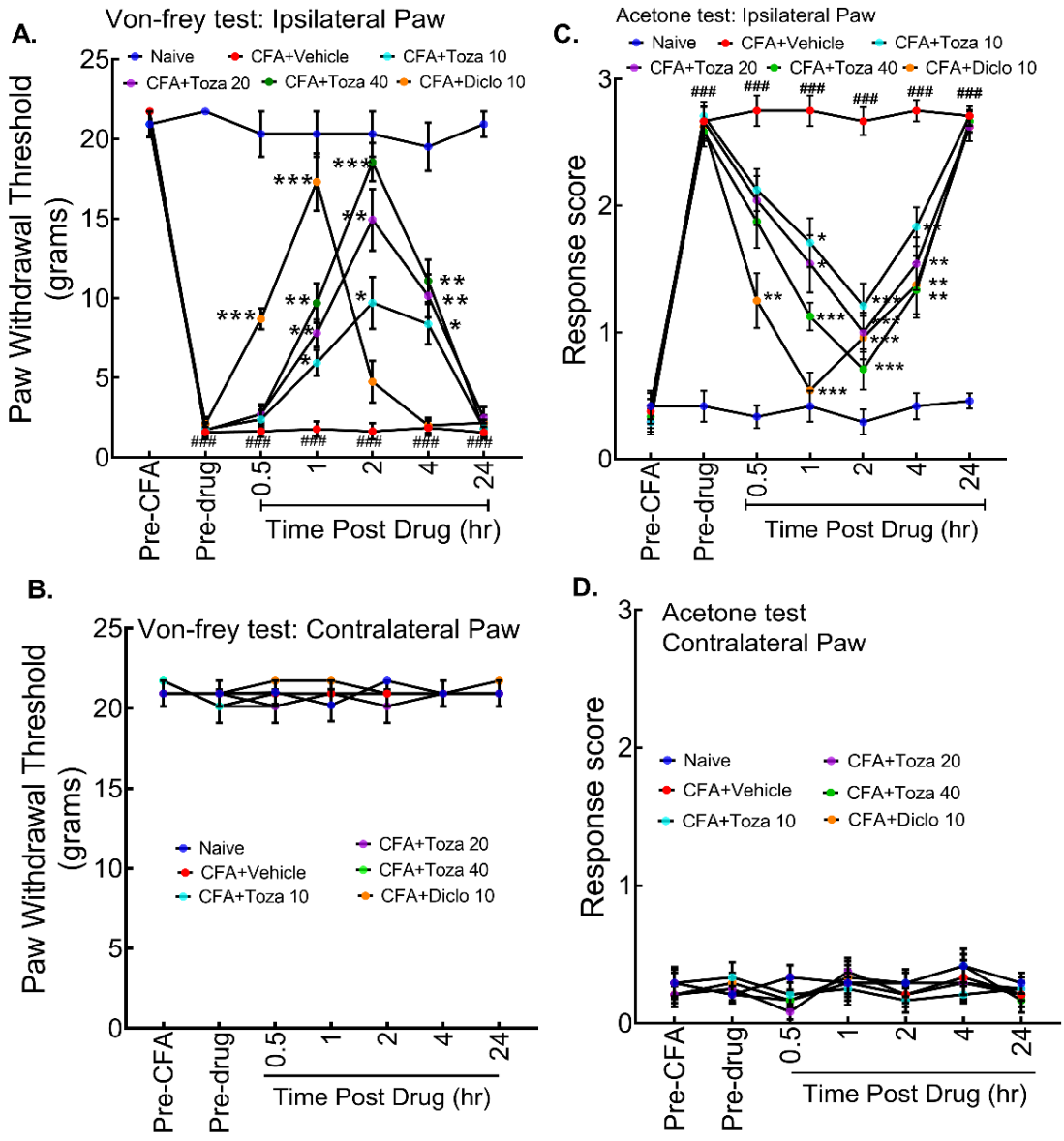

Figure 5.5 Effect of tozasertib on CFA induced mechanical and thermal allodynia in rats: von-Frey hair test: (A) Tozasertib (10, 20 and 40 mg/kg *i.p*) and diclofenac (10mg/kg *i.p.*) treatment inhibits CFA-induced response to non-noxious mechanical stimuli. **(B)** Contralateral paw recording showed no significant effect on paw withdrawal thresholds. **Acetone drop test: (C)** CFA induced cold allodynia was

significantly decreased by tozasertib (10, 20 and 40 mg/kg *i.p*) and diclofenac (10mg/kg *i.p.*) treatment. **(D)** No effect on contralateral paw recordings was observed. (n=8/group). A two-way ANOVA followed by Bonferroni's multiple comparison test was used. Data were presented as mean ± SEM. $^{###}P<0.001$ indicates statistical significance as compared to the Naïve rats. *p<0.05, **p<0.01, and ***p<0.001 indicates statistical significance as compared to the nerve injured rats. P<0.05 was considered statistically significant. Doses: Toza10, Tozasertib 10mg/kg; Toza 20, Tozasertib 20 mg/kg; Toza40, Tozasertib 40 mg/kg; Diclo 10, Diclofenac 10mg/kg; CFA, Complete Freund's Adjuvant.

-demonstrated a significant (p<0.0001; F (5, 42) = 149) effect across the groups on ipsilateral paw-withdrawal threshold in von-Frey hair test. CFA injection induce mechanical allodynia evident by significantly (p<0.0001) decreased ipsilateral paw-withdrawal threshold in rats as compared to the naïve group, pre-CFA baseline, and contralateral paws (Figure 5.5A). Tozasertib administration (10, 20 and 40mg/kg *i.p.)* significantly increased the innocuous stimuli induced ipsilateral paw-withdrawal threshold in CFA injected rats at 1 hr (p=0.0148, p=0.0010 and p=0.0031 respectively), 2 hr (p=0.0195, p=0.0025 and p<0.0001 respectively) and 4 hr (p=0.0141, p=0.0043 and p=0.0019 respectively) post-drug administration as compared to the vehicle treated rats. These findings demonstrate the anti-allodynic effect of tozasertib under CFA induced inflammatory pain condition in rats. Diclofenac (10mg/kg *i.p.*) treatment also attenuated the mechanical allodynia in CFA injected rats at 0.5 hr (p<0.0001) and 1 hr (p=0.0005) post drug-administration as compared to the vehicle treated rats. No effect was observed in the contralateral paw withdrawal threshold across the groups (Figure 4.5B).

5.3.2.5 Tozasertib attenuates cold allodynia in chronic inflammatory pain rat model

Acetone drop test was conducted to study the effect of tozasertib on cold allodynia like behavior in CFA injected rats. Two-way ANOVA followed by Bonferroni's multiple comparison test suggested a significant (p<0.0001; F (5, 42) =

194) effect on the response score of ipsilateral paws across the groups. CFA injection develops cold hypersensitivity indicated by significantly ($p<0.0001$) increased in ipsilateral paw response scores in rats as compared to their respective pre-CFA baselines, contralateral paw scores and naïve group rats (Figure 5.5C). Treatment with tozasertib (10, 20 and 40mg/kg) significantly reduced the response score at 1 hr ($p=0.0104$, $p=0.0105$ and $p<0.0001$ respectively), 2 hr ($p=0.0003$, $p<0.0001$ and $p<0.0001$ respectively) and 4 hr ($p=0.0045$, $p=0.0062$ and $p=0.0028$ respectively) post drug treatment. Moreover, diclofenac (10mg/kg i.p.) treatment also reduced cold allodynia at 0.5 hr ($p=0.0012$), 1 hr ($p<0.0001$) and 2 hr ($p<0.0054$) post-diclofenac administration in CFA injected rats.

5.3.3 Effect of tozasertib on CFA induced biochemical and molecular alterations

5.3.3.1 Tozasertib attenuates oxidative-nitrosative stress in the sciatic nerve of CFA-injected rats

Inflammatory pain is coupled with the activation of oxidative stress pathways and altered antioxidant enzymes activity which harms the neuronal integrity and facilitates pain signal transduction. Moreover, activation of NMDARs also induces oxidative stress, thereby producing synaptic excitotoxicity and apoptotic cascades in glial cells and neighbouring neurons [197]. To identify the involvement of the same, we have measured the oxidative stress profile in the sciatic nerve of CFA injected rats and found a significant increase in MDA and nitrite levels and a decrease in levels of reduced glutathione enzyme. CFA administration was found to enhance the oxido-nitrosative-stress which was attenuated by the treatment with tozasertib. We observed a significant effect across the groups on MDA ($p<0.0001$; $F(5, 18) = 10.9$) and nitrite ($p<0.0001$; $F(5, 18) = 9.19$) levels in one-way ANOVA test followed by Tukey's

multiple comparison. CFA injection significantly increased the MDA ($p<0.0001$) and nitrite ($p=0.0002$) levels in sciatic nerve of rats as compared to the naïve rats (Figure 5.6 A and B). Treatment with tozasertib (10, 20 and 40mg/kg *i.p.*) significantly restored the CFA-induced MDA ($p=0.0235$, $P=0.0027$ and $p=0.0002$ respectively) and nitrite ($p=0.0496$, $p=0.0048$ and $p=0.0003$ respectively) levels in sciatic nerve as compared to the vehicle-treated group. Diclofenac treatment also suppressed the MDA ($p=0.0019$) and nitrite ($p=0.0047$) levels in sciatic nerve of CFA injected as compared to the vehicle-treated rats. Further, one-way ANOVA test followed by Tukey's post hoc analysis suggested a significant effect on the antioxidant enzyme, GSH levels across the groups ($p<0.0001$; $F(5, 18) = 17.6$) (Figure 5.6C). Tozasertib (10, 20 and 40 mg/kg *i.p.*) treatment significantly restored the levels of GSH in the sciatic nerve ($p=0.0180$, $p=0.0026$ and $p<0.0001$ respectively) of CFA injected rats as compared to the vehicle-treated rats. Diclofenac (10mg/kg *i.p.*) treatment also ($p=0.0088$) restored the decreased GSH levels in the sciatic nerve of CFA injected rats (Figure 5.6C).

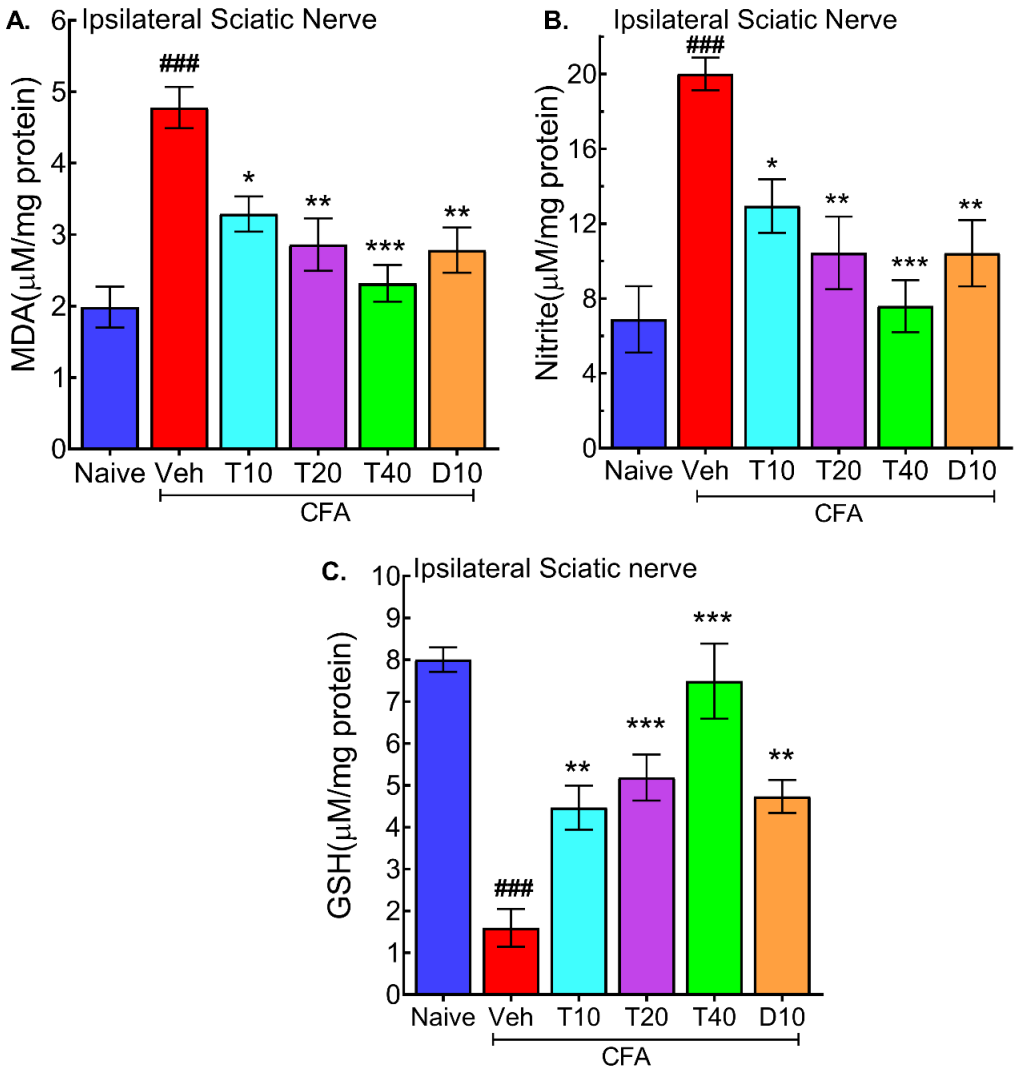

Figure 5.6 Effect of tozasertib on CFA-induced oxido-nitrosative stress in sciatic nerve of rats. (A) MDA: CFA injection induced a significant increase in sciatic nerve of rats which was significantly attenuated by tozasertib (10, 20, and 40 mg/kg *i.p*) and diclofenac (10 mg/kg *i.p*) treatment. **(B) Nitrite:** Tozasertib (10, 20 and 40 mg/kg *i.p*) and diclofenac (10mg/kg *i.p*) treatment significantly attenuates CFA-induced increased nitrite levels in sciatic nerve of rats. **(C) Glutathione:** CFA administration significantly reduced GSH levels in sciatic nerve of rats. Tozasertib (10, 20, and 40 mg/kg *i.p*) and diclofenac (10mg/kg *i.p*) treatment significantly restored GSH levels in sciatic nerve of rats administered with CFA. n=4 biological and n=3 technical replicates. A one-way ANOVA followed by Tukey's multiple comparison test was used. Data were presented as mean ± SEM. $P<0.05$ was considered statistically significant. Doses: Toza10, Tozasertib 10mg/kg; Toza 20, Tozasertib 20 mg/kg; Toza40, Tozasertib 40 mg/kg; Diclo 10, Diclofenac 10mg/kg; CFA, Complete Freund's Adjuvant.

5.3.3.2 Tozasertib inhibits glial cell activation in dorsal root ganglion and spinal cord of CFA injected rats

Neuroinflammation occurs in response to any tissue injury or activated immune system, and stimulates pain signal transduction across the nociceptors and dorsal horn of the spinal cord. Glial cells perform surveillance across the central and peripheral nervous system, but under the chronic pain condition, their morphological and functional characteristics are altered. Inflammation-mediated activation of NMDARs leads to the excitotoxicity in synapse which can be sensed by microglia via the TLR4 receptor system and thus release of various inflammatory cytokines occurs [198,199]. After nerve injury, microglial activation in the dorsal horn of the spinal cord is required for synaptic alterations and pain sensitization [200]. Blockade of NMDARs is found to inhibit the microglial activation and reduce the neuroinflammation, thereby attenuating the pain-like behavior in rats [201]. The previously published reports show that glial cell markers IBA1 and ICAM1 are highly expressed in the carrageenan-induced inflammatory pain model [202,203]. Thus we investigated two classical markers of glial activation and neuroinflammation i.e., IBA1 and ICAM1 in DRG and spinal cord of CFA injected rats. One-way ANOVA followed by Tukey's post-hoc analysis demonstrates a significant effect across the groups ($p<0.0001$) on IBA1 expression in DRG ($p<0.0001$; $F(5, 12) = 20.6$) and spinal cord ($p<0.0001$; $F(5, 12) = 10.0$) tissues. Vehicle administered CFA injected rats showed a significant increase in IBA1 protein expression in DRG ($p<0.0001$) and spinal cord ($p<0.0004$) of rats as compared to the naïve rats. This indicates the involvement of microglial activation and neuroinflammatory signaling in pathophysiology of CFA induced chronic pain.

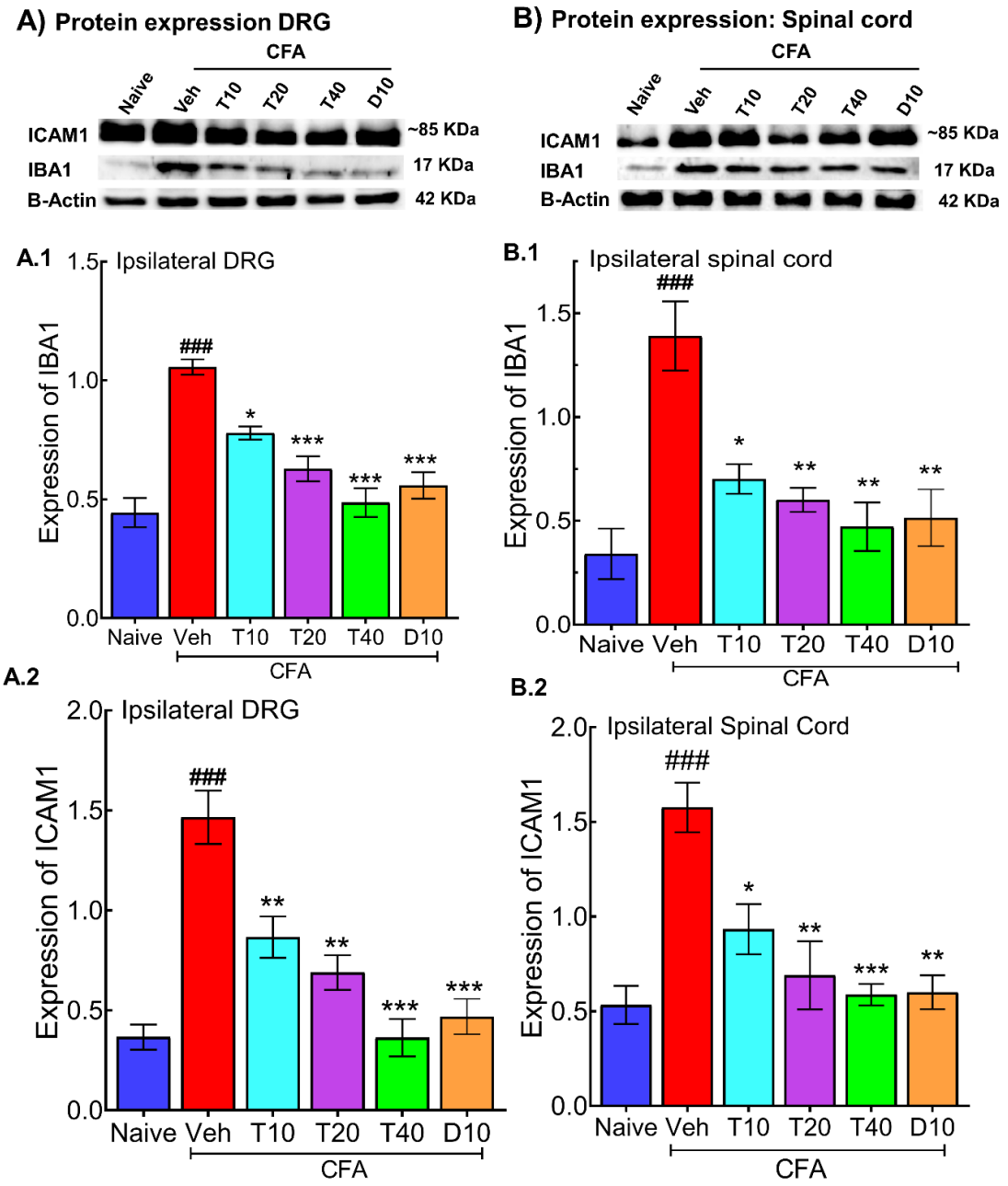

Figure 5.7 Effect of aurora kinase inhibitor on IBA1 and ICAM1 protein expressions in dorsal root ganglion and spinal cord of CFA injected rats. mRNA expressions: CFA injection increased protein expression of IBA1 and ICAM1 in L4-L5 DRG **(A)** and spinal cord **(B)** of rats which was significantly attenuated by tozasertib (10, 20 and 40 mg/kg *i.p.*) and diclofenac (10mg/kg *i.p*) treatment. (n=8/group). A one-way ANOVA followed by Tukey's multiple comparison test was used. Data were presented as mean ± SEM. P<0.05 was considered statistically significant. Doses: Toza10, Tozasertib 10mg/kg; Toza 20, Tozasertib 20 mg/kg; Toza40, Tozasertib 40 mg/kg; Diclo 10, Diclofenac 10mg/kg; CFA, Complete Freund's Adjuvant.

Administration of tozasertib (10, 20 and 40mg/kg *i.p.*) significantly attenuated the IBA1 protein expressions in both DRG ($p=0.0199$, $p=0.0007$ and $p<0.0001$ respectively) and spinal cord ($p=0.0136$, $p=0.0050$ and $p=0.0014$ respectively) of CFA injected rats as compared to the vehicle treated animals (Figure 5.7 A and B). Further investigations suggest a significant reduction in CFA-induced protein expressions of ICAM1 by tozasertib (10, 20, and 40mg/kg *i.p.*) in both DRG ($p=0.0091$, $p=0.0011$ and $p<0.0001$ respectively) and spinal cord ($p=0.0260$, $p=0.0025$ and $p=0.0010$ respectively) as compared to the vehicle treated group. These findings revealed the anti-inflammatory activity of pan aurora kinase inhibitor against CFA induced chronic pain condition. Diclofenac treatment also suppressed the IBA1 and ICAM1 expression in both DRG ($p=0.0002$ and $p=0.0001$ respectively) and spinal cord ($p=0.0022$ and $p=0.0011$ respectively) of rats as compared to the vehicle-treated group.

5.3.3.3 Tozasertib suppressed the KIF17/NR2B/mlin10 expression in dorsal root ganglion and spinal cord of CFA injected rats

NR2B subunit is essential for the functionalization of NMDARs on cell surface which causes the development of central sensitization, a classical feature of chronic pain [187,204]. Whereas, in the peripheral nervous system (dorsal root ganglion) the NR2B subunit is involved in the development of peripheral hyperalgesia and allodynia via regulating a series of cellular events including inflammatory sensitization [126,189]. The role of the NMDA receptor system is well evident in chronic pain pathophysiology, however direct pharmacological blockade of these receptors is coupled with severe side effects as it participates in the basal physiological role including synaptic plasticity [53,169]. Recent findings suggested that an indirect approach targeting NMDARs receptors during synthesis, maturation and trafficking

could provide safer therapeutics with potent analgesia [140,204,205]. Kinesins are the motor proteins that are involved in the trafficking of cargo in different cell types including neurons. KIF17 is a homodimeric kinesin motor protein that belongs to the OSM3/KIF17 family, involved in the trafficking of NR2B subunit of NMDARs from the cytosol to periphery. Moreover, reports suggesting the role of kinesins in the regulation of nociceptive response published in the last decade demonstrates their potential in the treatment of chronic pain disorders [131,145,206–208]. Studies have reported specific kinesin and their respective cargos upregulation such as KIF17/NR2B, KIF3A/NAV1.6 and KIF13B/TRPV1 during chronic pain conditions [125,131,145,165]. Monastrol, an inhibitor of kinesin-5 was found to protect against bortezomib-induced peripheral neuropathy [208]. Protein expression of NR2B and KIF17 in L4-L5 DRG and spinal cord tissues was measured using western blot analysis. We observed a significant effect across the groups in one-way ANOVA followed by Tukey's multiple comparison test on KIF17 and NR2B protein expression in L4-L5 DRG ($p<0.0001$) and L4-L5 spinal cord ($p<0.0001$) tissues (Figure 5.8A and B). CFA injection significantly increased protein expression of KIF17 and NR2B in L4-L5 DRG ($p=0.0007$) and spinal cord ($p<0.0001$) of rats as compared to the naïve group. Administration of tozasertib (10, 20 and 40 mg/kg *i.p.*) significantly attenuates CFA induced expression of KIF17 and NR2B proteins in L4-L5 DRG ($p=0.0136$, $p=0.0151$ and $p=0.0014$ respectively; $p=0.0019$, $p=0.0015$ and $p<0.0001$ respectively) as compared to vehicle treated rats. We further observed a significant decrease - in KIF17 and NR2B protein expression in L4-L5 spinal cord post-tozasertib (10, 20 and 40 mg/kg *i.p.*) administration ($p=0.0159$, $p=0.0060$ and $p=0.0005$ respectively; $p=0.0367$, $p=0.0160$ and $p=0.0018$ respectively) as compared to the vehicle treated rats (Figure 5.8). However, diclofenac (10mg/kg *i.p.*) treatment failed to produce any effect on-

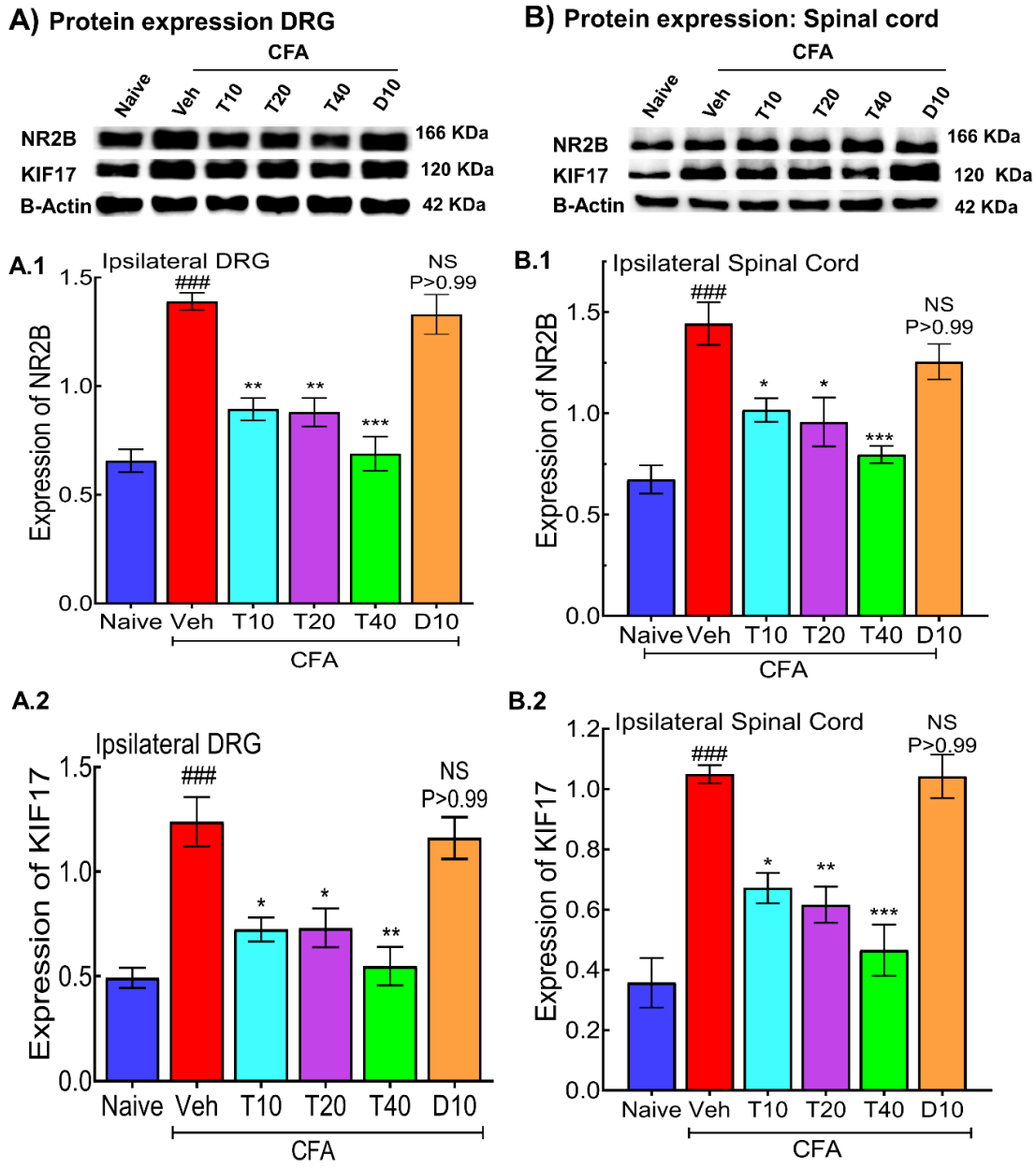

Figure 5.8 Effect of tozasertib treatment on CFA-induced KIF17 and NR2B expressions in DRG and spinal cord of CFA injected rats. (A) DRG protein expression: CFA injection significantly enhances expressions of KIF17 **(A1)** and NR2B **(A2)** in ipsilateral L4-L5 DRG of rats. Tozasertib (10, 20, and 40 mg/kg i.p.) treatment significantly reversed these changes as compared to the CFA injected rats. **(B) Spinal protein expression:** CFA injection significantly increase the ipsilateral L4-L5 spinal expressions of KIF17 **(B1)** and NR2B **(B2)** which was significantly attenuated on treatment with tozasertib (10, 20 and 40 mg/kg *i.p.*). (n=8/group). A one-way ANOVA followed by Tukey's multiple comparison test was used. Data were presented as mean ± SEM. P<0.05 was considered statistically significant. Doses:

Toza10, Tozasertib 10mg/kg; Toza 20, Tozasertib 20 mg/kg; Toza40, Tozasertib 40 mg/kg; Diclo 10, Diclofenac 10mg/kg; CFA, Complete Freund's Adjuvant.

-L4-L5 DRG and spinal cord expression of KIF17 and NR2B as compared to the vehicle treated rats. mLIN10 is a scaffolding protein that facilitates the binding of KIF17 with NR2B subunit, thus we examined the mRNA expressions of mLIN10 in L4-L5 DRG and spinal cord tissues. One-way ANOVA followed by Tukey's multiple comparison suggested a significant effect across the groups. mRNA expression of mLIN10 was significantly increased in L4-L5 DRG ($p<0.0001$) and spinal cord ($p<0.0001$) tissues of CFA injected rats as compared to the naïve rats (Figure 5.9 A and B) which was significantly attenuated on treatment with different doses of tozasertib (10, 20 and 40 mg/kg *i.p.*). However, diclofenac (10mg/kg *i.p.*) treatment did not produce any significant changes in mLIN10 mRNA expressions in L4-L5 DRG and spinal cord of CFA injected rats as compared to the vehicle-treated rats.

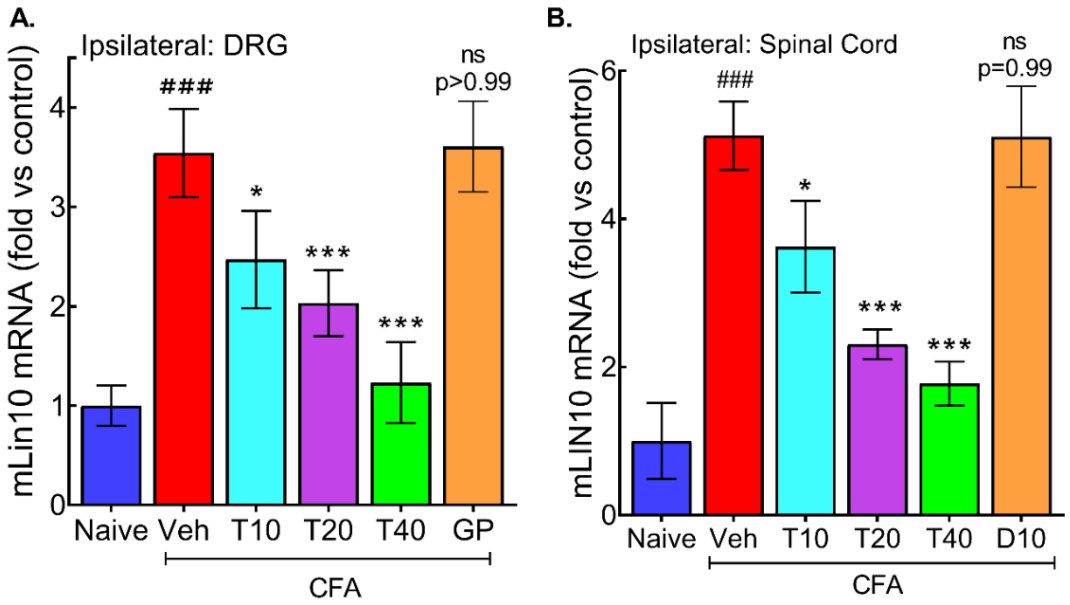

Figure 5.9 Inhibition of pan-Aurora kinase attenuates mLIN10 mRNA expressions in dorsal root ganglion and spinal cord of CFA injected rats. **(A) DRG mRNA expressions Tozasertib** treatment (10, 20 and 40 mg/kg *i.p.*) attenuates CFA-induced increase in protein expressions of KIF17 and NR2B in ipsilateral L4-L5 DRG of rats.

(B) Spinal mRNA expressions CFA injection induced a significant increase in mRNA expressions of mLin10 in ipsilateral L4-L5 spinal cord tissues of rats which was significantly attenuated on treatment with tozasertib (10, 20 and 40 mg/kg *i.p.*). Whereas diclofenac (10mg/kg *i.p*) treatment did not affect mLin10 expressions in ipsilateral L4-L5 DRG and spinal cord of CFA injected rats. (n=8/group). A one-way ANOVA followed by Tukey's multiple comparison test was used. Data were presented as mean ± SEM. $P<0.05$ was considered statistically significant. Doses: Toza10, Tozasertib 10mg/kg; Toza 20, Tozasertib 20 mg/kg; Toza40, Tozasertib 40 mg/kg; Diclo 10, Diclofenac 10mg/kg; CFA, Complete Freund's Adjuvant.

Our findings suggest that CFA injection led to significantly increased protein expressions of KIF17 and NR2B in both DRG and spinal cord of rats. These results support the evidence that kinesins and their cargo assembly is upregulated during chronic pain conditions and may drive the behavioral hypersensitivities. Tozasertib reversed these changes in DRG as well as spinal cord tissues of CFA injected rats. The binding of KIF17 to NR2B is facilitated by the scaffolding proteins such as mLin10 [104]. Here we have observed that mLin10 mRNA expressions were significantly increased in CFA injected rats which were significantly restored on tozasertib treatment. These findings suggest that tozasertib might interfere with KIF17/mLin10/NR2B mediated nociception during chronic inflammatory pain.

5.4 Outcomes

Pan aurora kinase inhibition using tozasertib attenuates CFA-induced pain hypersensitivities in rats through the KIF17/mLin10/NR2B signaling which in turn leads to the glial cell inhibition and suppression of neuroinflammatory and oxido-nitrosative cascades. The development of novel analgesics targeting the aurora kinase-mediated regulation of kinesins could lead to effective and safer therapeutics for the management of chronic pain.