Chapter-3

Materials & Methods

3.1 Drugs, chemicals and antibodies

Following is the list of drugs, chemicals and antibodies used in this work along with their catalog number and source of procurement.

Table 3.1 List of drugs, chemicals and antibodies

S. No	Drugs/chemical/antibodies	Source
1.	Tozasertib or VX-680	Goldenwing China (China)
2.	Diclofenac	Sir Sundar Lal Hospital Pharmacy (Banaras Hindu University), Varanasi, India
3.	Morphine	Sir Sunderlal Hospital Pharmacy (Banaras Hindu University), Varanasi, India
4.	Gabapentin	Sigma-Aldrich (St. Louis, MO, USA)
5.	NR2B antibody (ab28373)	Abcam (USA)
6.	β-actin antibody (ACTN05 (C4)	Abcam (USA)
7.	KIF17 antibody (SC137040)	SantaCruz Biotechnology (U.S.A)
8.	NF-κβ antibody (ab16502)	Abcam (USA)
9.	IBA1 (SC32725)	SantaCruz Biotechnology (U.S.A)
10.	ICAM1 (SC8439)	SantaCruz Biotechnology (U.S.A)
11.	Anti-Mouse IgG H&L (#ab6728)	Abcam (USA)
12.	Goat anti-rabbit IgG H&L (HRP) (ab6721)	Abcam (USA)
13.	Formaldehyde	Sigma-Aldrich (St. Louis, MO, USA)

14.	Complete freund's adjuvant	Sigma-Aldrich (St. Louis, MO, USA)
15.	TriZol	Thermo scientific (USA)
16.	Maxima SYBR Green/Fluorescein qPCR Master Mix (#K0241)	Thermo scientific (USA)
17.	RevertAid first-strand cDNA synthesis kit (#K1622)	Thermo scientific (USA)
18.	Nuclease Free Water	Sigma-Aldrich (St. Louis, MO, USA)
19.	Bovine Serum Albumin (BSA)	Sisco Research Laboratories Pvt Ltd (Mumbai)
20.	Bradford reagent	Sigma-Aldrich (St. Louis, MO, USA)
21.	Enhanced chemiluminescence reagent	Biorad (USA)
22.	(5,5'-dithio bis-(2-nitrobenzoic acid)	Sigma-Aldrich (St. Louis, MO, USA)
23.	Nitrocellulose membrane	Biorad (USA)
24.	Transfer buffer	Biorad (USA)
25.	Malondialdehyde	Sisco Research Laboratories Pvt Ltd (Mumbai)
26.	Griess reagent	Sisco Research Laboratories Pvt Ltd (Mumbai)
27.	Glutathione	Sigma-Aldrich (St. Louis, MO, USA)
28.	Povidone iodine solution	Win-Medicine (India)
29.	Acetone	Sisco Research Laboratories Pvt Ltd (Mumbai)
30.	Chloroform	Sisco Research Laboratories Pvt Ltd (Mumbai)
31.	Ethanol	Sigma-Aldrich (St. Louis, MO, USA)
32.	Tris-hydrochloric acid	Sisco Research Laboratories Pvt Ltd (Mumbai)
33.	EDTA	Sigma-Aldrich (St. Louis, MO, USA)
34.	Disodium hydrogen phosphate	Sigma-Aldrich (St. Louis, MO, USA)

35.	Potassium dihydrogen phosphate	Sigma-Aldrich (St. Louis, MO, USA)
36.	Potassium chloride	Sisco Research Laboratories Pvt Ltd (Mumbai)
37.	PMSF	Sigma-Aldrich (St. Louis, MO, USA)
48.	2- mercaptoethanol	Sigma-Aldrich (St. Louis, MO, USA)
39.	Bromophenol blue dye	Sigma-Aldrich (St. Louis, MO, USA)
40.	Sodium dodecyl sulphate (SDS)	Sisco Research Laboratories Pvt Ltd (Mumbai)
41.	Ammonium persulphate	Sisco Research Laboratories Pvt Ltd (Mumbai)
42.	Tetramethylethylenediamine (TEMED)	Sisco Research Laboratories Pvt Ltd (Mumbai)
43.	Glycerol	Sisco Research Laboratories Pvt Ltd (Mumbai)
44.	Tris-base	Sisco Research Laboratories Pvt Ltd (Mumbai)
45.	Glycine	Sisco Research Laboratories Pvt Ltd (Mumbai)
46.	Prestained protein ladder	Genetex biotech (India)
47.	DEPC-treated water	Qiagen, Germany
58.	Sodium chloride	Loba Chime (India)
49.	Sodium fluoride	Sigma-Aldrich (St. Louis, MO, USA)
50.	Triton x100	Loba Chime (India)
51.	Sodium orthovendate	Loba Chime (India)
52.	Sodium deoxycholate	Loba Chime (India)
53.	Acrylamide	Sigma-Aldrich (St. Louis, MO, USA)
54.	Bis-acrylamide	Sigma-Aldrich (St. Louis, MO, USA)
55.	HCL	Sigma-Aldrich (St. Louis, MO, USA)
56.	NaOH	Sigma-Aldrich (St. Louis, MO, USA)

3.2 Equipment and software

Following is the list of equipments and softwares along with their make, utilized in the present study:

Table 3.2 List of equipment & software:

S. No	Equipment/software	Source
1.	Chemidoc	Biorad (USA)
2.	Gel electrophoresis assembly	Biorad (USA)
3.	Transblot	Biorad (USA)
4.	Nanodrop	Thermo scientific (USA)
5.	Rotor-Gene Q 2plex HRM real-time PCR System	Qiagen (Germany))
6.	CO2 Incubator	Eppendorf (Germany)
7.	Biosafety Cabinet	Clean Air (India)
8.	pH meter	Eutech (UK)
9.	Cold centrifuge	Eppendorf (Germany)
10.	Microplate reader SpectraMax M5	Molecular Devices (USA)
11.	Micropipettes	Eppendorf (Germany)
12.	Refrigerator (4°C)	Remi (India)
13.	Deep freezer (-40°C)	Remi (India)
14.	Deep freezer (-80°C)	Thermo scientific (USA)
15.	Millipore system	Merk-Sigma (USA)
16.	Tissue homogenizer (MT-30K)	MIULAB (China)
17.	Hargreaves apparatus	Ugo Basile (Italy)
18.	Von-Frey filaments	Anesthesio (USA)
19.	Conditioned Place Preference Apparatus	Rolex (India)
20.	Ranjour	Fine Science Tools (USA)
21.	Temperature sensor	Aptech Deals (Indian)
22.	Von-Frey mesh	Workshop IIT (BHU), Varanasi
23.	Dry Bath	Precious Instrument techno (Delhi, India)
24.	Rocker	Precious Instrument techno (Delhi, India)
25.	Vortex	Remi (India)
26.	Spinwin	Abdos (India)
27.	Weighing balance	Sartorius (Germany)

28.	Surgical tools	Bharat Surgicals (Varanasi, India)/Fine Science Tools (USA)
29.	Matlab software	MathWorks (USA)
30.	Microsoft word	Microsoft (USA)
31.	Microsoft power point	Microsoft (USA)
32.	Microsoft excel	Microsoft (USA)
33.	Gpower software	Heinrich Heine University (Germany)
34.	Chemdraw Software	PerkinElmer (USA)
35.	Image Lab software	Biorad (USA)
36.	Rotor-Gene Q Series software	Qiagen (Germany)
37.	GraphPad Prism 5.0	GraphPad (USA)
38.	Schodinger	Schodinger (USA)
39.	SIM alignment tool	/iss Institute of Bioinformatics
40.	Mutalin Software	Florence Corpet

3.3 *In-silico* studies

Alignment of the amino acid sequences of human and rat Aurora kinase A was performed using the Multalin software [134] and SIM alignment tool. The primary sequences from the UniProt database [135] were used (the code O14965 for human and P59241 for rat Aurora kinase A).

3.3.1 Molecular dynamics simulation study

The general limitation of any grid-based docking algorithm is that it treats the receptor as a rigid entity and therefore it provides a still picture of the protein-ligand interaction [136]. However, in the physiological system, this interaction is dynamic. Therefore, to better understand the interaction between the protein-ligand complex of tozasertib and aurora kinase A, we performed molecular dynamics simulation using the Desmond molecular dynamics program [137]. In general, the protein-ligand complex was placed into an orthorhombic box of size 10 x 10 x 10 Å and solvated explicitly with TIP3P. OPLS3e was used as a force field for the system. The system was neutralized

by Na^+ and Cl^- ions at a final concentration of 0.15 M. The system was minimized and pre-equilibrated using the standard equilibration protocol implemented in Desmond which contains several steps before the final production run. Beginning with a Brownian Dynamics NVT simulation for 100 ps at 10 K temperature, with small timesteps and restraints on solute heavy atoms followed by NVT simulation for 12ps at 10 K temperature with small timesteps and restraints on solute heavy atoms. Then a 12 ps NPT simulation at 10K temperature with restraints on solute heavy atoms and a 12 ps NPT simulation with restraints on solute heavy atoms, and finally a 24 ps NPT simulation without any restraints. After all these equilibration steps, with default relaxation settings pre-simulation a final production simulation was performed for 50 ns using NPT (normal Pressure and Temperature) ensemble at 300 K and 1.013 bars. The pressure and temperature were maintained using Martyna–Tobias–Klein barostat and Nose-Hoover Chain thermostat respectively [138]. The RESPA integrator was used with a time step of 2 fs during the simulation with a smooth PME method for the calculation of long-range electrostatic interaction. The energy and coordinates were saved in the trajectory at every 10 ps. The final simulation trajectory was analyzed using a simulation interaction diagram available in Maestro.

3.4 *In-vivo* studies

3.4.1 Experimental animals

Male Sprague Dawley rats (200-250 gm) were used in the study. Animals were housed four per cage in standard housing conditions; temperature 21 ± 2°C; 12-hour light-dark cycle. Animals were provided with standard laboratory food *ad-libitum* and sterile water. All the animals were randomly assigned to the different experimental groups (n=8/group).

3.4.2 Ethical committee approval

All experiments were performed in accordance with the guidelines by International Association for The Study of Pain and the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, New Delhi. All the experimental protocols were approved by the Institute Animal Ethics Committee of Banaras Hindu University, Varanasi, UP, India (Dean/2019/IAEC/1617).

3.4.3 Animal model of neuropathic pain and experimental design

Chronic constriction injury in the left sciatic nerve of the rats was performed for the development of neuropathic pain as per the method described earlier [139–141]. The method was originally described by Bennet & Xi in 1988 and this model is widely used for the investigations on neuropathic pain [139]. Rats were acclimatized in the laboratory conditions and weighed with a digital weighing balance for the dose calculation of anesthesia and to perform exclusion criterion on the basis of body weight. Rats were anesthetized using ketamine [90 mg/kg intraperitoneally (i.p.)] and xylazine (10 mg/kg i.p.).

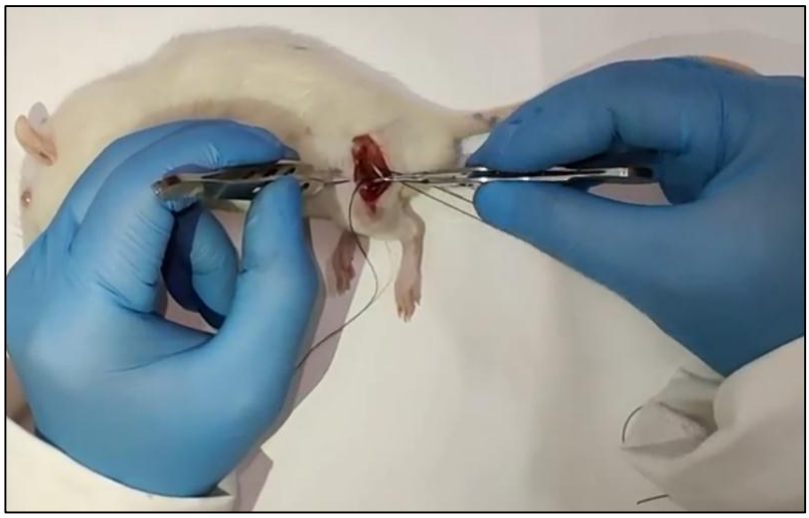

Figure 3.1 CCI model being performed in our lab at IIT (BHU) Varanasi.

Under aseptic conditions, the left sciatic nerve of the rats was exposed through the incision over bicep femoris muscle. The nerve was then made free from the surrounding tissue using a fine forcep tool and ligated with a 6-0 braided silk suture (Teleflex Medical, USA). Each ligation was confirmed with the very short reflex of the ipsilateral paw. After the ligation of the sciatic nerve, the muscles followed by skin were sutured using 4-0 silk threads. Finally, povidone-iodine solution 10% w/v was applied to the sutured skin of rats. Animals were closely monitored until they recovered from anesthesia and then placed in their respective home cages. Post-surgical care was performed to avoid any infection from the injury with appropriate application of povidone-iodine solution till the skin was recovered from incisions. Pain behavior testing was performed before and after sciatic nerve ligation to confirm the neuropathic pain phenotype and its development in rats. Dose for tozasertib (10, 20 and 40 mg/kg *i.p.)* was selected on the basis of our preliminary study which suggested that this compound produced antinociceptive effect at 10 and 20 mg/kg but not at 5 mg/kg *i.p.*

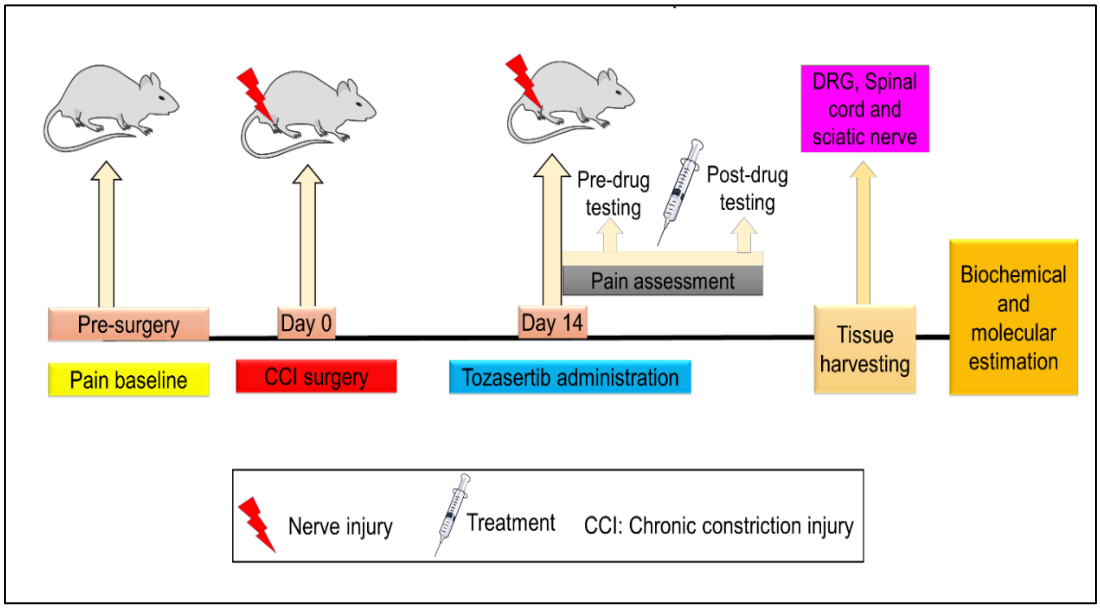

Figure 3.2 Experimental timeline. Reprinted with permission by American Chemical Society from own source reference [140].

3.4.4 Formalin induced acute inflammatory pain model

Formalin test is widely used to model acute inflammatory pain in laboratory rodents. Rats were injected with 50 µl of 2.5 % formalin by intraplantar route (Thermo Scientific: Q23005) as described in the previously published study [142]. Tozasertib at different doses (10, 20, and 40mg/kg), diclofenac (10 mg/kg), and vehicle were administered 30 min before the formalin injection in rats through the *i.p.* route. Following formalin injection, rats were immediately placed in a transparent plexiglass chamber for recording and scoring of flinching behavior by a blind observer. The test lasted for a total duration of 90 mins in which the flinching and licking duration was observed for consecutive five min intervals of the total duration. Rats were returned to their home cages after the experiment.

3.4.5 Complete Freund's adjuvant induced chronic inflammatory pain in rats

To induce chronic inflammatory pain, rats were administered 100 µl complete Freund's adjuvant (CFA) in the left hind paw by intra-planter injection [143]. CFA administration led to the development of inflammatory hypersensitivities which is evident from swelling and redness in the injected hind paws (Figure 3.3). A battery of pain behavioral assays was done before and after CFA administration to confirm the pain hypersensitivity and at different time points post-drug and vehicle administration to evaluate the effect of tozasertib treatment. The treatment started at day four post-CFA injection with the vehicle, tozasertib (10, 20, and 40mg/kg *i.p.),* and diclofenac (10mg/kg *i.p.*). Behavioral tests including heat hyperalgesia, cold allodynia and hyperalgesia, mechanical allodynia, and hyperalgesia were carried out before and after drug treatment.

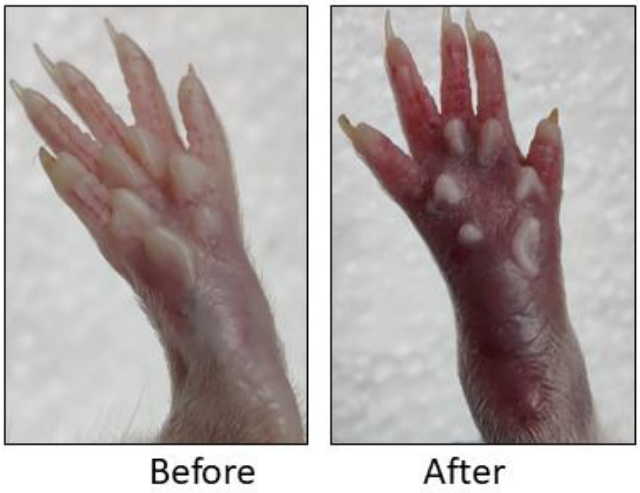

Figure 3.3 Redness and swelling in rat paw before and after CFA injection.

3.4.6 Animal pain behavior tests

3.4.6.1 Tail flick test: Analgesic assay

The test was performed to check the effect of tozasertib on the thermal threshold in naïve rats [144]. Rats were restrained in restrainers and a thermal source was applied to their tail (Ugo Basile, Italy). The cut-off time was set at 10 sec to avoid any damage to the tissue of rats. The heat intensity was standardized by maintaining the average response time between 3-4 seconds. The response time was measured using an automatic detector device (Ugo Basile, Italy).

3.4.6.2 Tail clip test

The test is used for the measurement of mechanical threshold by application of noxious stimulus on the tail of rodents. Alligator clips were applied to the tail of rats [145]. The latency of the rat to reach the clip by mouth was considered as the end point. Generally, animals reach and start licking the tail in 3-4 sec. Further, the effect of the drug was observed if it can increase the threshold of pain in rats.

3.4.6.3 Pinprick test

The test was used to measure the mechanical hyperalgesia in rats. A 22-gauge needle was glued on the von-Frey hair [145]. The pin was applied to the hind paw of rats. Care was taken while applying the pin to the paw to avoid puncture of the skin. Ten trials were performed for each paw. The frequency of paw withdrawal per ten trials was recorded by a blind observer.

3.4.6.4 Hargreaves test: Thermal hyperalgesia

Thermal hyperalgesia was measured in nerve-injured rats by using Hargreaves apparatus (Ugo Basile, Italy). The procedure was performed in accordance with the previously published study [146,147]. Animals were enclosed in transparent acrylic chambers and allowed to acclimatized on Hargreaves apparatus. On test day heat was applied using an automated infrared (IR) beam source and the cut-off time was set at 20 seconds. The heat was applied to the plantar surface of the hind paw and readings of both ipsilateral and contralateral paws were taken in triplicate. All the experimental groups were tested for thermal hyperalgesia pre-injury baseline followed by pre-drug baseline and at different time-points post drug and vehicle administration.

3.4.6.5 von-Frey hair Test: Static allodynia

von-Frey hair test was used to assess the dynamic mechanical allodynia in nerve-injured rats. The mechanical sensitivity was assessed by the application of von-Frey filaments (Anesthesio, USA) of different forces ranging from 0.40 gram (gm) to 13gm on the dorsal surface of hind paws [65]. Animals were placed in the plexiglass chambers on the top of the wire-mesh table and the von-Frey filaments were applied in the sub plantar region of rats paws from the bottom of the mesh. The up-down method

was used to measure the mechanical sensitivity before and after nerve injury and post-drug treatment in rats. von-Frey hairs were applied at the middle of the sub-plantar region of the hind paw of rats. Paw withdrawal, licking, and shaking of the paw in response to von-Frey filament was considered as a positive response. The test was started with a lower force of 1.8 gm filament. If there was no response, then filament of higher force was used. When the response was positive then testing with lower force filament was done. The test was performed till five positive responses were observed or the test reaches the withdrawal maximum and the minimum threshold at higher or lower forces. Pre-injury baseline and 0.5 hr, 1 hr, 2 hr, 4hr, and 24 hr post-drug/vehicle administration thresholds were measured in nerve-injured rats.

3.4.6.6 Cotton swab test: Dynamic mechanical test

A cotton swab test was performed to assess the dynamic mechanical allodynia in rats after nerve injury and at different time points post-drug treatment in accordance with a previously published study [148]. Rats were kept in plexiglass chambers placed on an elevated mesh floor and acclimatized to the gentle stroking with a cotton swab. Using a puffed cotton swab, the hind paws of rats were gently stroked and latency of the paw withdrawal was recorded with a cut-off time of 15 sec. Care was taken while performing this test so as to avoid considering the normal movement of the rat. Paw withdrawal latency was plotted against the time points of respective drug treatment.

3.4.6.7 Ice floor test

Rats were placed in a clear plexiglass chamber with an ice floor [149]. A temperature sensor was placed on the surface to monitor the temperature changes. The temperature was maintained at 4°C ± 2°C. Moderate illumination was made for the

whole duration of the test. The test was video recorded for 1 minute and scoring of paw latency or licking was measured by a blind observer. Pre-injury baseline and 0.5 hr, 1 hr, 2 hr, 4hr, and 24 hr post-drug/vehicle administration paw lifts were counted in nerve-injured rats.

3.4.6.8 Acetone evaporation test

The cold allodynia was measured by the acetone evaporation cooling test. Rats were acclimatized to the plexiglass chambers kept on Von-Frey mesh. A drop of acetone (100 μl) was applied to the dorsal surface of the hind paw without touching the skin. Three trials were performed to evaluate the cold allodynia. The test was video recorded for 1 min and scored by a blind observer. Scoring was graded in four-point response 0, no response; 1, flicking of the paw; 2, repeated flicking of the paw; 3, repeated paw flicking with licking [150].

3.4.6.9 Conditioned place preference: Spontaneous ongoing pain assay

A three-chambered (A, B, and C) conditioned place preference (CPP) apparatus [141,151,152] was used where C was the central corridor section giving entries to the adjacent chambers A and B, both with different visual and tactile cues for the nerve-injured rats treated with vehicle or drug. We used Any Maze software (Version 7.0 Stoelting, USA) to video-track and analyze the time duration spent by each rat in different chambers. Animals were habituated in the test room for 30mins followed by acclimatization in the CPP apparatus for 15 min. The chambers were thoroughly cleaned with 70% ethanol prior to and after testing each animal in the apparatus. On the next day, preconditioning baselines were video recorded for each rat to measure their pre-drug compartment preference. During preconditioning, animals were allowed to

freely explore all the compartments and video tracking was done for 15 mins to find out the time spent by rats in each chamber. Rats showing preferences less than 20% and more than 80% for any of the chambers were excluded from the test. Counter balancing was performed to avoid any biases to the chamber and the average preference o chambers for drug and saline paired chamber was kept at minimal variation. Further, conditioning was done for two days involving two sessions. In the morning session animals were administered with the vehicle and restricted in the paired chamber for a period of 30 mins. Four hours later, a second session was performed where animals were given drugs and restricted to the paired chamber for 30 mins. On trial day, post-conditioning was performed where rats were gently placed in the corridor and were allowed to freely move across all the compartments. The post-conditioning trial was video- recorded for 15 mins and time spent by rats in each chamber was measured. Conditioned place preference was calculated and plotted in graphical forms [152].

3.4.7 Behavioral neurotoxicity assays

3.4.7.1 Rota-rod test

Rota-rod test was performed to measure the motor-coordination activity of rats before and after drug treatment. The test was performed in accordance with previous studies [65,153]. On the first day, rats were trained on rota-rod apparatus for 120 sec at the lower speed of 5 revolutions per minute (rpm). On the trial day, the performance of rats was measured before and after drug administration at a constant speed of 25 rpm. Cut-off time was set at 120 seconds and the time for which rats remained on the accelerating rod was noted down as time spent on rota-rod. Fall latencies were plotted in graphs as an indicator of the grip strength or motor coordination in rats.

3.4.7.2 Open field test

The test was performed to evaluate the effect of the drugs on the locomotor behavior of rats [152,154]. The method used has been described in our previously published studies [152]. Habituation was performed one day before the test in order to make the rats familiar with the novel environment. On testing day, rats were placed in the open filed apparatus (45x45x75cm) and the session was video recorded for 10 minutes. Parameters such as total distance traveled and average speed was measured using Any Maze software (Version 7.0 Stoelting, USA).

3.4.8 Tissue harvesting and storage

Euthanasia was performed after the completion of the behavioral studies and the animals were sacrificed humanely using deep isoflurane anesthesia followed by cervical decapitation. Ipsilateral and contralateral lumber L4-L5 dorsal root ganglion were dissected out by cutting through the muscles and pulling out the transverse processes of the spinal cord [140,141]. Further, the deep cut was made through the skin above the spinal cord. The vertebral column was removed using a rongeur and Dumont forceps and the spinal cord was exposed fully. The L4-L5 lumbar region was identified and cut from the middle section to divide the ipsilateral and contralateral spinal cord into two equal halves. The sciatic nerve was also harvested by dissecting through the bicep femoris muscle. All tissues were stored at -80°C for further biochemical and molecular analysis

3.4.9 Biochemical assays

3.4.9.1 Lipid peroxidation

The lipid peroxidation was assessed by the spectrophotometric based assay for the malondialdehyde (MDA) [155]. Tissue lysate from sciatic nerve was added to the

microcentrifuge tube (Tarsons) followed by addition of the 0.01M Tris HCl and incubation at 37°C for 1 hr. To this mixture 10% trichloroacetic acid was added and centrifugation was performed at 1000 g and supernatant was isolated by pipetting. Further, 0.67% of thiobarbituric acid was added and samples were incubated for 10 min at 95°C. Finally, after cooling the samples were read under multimode spectrophotometer at 532 nm. A predetermined standard curve equation was used to calculate the amount of MDA present in per mg of tissue protein.

3.4.9.2 Nitrite estimation

Griess reagent-based estimation of nitrite was performed in sciatic nerve tissue samples [140,154]. 50µL of sample was added to the equal amount of Griess reagent in 96 well plate. The mixture was incubated at room temperature for 30 mins followed by the spectrophotometric analysis at 540 nm. Levels of nitrite per mg of protein were calculated using an equation that has been derived from the standard curve.

3.4.9.3 Glutathione estimation

The glutathione was measured using the protocol taken from the previously published study [156]. Briefly, buffers (pH 7.4 and 8.0) were prepared using sodium hydrogen phosphate and sodium dihydrogen phosphate. The DTNB (5,5'-dithio bis-(2-nitrobenzoic acid) was added to the phosphate buffer with pH 8.0. The buffer with DTNB and pH 7.4 buffer were added to the tissue lysate in a 96 well plate. Incubation at 38°C was performed for 10 min and absorbance was taken at 412 nm using a multimode plate reader. The amount of GSH was calculated using a predetermined standard curve equation.

3.4.10 Molecular biology studies

3.4.10.1 Western blot analysis

DRG and spinal cord tissue lysates from rats were prepared using radioimmunoprecipitation assay (RIPA) buffer. The estimation of protein in each tissue sample was done by the Bradford method. Briefly, the 198µl of Bradford reagent was added to the 2µl of sample in 96 well plates followed by 10min incubation at room temperature and reading was taken at 496nm using a multimode plate reader. The protein concentration in each sample was determined using the standard curve plotted with bovine serum albumin (BSA).

Table 3.3 Composition of RIPA buffer

S.N	Chemical	Concentration
1	Tris-HCL (pH 8.0)	50mM
2	NaCl	150mM
3	Triton X	0.1%
4	Sodium deoxycholate	0.3%
5	SDS	0.1%
6	Sodium fluoride	1mM
7	Sodium orthovanadate	1mM
8	EDTA	2mM
9	EGTA	2mM
10	PMSF	1mM
11	Protease inhibitor	5µl/100 mg of tissue

The samples were prepared by mixing the 20µg of protein with loading buffer (Recipe shown in table 3.4) and volume was made up to 20µl with deionized water. Next, the samples were heated on a dry bath at 100°C for 5 mins and briefly centrifuged at room temperature. The gel was set for electrophoresis and an equal amount of sample

(20µl) and protein ladder (3µl) were loaded into the wells of polyacrylamide gel and protein were allowed to separate using Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE) with running buffer (Recipe is given in table 3.5). The initial voltage was maintained at 70 V till the lanes reached the resolving buffer and then made run at 90 V.

Table 3.4 Loading buffer recipe

SN	Composition	Concentration
1	SDS	4%
2	2-mercaptoethanol	10%
3	Glycerol	20%
4	Bromophenol blue	0.004%
5	Tris HCl	0.125 M

Table 3.5 Running buffer recipe

SN	Composition	Concentration
1	Tris	25mM
2	Glycine	190mM
3	SDS	0.1%

After the gel electrophoresis, the proteins were transferred to nitrocellulose/PVDF membranes using transfer buffer (Biorad, USA). Briefly, the transfer stacks and nitrocellulose/PVDF membranes were soaked into the transfer buffer and a sandwich was prepared. The cassettes were loaded into the transblot system and protocol was made run 0.7amp, 15V for 45 mins. After the completion of the protein transfer, the membranes were blocked to reduce the nonspecific binding using 3% BSA solution prepared in TBST (tris buffer saline-tween-20) over the rocker system for 2hr. Membranes were washed with TBST for 3X using an orbital shaker for 5 min each. Further, the membranes were incubated with primary antibodies at 4°C for

overnight. Following incubation, membranes were washed with TBST and incubated with their respective secondary antibodies for 1hr. Immune complexes formed were detected using ECL (chemiluminescent agent) (Invitrogen, Carlsbad, California, United States). Blots were visualized in the gel documentation system (ChemiDoc^{TM}, BioRad, Hercules, California, United States) and quantified using Image J software.

3.4.10.2 Reverse transcription polymerase chain reaction (rtPCR) analysis

Total RNA was isolated from the spinal cord and DRG tissues using the Trizol reagent method. Briefly, trizol was added to the samples, and tissue was homogenized using an automated homogenizer at an ice-chilled platform. The resulting mixture was kept at room temperature for 10 mins and then chloroform was added followed by centrifugation at 12000g at 4°C. Next, the supernatant was removed and an equal amount of ethanol was added to the same followed by incubation for 10 min and centrifugation at 12000g at 4°C for 15 min. The pallet was RNA pallet was observed and washed with 75% ethanol twice. Finally, the resulting pallet was dissolved in nuclease-free water and RNA was quantified using NanoDrop (Thermo Scientific).

Further, cDNA was prepared using commercially available cDNA Synthesis kit (K1622 Thermo scientific) by mixing the template RNA, random primers, reaction buffer, RNAse inhibitor, dNTP mix, revert aid RT and final column was made up using nuclease-free water. The cDNA synthesis reaction was made run for 60 min at 45°C and terminated at 70°C for 5 min. A reaction master mix of 15µl was prepared by mixing Maxima SYBR Green/Fluorescein qPCR Master Mix (K0241), forward primer, reverse primer, template DNA, and nuclease-free water (Sigma W4502). The run was performed on Rotor-Gene Q (Qiagen, Germany). Table S1 (supplementary file) shows the list of used primers for rtPCR.

Table 3.6 Primers used in rtPCR analysis

SN	Gene	Sequence	Primer 5'<-----Sequence----->3'
1	GAPDH	Forward	CAGTGCCAGCCTCGTCTCAT
		Reverse	CAAGAGAAGGCAGCCCTGGT
2	NR2B	Forward	GGCAGGGGCGTCAAAAACAA
		Reverse	CACACAGGGGTTGGACTGGT
3	KIF17	Forward	CAGCCATCCTCCACTGACCT
		Reverse	ACCACCTCCTCAGCCACTTT
4	TNFa	Forward	GTAGCCCACGTCGTAGCAAAC
		Reverse	ACCACCAGTTGGTTGTCTTTGA
5	NFKB1	Forward	ACGACGATCCTTTCGGAACT
		Reverse	TCCTCTCTGTTTCGGTTGCT
6	IL1B	Forward	CCTATGTCTTGCCCGTGGAG
		Reverse	CACACACTAGCAGGTCGTCA
5	IL6	Forward	TCTGGTCTTCTGGAGTTCCGTT
		Reverse	GAGAGCATTGGAAGTTGGGGT
6	mLin10	Forward	CGCATGGACAGTTATGAGC
		Reverse	TAAGGCGAACGGATGGTC

3.5 Acute toxicity study in rats

3.5.1 Animals

Female Sprague Dawley (SD) rats weighing 200 to 250 gm (8-12 weeks old) were used for acute toxicity studies. The protocols were approved by the Institute of Medical Science (IMS), Banaras Hindu University, Varanasi, India (Dean/2019/IAEC/1617). Animals were housed in the animal house facility of the Department of Pharmaceutical Engineering & Technology, Indian Institute of Technology (Banaras Hindu University), India. The animals were housed under optimal conditions (temperature 22 ± 3 °C) with a 12-hour light/dark cycle and free access to

food and water. Animals were acclimatized to the laboratory room before the commencement of the experimental procedure.

3.5.2 Animal grouping and experimental design

The protocol was designed in accordance with Organization for Economic Co-operation and Development (OECD) 420 fixed-dose method with some modifications as per the previously published literature [157,158]. Experimental animals were randomly assigned into two groups with 6 animals each. The first group received the vehicle and the second group received tozasertib at the single dose of 40 mg /kg intraperitoneally (*i.p.)*. Tozasertib was purchased from Wuhan Goldenwing, China, and was prepared freshly in 1% dimethyl sulfoxide (DMSO; 0.5%). The detailed cage-side observations were conducted including changes in eyes and mucous membranes, skin and fur, respiratory, circulatory, autonomic, and central nervous systems, somatomotor activity, and behavior patterns with aforementioned time points [159].

3.5.3 Body weight and food water consumption

Rats were returned to the animal house and were maintained for normal food and water. The food and water intake were recorded daily and average weekly consumption was calculated. Individual body weights were recorded once a week [159].

3.5.4 Gross observations and mortality

The animals were observed for gross general behavior (like gait, skin color, lacrimation, sleepiness, writhing, etc.) continuously for the first hour after dosing, periodically during the first 24 h (major observation during the first 4h), and daily thereafter for a total of 14 days for mortality and any other observable toxicity. Special focus was given to observations of convulsions, tremors, diarrhea, salivation, lethargy, sleep, and coma. All

animals were observed for toxic signs and any pre-terminal deaths daily and finally, the number of survivors was noted.

3.5.5 Hematological index

After the 14th-day animals were fasted and blood was collected in separate tubes with or without the addition of the anticoagulants. Complete hematological profiling was performed from the blood samples collected and parameters were different measured including WBC (white blood cells), lymphocytes, Monocytes, Granulocytes, RBC (Red Blood Cells), Hemoglobin, Hematocrit, MCV (Mean Corpuscle Volume), MCH (Mean Corpuscle Hemoglobin), MCHC (Mean Corpuscle Hemoglobin Concentration), RDW (Red Blood Cell Distribution Width, Platelet count, Thrombocytocrit (PCT) [160].

3.5.6 Blood biochemical analysis

The blood was immediately centrifuged after collection at 4000 RPM for 10 min and processed for the biochemical analysis [158]. The Major Biochemical assessment included the estimation of total bilirubin (tbil), direct bilirubin (DBIL), total protein (tp), serum albumin (ALB), globulin (GLO), alanine transferase (ALT), aspartate aminotransferase (AST), alkaline phosphate (ALP), triglyceride (TG), total Cholesterol (TCH), blood sugar (GLU), urea nitrogen (BUN), creatine (CR), uric acid (UA), lactate dehydrogenase (LDH), creatine kinase (CK) [159].

3.5.7 Histopathological analysis

Histopathological analysis of liver and kidney was performed. Organs were harvested and fixed in 10% formalin followed by a dehydration process using ethanol and finally, tissue was fixed using paraffin [161]. Using microtome thin section 5μm

were sliced and stained using hematoxylin & eosin dye. The sections were analyzed under the microscope with a magnification of 10X.

3.6 Statistical analysis

Behavioral data was analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test and two-way ANOVA followed by Bonferroni's multiple comparisons. For two-way ANOVA we used a repeated measure model of statistical analysis. Student t-test was performed for analyzing the data from the acute toxicity study. Microsoft Excel was used to interpret the raw data and calculate the mean, standard error mean (SEM), % maximum possible effect (MPE), fold change for rtPCR and western blot data. For acute toxicity studies t-test was performed. Molecular studies data was analyzed by using one-way ANOVA followed by Tukey's multiple comparison test. GraphPad Prism 8.0 was used to perform the statistical analysis. Data was presented as mean ± SEM. $P<0.05$ was considered statistically significant. The sample size was calculated using Gpower statistical analysis tool and on the basis of our previous studies [140,162].