Molecular Pharmacological Investigations on New Chemical Entities useful in the Treatment of Neuropathic Pain

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CERTIFICATE

This is to certify that the thesis entitled **"Molecular Pharmacological Investigations on New Chemical Entities useful in the Treatment of Neuropathic Pain"** and submitted by **MATHARASALA GANGADHAR** ID No.**2011PHXF0421H** for award of Ph.D. of the Institute embodies original work done by him under my supervision.

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ABSTRACT

Pain is an ill-defined, complex unpleasant phenomenon, usually evoked by an internal or external noxious stimulus (nociception). An analgesic is the drug which relieves pain by acting in the peripheral or on central pain mechanisms, without significantly altering consciousness. Neuropathic pain is a neurological disorder, characterized by the recurrent appearance of spontaneous pain, tingling like sensation and numbness due to neuronal hyperactivity and increased sensitivityinitially in the peripheral neurons and in the neurons of brain in advanced stages. Most of the existing drugs for the treatment of neuropathic pain possess many adverse side effects such as sedation, drowsiness, dizziness and nausea. This stimulated us to conduct screening studies to find out suitable molecules for neuropathic pain treatment with least side effects. Several extensive research reports revealed that semicarbazone, triazole and curcumin derivatives have their effects on CNS disorders like epilepsy, acute pain and inflammation. Hence, in the present thesis we mainly focused on CNS disorders like neuro-inflammation and neurodegeneration which are commonly observed in neuropathic pain states.

The selection of above three core moieties for carrying out screening studies was based on their earlier reported activities. All the three moieties, semicarbazone, triazole and curcumin were lipophilic in nature with potent anti-oxidant and anti-inflammatory properties. These molecules had proven antiepileptic activities which could calm down neuronal hyperexcitability. All these properties are essential for any compound to act as an anti-neuropathic agent. For this thesis work we had selected fifteen semicarbazone derivatives, six triazole derivatives and four curcumin derivatives from BITS database, synthesized and characterized before screening on animal models. Most of the compounds were active in preliminary screening studies. The compounds MG2, MG4 and MG9 were active in alleviating pain responses, improving nerve conduction velocity and blocking voltage gated sodium channels >50% (MG9). The compounds MG2, MG17 and MG24 from each series were extensively screened as they emerged as the most potent compounds from each series. These three compounds showed very good Rho kinase inhibition at IC_{50} of < 1μM with other activities such as antiallodynic, anti hyperalgesic and anti-inflammatory properties.

These three compounds MG2, MG17 and MG24 were also found to normalize dysregulated pro-inflammatory cytokine mRNA expression levels in the spinal nervous tissue of neuropathy induced rats. These compounds were able to reduce mRNA expression levels of TNF-α, IL-6 and NF-kB but were unable to reduce IL-1β expression levels at 30 $mg.kg^{-1}$ dose. In addition to the above properties all the three compounds showed neuroprotective effects on brain tissue of methyl mercury induced neurodegeneration in rats.

Thus the identified active molecules **MG2, MG17** and **MG24** constituted as a prototypical molecules for further optimization and development as anti-neuropathic agents. Thus, these molecules upon further optimization through lead optimisation using the knowledge of medicinal chemistry, in-vivo studies, and pharmacokinetic approaches could lead to generation of multipotent agents for the treatment of neuroinflammation related diseases.

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Abbreviations

AD	Alzheimer's disease
ADMET	Absorption, Distribution, Metabolism, Elimination and Toxicity
AEDs	Antiepileptic drugs
ANOVA	Analysis of variance
BBB	Blood brain barrier
BDNF	Brain derived neurotrophic factor
BFGF	Basic fibroblast growth factor
CA	Cold Allodynia
CRD	Cysteine rich domain
CC_{50}	Cell cytotoxicity 50%
CCI	Chronic constriction injury
CIDP	Chronic inflammatory demyelinating poly neuropathy
CT	Computed tomography
cDNA	Complementary DNA
CNS	Central Nervous System
DAD	Diode array detector
DRG	Dorsal root ganglion
DPN	Diabetic peripheral neuropathy
DRC	Dose response curve
ED	Effective Dose
EC_{50}	Effective concentration 50
EMG	Electromyography
ERK	Extracellular signal regulated kinase
FBS	Fetal bovine serum
FPBS	Flourophenoxy benzaldehyde semicarbazone
GABA	γ-aminobutyric acid
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GLIDE	Grid based Ligand Docking and Energetics
HEK-293	Human embryonic kidney cells-293
IC_{50}	Inhibitory concentration 50%
IL-1β	Interleukin-1β
IL-6	Interleukin-6
I.P	Intra Peritoneal
IFMs	Inflammatory mediators
ISO	Isoprenaline
MEM	Minimum Essential Medium
mg	Milligram
ml	Milliliter
mmol	Millimole
MRI	Magnetic resonance imaging
MES	Maximal electro shock
mRNA	Messenger RNA

MTT	((3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide)
MH	Mechanical Hyperalgesia
NaCl	Sodium chloride
NCCS	National Centre For Cell Science
NFκB	Nuclear factor kappa B
NCS	Nerve conduction study
NCV	Nerve conduction velocity
NIH	National Institutes of Health
NGF	Nerve growth factor
NSUN	Non systemic vasculitic neuropathy
NINS	Non inflammatory neuropathies
NT	Neurotoxicity
NYHA	New york heart association
Oligo-dT	Short sequence of deoxy-thymine nucleotides
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
P.O	Per oral
PI	Protective Index
PSNL	Partial sciatic nerve ligation
ppm	Parts per million
rpm	Rotation per minute
RPMI	Roswell Park Memorial Institute medium
rt	Room Temperature
RT	Retention transcriptase
RH	Relative humudity
RT Enhancer	Reverse transcriptase enhancer
RT-PCR	Real Time Polymerase Chain Reaction
SCSTY	Subcutaneous strychnine
SCPTZ	Subcutaneous pentylenetetrazole
SCBIC	Subcutaneous bicuculline
SAR	Structure-Activity Relationship
SEM	Standard error of mean
SMP	Sympathetically maintained pain
SP	Spontaneous pain
TA	Tactile allodynia
THD	TNF homology domain
Tm	Melting temperature
TTX	Tetrodotoxin
TNF-α	Tumor Necrosis Factor A
TNFRSFP	TNF receptor super family proteins
TRI reagent	TRIzol reagent
VAS	Visual analogue scale

List of symbols used

%	Percent
˚C	Celsius
cm	Centimeter
g	Gram
h	Hour
m	Meter
mg	Milligram
MHz	Megahertz
mL	Milliliter
mm	Millimeter
nm	Nanometre
nM	Nanomolar
W	Watt
α	Alpha
β	Beta
δ	Delta
ζ	Zeta
λmax	Wavelength of maximum absorption
μL	Microliter
Å	Angstrom
μM	Micromole
μm	Micrometer

CHAPTER-1

Introduction to Neuropathic pain and Neurodegeneration

“These pains you feel are messengers. Listen to them”

- JalaluddinRumi

(An Islamic scholar)

1.1 Pain and types of pain

Pain is a complex phenomenon and also subjective which makes it difficult to define. The International Association for the Study of Pain (IASP) has defined it most appropriately as: "Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage". Pain is a symptom rather than a disorder in medical diagnosis [Pain definitions (IASP), 2015]. Pain protects an individual or living being from damaging and noxious stimulations or conditions and helps body parts to heal from repetitive insults. Pain also warns an individual by recollecting prior similar experiences to avoid damaging situations in the future [The neurobiology of pain, 1984]. Mostly pains get suppressed when the damaging situations are removed and the damaged part is healed but some still remain even after the stimulus is removed [The Handbook of Chronic Pain, 2007].

Pain perception helps an individual to protect his body part from potential injury or damage. Pain is classified as acute and chronic based on duration of pain perception. Acute pain lasts for 3-6 months and chronic pains lasts for more than 6 months. Chronic pains may have an underlying cause while acute pains have direct cause and usually manifests in ways that can be easily described. Based on the location in the body, pain is again classified into three types, somatic, visceral and neuropathic [Moore *et al*., 2015].

Pain classification helps in assessment and treatment. In general, the common types of pain include nociceptive, neuropathic and inflammatory pain. Nociceptive pain represents the normal response to noxious insult or injury of tissues such as skin, muscles, visceral organs, joints, tendons, or bones such as somatic-musculoskeletal (joint pain, myofascial pain), cutaneous-localised to skin (periphery), visceral-to internal organs and smooth muscle. Second is neuropathic pain initiated or caused by a primary lesion or disease in the somatosensory nervous system. Symptoms related to sensory abnormalities include numbness, tingling and loss of perception which usually arises due to underlying diseases or conditions such as diabetes, cancer, postherpetic neuralgia, spinal cord injury and phantom limb pain. Third type of pain (inflammatory) is a result of activation and sensitization of the nociceptive pain pathway by a

variety of mediators released at the site of tissue inflammation. The mediators that have been implicated as key players are proinflammatory cytokines such as IL-1-alpha, IL-1-beta, IL-6 and TNF-alpha, chemokines, reactive oxygen species, vasoactive amines, lipids, ATP, acid, and other factors released by infiltrating leukocytes, vascular endothelial cells or tissue resident mast cells. Examples of inflammatory pain include appendicitis, rheumatoid arthritis, inflammatory bowel disease and *herpes zoster* infection.

There are well-recognized pain disorders that are not easily classifiable. Our understanding of their underlying mechanisms is still rudimentary though specific therapies for those disorders are well known; they include cancer pain, migraine and other primary headaches and wide-spread pain of the fibromyalgia type.

1.2 Neuropathic pain

Neuropathy and neuropathic pain overview

Dysfunctioning of nerves leading to loss of sensation is called neuropathy. People with this condition experience pain related to sensory system called painful neuropathy and is described as neuropathic pain. Since pain is an unique experience of individual, its description varies based on severity of perception. The common descriptors for neuropathic pain include tingling, pricking, throbbing, burning, stabbing and aching pain. Neuropathic pain may be present continuously but its intensity could increase and decrease alternatively.

1.3 Causes of neuropathic pain

Exact reason for neuropathic pain is unknown. According to one theory, inability of nerve cell to conduct sensory impulses or messages leads to spontaneous nerve activity which is interpreted by brain as pain. Neuropathic pain could also occur without any stimulation. It may be associated with an exaggerated or heightened sensitivity to normal stimulation such as a light touch or the sensation of clothing. The multiple causes, ranging from diabetes mellitus to toxin exposure are associated with neuropathic pain. Other illness like HIV and kidney failure is also associated with neuropathy. A peripheral nerve injury, alcohol and tobacco consumption and certain prescription drugs have been shown to cause neuropathy. Rash caused by shingles (*herpes zoster*) can lead to nerve pain. Pain may begin at any point after the development of neuropathy. Currently, it is difficult to predict who could develop neuropathic pain, but researchers have identified potential factors which might be involved in generation of peripheral neuropathy (**Table 1.1**). In fact, many people are unaware of the presence of neuropathy until pain begins.

Table 1.1.*Potential factors thought to be involved in peripheral neuropathy:*

1)	Diabetes mellitus	9)	Vasculitis/blood vessel disease
2)	Injury/trauma	10)	Tumor formation/cancer
3)	Disc herniation/spinal stenosis	11)	Repetitive stress injury
4)	Kidney disease	12)	Toxin exposure
5)	Thyroid hormone abnormality	13)	Prescription medications
6)	Excess growth hormone	14)	Infections
7)	Vitamin deficiency	15)	Heredity
8)	Alcoholism	16)	Small fibre neuropathy

The risk of developing neuropathy could be reduced by limiting alcohol, tobacco, and environmental toxin exposure, as well as maintaining general good health.

1.4 Symptoms of neuropathic pain

Chronic pain in the feet, legs and upper extremities is the most common complaint of neuropathic pain patients. There is no obvious cause for the pain and often the diagnosis is delayed. The severity of pain might differ individually. Burning, stabbing, prickling, or tingling are words commonly used to describe its symptoms. As the entire hands and feet are commonly affected, the symptoms are often described as being in a "stocking-glove distribution".

1.5 Diagnosis and screening tools of neuropathic pain

1.5.1 Neuropathic pain diagnosis

No objective test for pain has been developed. As such, when patients present with complaints of neuropathic pain, testing for evidences or causes of neuropathy is performed. Physical examination may reveal decreased ability of a patient to sense temperature or light pinpricks. Strength and reflexes are usually normal. Patients may be unable to identify if someone is moving their foot or toe and may be unable to sense a vibrating tuning fork. If there is evidence of neuropathy on a physical examination, a nerve conduction study with electromyography (NCS and EMG) may be recommended. This test is used to determine the severity of the neuropathy, and may offer additional clues as to the cause of neuropathy. Blood tests could be used to identify vitamin deficiencies or other metabolic abnormality which are contributing or causing

the neuropathy. In some cases, MRI or CT scans may be needed to further define the possible causes of the neuropathy. Skin or nerve biopsies are done infrequently but can provide further information in some situations, such as small fiber neuropathy.

1.5.2 Neuropathic pain screening tools

After diagnosis of neuropathy, further evaluation of the discomfort need to be done. A number of tools have been identified [Bennett MI *et al*., 2007] and comprise of: neuropathic pain scale, neuropathic pain questionnaire, pain DETECT, ID-pain, Leeds assessment of neuropathic symptoms and signs (LANSS), and the Douleur neuropathique (DN4). The LANSS assess the neuropathic pain and comprises of five symptoms and two examination items (allodynia and pin-prick testing) and has been developed into a patient self-report tool (S-LANSS) [Bennett M, 2001]. Screening tools are not designed as diagnostic tools but they can be useful in highlighting the need for a more detailed clinical assessment. Visual analog scales (VAS) are frequently used to gauge the pain severity [Danette *et al*., 2016] (**Fig. 1.1**).

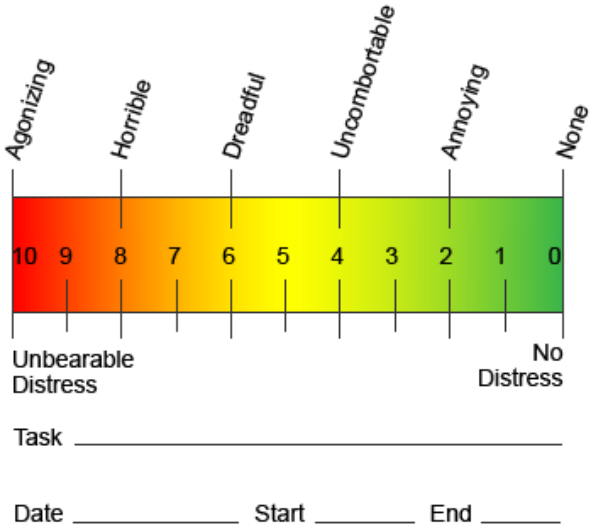


Figure 1.1: Visual analog scale used to quantify the range or severity or intensity of pain experienced. Pain severity is measured on scale from 0 (no pain) to 10 (unbearable pain) [Haefeli M *et al*. 2006].

1.6 Pathophysiology of neuropathic pain

Pain is a protective function and is a normal response to injury or disease. There may also be other manifestations of pain related to tissue injury including hyperalgesia, an exaggerated response to a noxious stimulus, and allodynia, the perception of pain from normally innocuous

stimuli. Both are the result of changes in nervous systems, and are referred to as peripheral or central sensitization.

Nociceptors not only signal acute pain, but when chronically sensitized, contribute to persistent pathological pain disorders from previous injury or ongoing disease. Genetic and environmental factors contribute to sensitization resulting in persistent (chronic) pain. Chronic pain is also characterized by the abnormal state and function of hyperactive spinal cord neurons. Increased transmitter release by spontaneously active primary afferent neurons and an increased responsiveness of postsynaptic receptors leads to hyperactivity. Release of biologically active factors from activated glia maintains a hyperexcitable state of synaptic transmission at the dorsal horn. Hyperexcitability is aggravated by the loss of inhibitory interneurons. Under normal circumstances the nociceptive sensory system returns to a normal functional state after healing. Many features of sensitization persists and are manifested as chronic pain and hyperalgesia. Studies have shown that chronic pain is accompanied by permanent structural alterations in specific brain areas leading to nociception (**Fig: 1.2**).

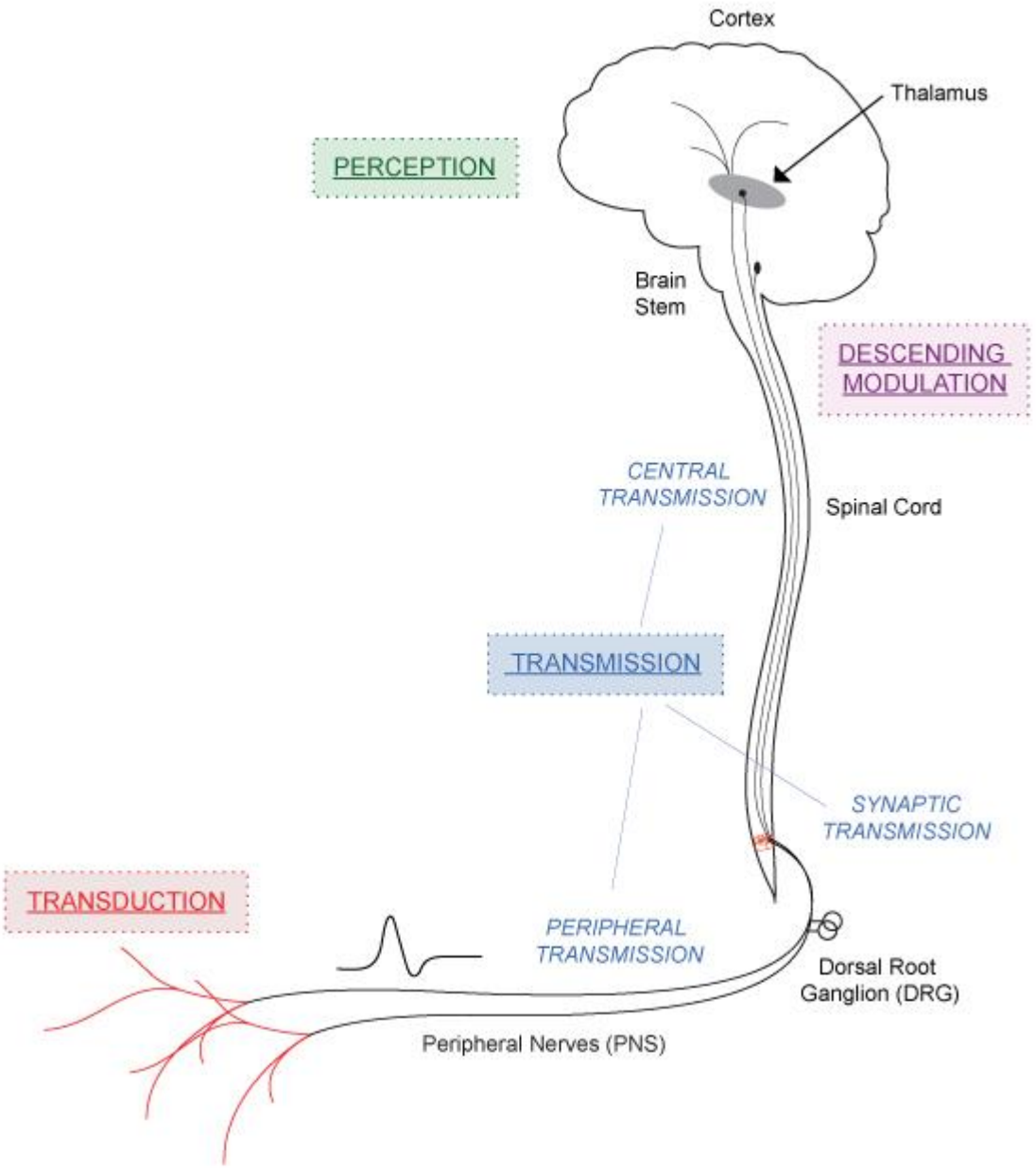


Figure 1.2: Peripheral and central transmission of pain responses

[Board of Regents of the University of Wisconsin System (2010)]

1.6.1 Peripheral sensitization

Peripheral mechanisms of neuropathic pain

In normal condition, unmyelinated C-fibres and myelinated Aδ-fibres of primary afferent neurons produce pain sensations. Without adequate stimulation, these nociceptors do not produce sensation. Noxious, high threshold stimuli are causes for nociceptors responses. In peripheral nerve injury, the primary afferent nerve undergoes alterations in function and structure. In both injured and uninjured conditions, neurons may become hyperexcitable because of various pathophysiological mechanisms [Woolf *et al*., 1999; Baron *et al.*, 2006].Peripheral nerve damage causes release of chemical substances like bradykinin, prostaglandins, serotonin, epinephrine, tumor necrosis factor (TNF-α), nerve growth factor (NGF) and cytokines, This in turn cause an upregulation and morphological changes of voltage-gated sodium and calcium channels in the affected and non-affected neurons, leading to decreased thresholds and an increased response to stimulation [Woolf *et al.*, 2000; Baron *et al.*, 2006]. These modifications in ion channels become ectopic pacemakers. This mechanism is unclear, eventhough the release of neurotrophins is thought to play a crucial role [Bridges *et al.*, 2001, Woolf *et al.*, 1999].

Regeneration of damaged nerves causes formation of new nerve projections on uninjured neighbouring neurons because of the release of nerve growth factor (NGF) [Reynolds and Woolf, 1993; Muller and Stoll, 1998; Watkins *et al*., 2003]. Damaged nerves may become demyelinated and lead to ectopic firing and ephaptic cross-talk between adjacent demyelinated axons (**Fig: 1.3**). For the regeneration of nerves, neuromas are formed at the distal end of the damaged axon. Neuromas consist of abnormally sprouting axons and many hyperexcitable calcium and sodium ion channels [Macres *et al*., 1999; Woolf and Mannion, 1999; Bridges, 2001].

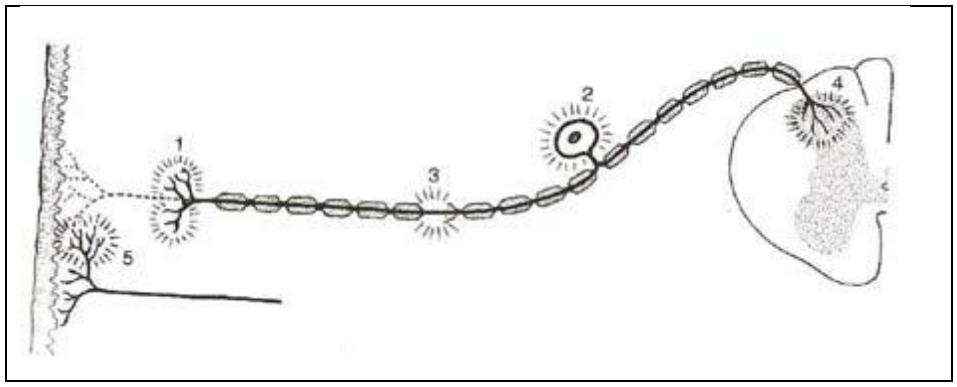


Figure 1.3: Schematic representation of defects in the primary afferent neuron. 1. Peripheral regenerative sprouting. 2. Ectopic activity in dorsal root ganglion. 3. Demyelinisation and ephaptic cross-talk. 4. Sprouting in the dorsal horn. 5. Peripheral collateral sprouting. [Cranenburgh *et al*., 2002]

In some patients, the sympathetic nervous system is involved in the maintenance of neuropathic pain [Woolf and Mannion, 1999; Macres, 1999; Gilron *et al*., 2006]. Multiple valid mechanisms were proposed to contribute to this sympathetically maintained pain (SMP). Firstly, as mentioned earlier, the expression of α-adrenergic receptors on both injured and uninjured is increased [Woolf and Mannion, 1999]. Secondly, direct pairing of peripheral noradrenergic and sensory nerve terminals and third, ephaptic cross talk between sensory and sympathetic nerve fibres are reported [Bridges *et al*., 2001]. Finally, nerve injuries induce sprouting of sympathetic axons [Woolf and Mannion, 1999]. Low threshold stimulation may actuate nociceptive second order neurons in the spinal cord, leading to phenomena such as allodynia [Woolf *et al*., 1992; Macres, 1999; Woolf and Mannion, 1999,Woolf *et al*., 1995,Wang *et al*., 2002, Zubieta *et al*., 2003] [**Table. 1.2**].

Table 1.2. Peripheral mechanisms of neuropathic pain

Peripheral mechanisms of neuropathic pain
➢ Damaged peripheral nerve endings induces release of excitatory chemical substances
➢ Upregulation and morphological changes of voltage-gated Na^+ and Ca^{2+} channels
➢ Formation of neuromas, cross-excitation of uninjured neighbouring nerves
➢ Ephaptic cross-talk between demyelinated nerves and collateral sprouting in the dorsal horn
➢ Modification of type and amount of neurotransmitters from affected nerve fibres
➢ Participation of the sympathetic nervous system and upregulation of α-adrenergic receptors in the peripheral nerve

1.6.2 Central sensitization

C*entral, spinal mechanisms of neuropathic pain*

Spinal sensitization is due to sustained painful stimulation. Nerve injury leads to changes in the central nervous system that cause pain transmission, and lead to hyperalgesia and allodynia. This is termed as central sensitization, and is characteristic of both nociceptive and neuropathic pain. In the 1960's it was discovered that repetitive stimulation of a nerve at a constant C-fibre strength resulted in a steady increase in action potential firing in the dorsal horn [Mendell LM, 1965]. This was an example for plasticity in pain pathways of the CNS and was termed 'wind-up'. Later on, in dorsal horn, it was discovered that activation of peripheral nociceptors increased synaptic efficacy [Cook AJ, 1987; Woolf CJ, 2011]. Moreover, it was the conditioning input that was amplified and non-stimulated input (both nociceptive and non-nociceptive) from other nerve fibres [Stubhaug A, 1997]. This homosynaptic and heterosynaptic potentiation is called 'central sensitization'. Excitatory amino acids, modifications in ion channel properties, increased receptor density and activation of presynaptic and postsynaptic kinases were found to be responsible for increase in dorsal horn synaptic strength. Immune cells (microglia), astrocytes and modification in gene transcription helped to keep the sensitized state, where normal inhibitory inputs were decreased, and excitatory activity increased [Stubhaug A, 1997]. Nociceptive event triggered the changes in the central nervous system itself, distorting or amplifying pain even when the original noxious stimulus disappeared. Pain in this

statedissociated from peripheral nociceptive stimuli and was driven to central. As like in neuropathic pain changes occurred in dorsal horn, amplification and strengthening of afferent inputs is such that normally innocuous inputs was such that normally innocuous inputs such as Aβ touch sensations could activate pain circuits resulting in allodynia [Stubhaug A, 1997, Kupers R, 2009, Aasvang EK, 2010]. In general, patients with central sensitization suffered with dynamic tactile allodynia, secondary hyperalgesia and temporal summation. Central sensitization, eventhough was caused by nociceptive pain, was not termed as 'nociceptive pain', because it happened in the absence of noxious stimuli [Woolf CJ, 2011].

Central sensitization has been concerned in surgery induced chronic pain. For example, experience of secondary hyperalgesia after post-operative abdominal surgery related chronic pain, [Lavand P *et al*., 2006; Wilder-Smith OH *et al*., 2010]. In 51% of patients with chronic post surgical pain was observed the signs of central sensitization, compared to 15% of pain free patients [Aasvang E.K et al, 2010; Aasvang EK, Kehlet H, 2010].

1.7 Identification, assessment and complications

The first step was to determine the site of pain, precipitating events and other clinical parameters. Identification of red flags requiring urgent interventions (e.g. spinal cord compression) was important.

Sometimes the clinical findings were well defined such as post-herpetic neuralgia, but they were less clear in other situations. Investigations are needed to find evidence of nerve dysfunction. Techniques like CT and MRI scans could identify causes of nerve compression or infiltration that may require further treatment. Nerve conduction studies and somatosensory-evoked potentials can confirm neuropathy but only was a measure of function in large myelinated fibres. Quantitative sensory testing could detect small-fibre neuropathies; however, they were expensive and, as yet, not widely available. Other investigations which were not used in routine practice but may be useful include biopsies/skin punch biopsies. Procedures which are currently used only in research involve functional neuroimaging, micro-neurography to assess peripheral nervous system responses and laser evoked potentials which test central responses to laser evoked stimuli in the periphery [Cruccu G *et al*., 2004].

Neuropathy is not necessarily the cause of the patient's pain. It is also important to remember that pain with neuropathic features could still occur without any identifiable nerve damage.

Patients with chronic nerve pain also suffer from sleep deprivation or mood disorders. Additionally, underlying neuropathy and lack of sensory feedback, patients are at risk of developing injury or infection or unknowingly causing an escalation of an existing injury.

1.8 Animal models of neuropathic pain

Various animal models have been established to mimic peripheral and central pain models of neuropathy. Chemotherapeutic drugs-induced cancer and HIV-induced pain; post-herpetic neuralgia (PHN); diabetes and chronic ethanol-induced; trigeminal neuralgia, and orofacial pain models are some animal models. In this thesis we tried to provide an overview on these different animal models of neuropathic pain in brief.

1.8.1. Peripheral nerve injury models

The early animal models of pain focused only on the acute behavioral and electrophysiological reactions. The tail flick and the hot plate tests were the only tests employed for screening preclinical pharmaceutical analgesics [D'Amour FE, 1941]. Early models were relevant only for testing acute nociceptive pain and were able to assess the hypersensitivity changes associated with chronic pain. Wall and co-workers in the 1970s developed a chronic pain model by producing injury to a peripheral nerve, which was quite distinct from acute noxious pain [Wall PD, 1974]. This was the oldest model of neuropathic pain in animals and involves complete transection of sciatic nerve at mid-thigh level [Wall PD, 1979]. In this model, anesthetized rat's common sciatic nerve was exposed and was tightly tied by nylon suture, proximal to its bifurcation into the tibial and the peroneal divisions, at two locations about 1-cm apart. The nerve was then completely transected between the pair of ligatures, and 5 mm of the nerve between the ligatures was removed to prevent nerve re-joining due to regeneration. Following complete nerve transection, a neuroma develops at the proximal nerve stump consisting of regenerative nerves sprouting in all directions [Devor M, 1976; Amir R, 1993; Muthuraman A, 2008]. The model produced anesthesia dolorosa, i.e. pain in the area in the absence of any sensory input in that area. Autotomy was observed in this model which was often considered as a marker of neuropathic pain [Wall PD, 1976]and the intensity depended upon the method and location of neurectomy [Zeltser R, 2000]. It was not clear whether autotomy was a reflection of

spontaneous pain or was a result of excessive grooming in the absence of sensory feedback [Katz *et al*., 1992]. A major limitation of this model was that a complete nerve transection or lesion was relatively uncommon in patients. Moreover, ethical considerations were also the key issues in this model as animals demonstrated excessive autotomy in this model [Kauppila T, 1998].

Table 1.3: Animal models of neuropathic pain

S. no.	Name of model	Principle of injury	Species
1	Axotomy (complete sciatic nerve transection)	Complete transection of sciatic nerve	Rats
2	Chronic constriction injury	Four loose ligatures around sciatic nerve	Rats, mice
3	Partial sciatic nerve ligation (Seltzer Model)	Tight ligation of one-third to half of sciatic nerve	Rats, mice
4	Spinal nerve ligation	Tight ligation of L5, L6 spinal nerves	Rats
5	Spared nerve injury	Axotomy of tibial and common peroneal nerves	Rats, mice
6	Tibial and sural nerve transection	Axotomy of tibial and sural nerves	Rats
7	Ligation of common peroneal nerve	Ligation of common peroneal nerve	Mice
8	Sciatic cryoneurolysis	Freezing of the sciatic nerve	Rats
9	Caudal trunk resection	Resection of caudal trunk	Rats, mice
10	Sciatic inflammatory neuritis	Injection of zymosan, HMG, TNF-alpha around sciatic nerve	Rats, mice
11	Cuffing-induced sciatic nerve injury	Implantation of polyethylene cuff around sciatic nerve	Rats, mice
12	Photochemical-induced sciatic nerve injury	Thrombosis in small vessels supplying sciatic	Rats, mice
		nerve by photosensitizing dye and laser	

13	Laser-induced sciatic nerve injury	Radiation mediated reduction in blood supply to sciatic nerve	Rats
14	Weight drop or contusive spinal cord injury	Dropping a weight over the exposed spinal cord	Rats, mice
15	Excitotoxic spinal cord injury	Intraspinal injections of excitatory amino acids	Rats, mice
16	Photochemical spinal cord injury	Thrombosis in blood vessels supplying the spinal	Rats
		cord by photosensitizing dye and laser	
17	Spinal hemisection	Laminectomy of T11–T12 segments	Rats
18	Drugs-induced	Direct injury of drugs to the nerves of peripheral nervous system	Rats, Mice
19	Diabetes-induced neuropathy		
	a) Streptozotocin-induced	Persistent hyperglycemia-induced changes in the nerves	Rats, mice
	b) Genetic models	High fat diet	Rats, mice
20	Bone cancer pain models		
	a) Femur, calcaneus, tibial, humerus	Inoculation of cancerous cells into respective bones	Rats, mice
	b) Neuropathic cancer pain	Growing a tumor in vicinity of sciatic nerve	Mice
	c) Skin cancer pain	Injection of melanoma cells in plantar region of hind paw	Mice
21	HIV-induced neuropathy	Delivery of HIV-1 protein gp120 to sciatic nerve	Rats
22	Post-herpetic neuralgia	Injection of viral infected cells in the footpad	Rats, mice
23	Chronic ethanol consumption/withdrawal	Administration of ethanol over extended period	Rats

		(around 70 days)	
24	Pyridoxine-induced	Administration of high dose pyridoxine for long period	Rats
25	Trigeminal Neuralgia	Compression of trigeminal ganglion, chronic	Rats
		constriction injury to infra-orbital nerve	
26	Orofacial pain	Injection of formalin, carrageenan into	Rats, mice
		temporomandibular joints and maxilla	
27	Acrylamide-induced	Administration of acrylamide for prolonged period	Rats

[Fundamental and clinical pharmacology, 2009] [Jaggi AS *et al.*, 2011]

1.9. Neuropathic pain prevention and epidemiology

The best way to prevent neuropathic pain is to avoid development of neuropathy, if possible. This includes identifying and modifying risk factors for diabetes, avoiding exposure to environmental toxins when possible, and limiting use of alcohol or tobacco. Utilizing good ergonomic form at work or when practicing hobbies can decrease the risk of repetitive stress injury.

A variety of disorders present symptoms related to neuropathic pain which is peripheral or central. Below is the prevalence of neuropathic pain conditions experienced by individuals suffering from various diseases.

Table 1.4: Reported medical conditions with prevalence of neuropathic pain

Condition	Neuropathic pain prevalence %
Diabetes [Sadosky A *et al*., 2008]	11-26
Cancer [Davis MP et al.,2004; Bennett MI et al.,2012]	18-39
HIV [Schifitto G et al., 2002; Morgello S et al.,2004]	35-53
Back Pain [Philip A et al., 2011]	37
Infection [Freynhagen R et al., 2006]	10-25
Stroke [Andersen G et al., 1995]	8
Spinal cord injury [Siddall PJ et al., 2003]	75
Multiple Sclerosis [Grau-Lopez L et al., 2011]	58

Neuropathic pain comprises of a variety of disorders affecting peripheral and central nervous systems. **Table 1.4**.shows the prevalence of neuropathic pain among individuals with different medical problems.

Neuropathic pain plays a role in the experience of pain symptoms for a wide variety of disparate medical conditions. Neuropathic pain is not only associated with diseases of the nervous system, but also disease processes where a mixed pathology is more likely. For example, neuropathic pain is surprisingly prevalent amongst cancer patients [Davis MP et al., 2004]. In a recent international study of over 1000 cancer patients, 79.7% presented clinical diagnosis of nociceptive pain, and 16.9% were considered to have neuropathic pain [Rayment C *et al*., 2012]. Of note, up to 39% of cancer pain patients had a dual pain mechanism, both nociceptive and neuropathic, a common feature of other conditions such as lower back pain [Bennett MI et al., 2012; Freynhagen R *et al*., 2006].

The prevalence of neuropathic pain in the general chronic pain population is approximately 17% [Torrance N et al., 2006]. Surprisingly the prevalence of neuropathic pain in the general population, with an estimated prevalence of 6-8% was found using screening questionnaires [Torrance N *et al*., 2006; Bouhassira D *et al*., 2008]. In general, neuropathic pain seems to be associated with poor health related quality of life, with mixed neuropathic pain having a similar impact on the EQ-5D as NYHA Class IV heart failure [Doth AH *et al*., 2010]. Patients also seem to suffer greater pain intensity, and report greater impact on daily living compared with nociceptive pain [Smith BH *et al*., 2007]. Patients also seem to suffer mental and physical health

problems, implying that the nature, and not simply the intensity of neuropathic pain is contributory [Freynhagen R *et al*., 2009]. The impact of neuropathic pain on quality of life is in part likely to reflect the chronic nature of such pain in many conditions. Although there is a paucity of longitudinal data describing the natural history of neuropathic pain, for some conditions such as diabetic peripheral neuropathy, symptoms are unlikely to improve with time [Daousi C *et al*., 2006]. In contrast, symptoms may be more likely to improve over time where the initiating disease or lesion resolves, such as post herpetic neuralgia, [Thyregod HG *et al*., 2007].

1.10 Neuropathic pain-Pharmacological management:

Treatment of the underlying cause when possible is the key. Many different medications are used to treat neuropathic pain, however, it should be noted that no specific medication has been approved to treat all types of painful neuropathy. In terms of pharmacotherapy, traditionally conditions thought to be associated with a predominantly nociceptive pain component, such as arthritis, have been treated with analgesics such as paracetamol, non-steroidal anti-inflammatory drugs (NSAIDS) and weak or strong opioids [Fitzcharles MA *et al*., 2011]. Similarly, this approach has been the cornerstone of analgesic management of cancer pain for over two decades following the publication of the WHO analgesic ladder [Geneva: WHO, 1996]. In contrast, neuropathic pain tends to be managed with entirely different types of medications, designed to act as pharmacological modulators of nerve pain. The most commonly used classes of drugs for neuropathic pain are the antidepressants (including tricyclic antidepressants and serotonin and noradrenaline reuptake inhibitors [SNRI]), and the anticonvulsants (including the gabapentinoids and older drugs such as carbamazepine). Antidepressant drugs act by enhancing the descending inhibitory pain pathways from the brain to the spinal cord. Anticonvulsants have a variety of mechanisms including blockade of sodium and calcium channels in the central nervous system. Current National Institute for Clinical Excellence (NICE) guidance for the management of neuropathic pain includes tricyclic antidepressants, gabapentinoids and SNRI medications as first and second line management. Although these medications are relatively efficacious for managing neuropathic pain, they seem to have little benefit in conditions where nociceptive pain predominates. For example, a recent Cochrane systematic review of the efficacy of antidepressants in inflammatory arthritis concluded that it was not possible to draw firm

conclusions about the efficacy of these drugs [Richards BL *et al*., 2012]. Similarly, six unpublished trials investigating the efficacy of gabapentin for the treatment of nociceptive pain failed to show any benefit [Vedula SS *et al*., 2009].

Though, antidepressant and antiepileptic medications are effective for neuropathic pain, they are not effective in treating nociceptive pain. When these drugs were used in populations with high levels of mixed pain (such as cancer pain) systematic reviews demonstrated that such adjuvants were helpful, although the effect size was much smaller than that found in patients with non-cancer neuropathic pain [Bennett MI *et al*., 2011]. Additionally, analgesics commonly used to treat nociceptive pain were also effective treating neuropathic pain. For example, tramadol, with its dual mode of action (opioid agonism and enhancing descending inhibitory pain pathways) is considered a third line anti-neuropathic agent by NICE, and is commonly used to treat nociceptive pain [McQuay HJ et al., 2012; Howes F et al., 2011]. Similarly, opioids such as morphine or oxycodone also showed efficacy in treating neuropathic pain, although outside the cancer pain population were rarely considered first or second line agents due to adverse effects [Freynhagen R *et al*., 2009].

Neuropathic and nociceptive pain differs in health impact and in responding to different modes of analgesia. Curing neuropathic pain is dependent on the underlying cause. If the cause is reversible, then the peripheral nerves may regenerate and pain will abate; however, this reduction of pain might vary from months to years.

1.11 Neurodegeneration

Neurodegeneration is a feature of several neurological pathologies and can thus both be secondary or causative to disease. Primarily neurodegenerative disorders include AD, leading to dementia due to loss of cholinergic neurons, PD, characterized by loss of nigrostriatal dopaminergic neurons and amyotrophic lateral sclerosis (ALS), where motoneurons are lost. Patients may also display mixed clinical phenotypes.

Neurodegeneration leads to gradual deterioration in a person's cognitive abilities. This loss is either due to structural changes that prevent neurons from functioning normally, or due to cell death. Neurodegenerative diseases are characterized by degeneration of distinct neuronal population, factors affecting inherent vulnerability and are probably involved in disease susceptibility. Such factors include nerve cell vulnerability, glial function and local

microenvironment. Todate, existing treatment options are mainly symptomatic. To compensate for the loss of cell function or transmitter release, efficient therapy for these diseases should aim at slowing or stopping the degenerative processes. To do so, knowledge of the target cell susceptibility and the local cellular interactions is needed.

1.12 What causes neurodegeneration?

Genetic mutations cause a small proportion (~5%) of neurodegenerative diseases. The remaining cases are thought to be caused by: (a) the accumulation of toxic proteins in the brain, and (b) brain's mitochondria not being functioning efficiently and creating toxic molecules that damage neurons. Although the causes could vary, scientists agree that the net result is cell's "programmed cell death" pathway, a sort of deliberate cell suicide that is supposed to protect the surrounding brain cells from toxic molecules.

Treatment should aim to decrease the intensity of acute pain to reduce permanent changes in the nervous system that may result in chronic pain. Multimodal therapy involving drug and non-drug treatments and rational combinations of drugs working by different mechanisms could provide optimal relief of pain.

Neurodegeneration is not well-understood as yet, and hence no cures. In the search for effective treatments, investigators employ animal models of disease to test potential therapeutic agents. Model organisms provide an inexpensive and relatively quick means to perform two main functions: target identification and target validation. Together, these show the value of any specific therapeutic strategies and drugs when attempting to ameliorate disease severity.

CHAPTER-2

Review of Literature

2.1 Overview

The literature review is structured and described briefly as, versatile pharmacophores (Semicarbazone, Triazole and Curcumin moieties) with multiple biological activities, why they are selected for the current neuropathic pain screening studies. A section is devoted to the proteins upregulated in neuropathic and neurodegenerative states and their role in disease progression is described.

2.2 Neuropathic pain

Neuropathic pain is one of the main and predominant discomfort arise as a consequence of neurodegenerative changes in the nervous system which include inflammation, altered plasticity, demyelination and finally complete loss of neurons. Neurons are the only connective tissue which generates and conducts impulses. Any disease arising as a result of damage to neurons will cause altered sensation and each individual with such disease experience variety of symptoms as explained in the introduction chapter.

Neuropathic pain (NP) is a complex, chronic pain state generally accompanied by tissue injury. The nerve fibres in neuropathic pain might be damaged, dysfunctional, or injured which propels inappropriate signals to other pain centres consequently leading to changes in nerve function at the site and areas around the injury. NP could be caused by numerous diseases, like diabetes, herpes zoster infections, trauma, cancer, and neurotoxins. Although several genes and proteins have been identified being involved in neuropathy [Komori N *et al.,* 2007], the underlying molecular pathway is poorly understood [Ossipov MH *et al.*,2005]. Many animal models have been established to mimic the diverse symptoms of NP, which are classified into different types based on clinical demand viz. peripheral nerve injury models, central pain models, drug-induced neuropathy models, and disease-induced neuropathy models [Jaggi AS *et al.,* 2011]. Peripheral nerve injury models are most commonly used for NP studies [Calvo M *et al.,* 2012]. Peripheral nerve injury leads to abnormal signalling from the peripheral nerves through spinal cord to brain subsequently generates reproducible pain-related behaviour within defined time course. Inducing injury to different sites of the peripheral nerve results in several abnormal pain responses in the animal, which can mimic different clinical situations.

Chronic constriction injury (CCI) model (Bennett and Xie, 1988) (**Fig 2.1**) involves, a partial lesion of the sciatic nerve (common sciatic nerve formed from the fusion of L4, L5 and L6 spinal nerves) inducing four loose ligations from either left or right side around the sciatic nerve. CCI leads to a quick development of hyperalgesia (abnormally increased pain sensation) and allodynia (exaggerated pain sensation to a stimulus, which normally would not cause pain) within 72h and reaches to its peak in 12- 14 days and pain could last approximately for 1 month. The chronic constriction injury (CCI) model of neuropathic pain results in slow, edematousaxotomy of predominantly large diameter myelinated axons (90% of Aβ and Aδfibers and 30% of C fibers) by three days post-ligation (Kajander and Bennett, 1992) while the uninjured, smaller axons remain intact, but exposed to the Wallerian degenerating nerve in which many pro-inflammatory molecules are produced – a state akin to that observed in human (Bennett and Xie, 1988). The CCI model, similar to pain sensations seen in human, generates a reproducible thermal hyperalgesia and mechanical allodynia (Bennett and Xie, 1988).The CCI model mimics the clinical condition of a patient suffering from causalgia (pain related to partialperipheral nerve injuries), developed from an injury to the nerve plexus or the dorsal root.

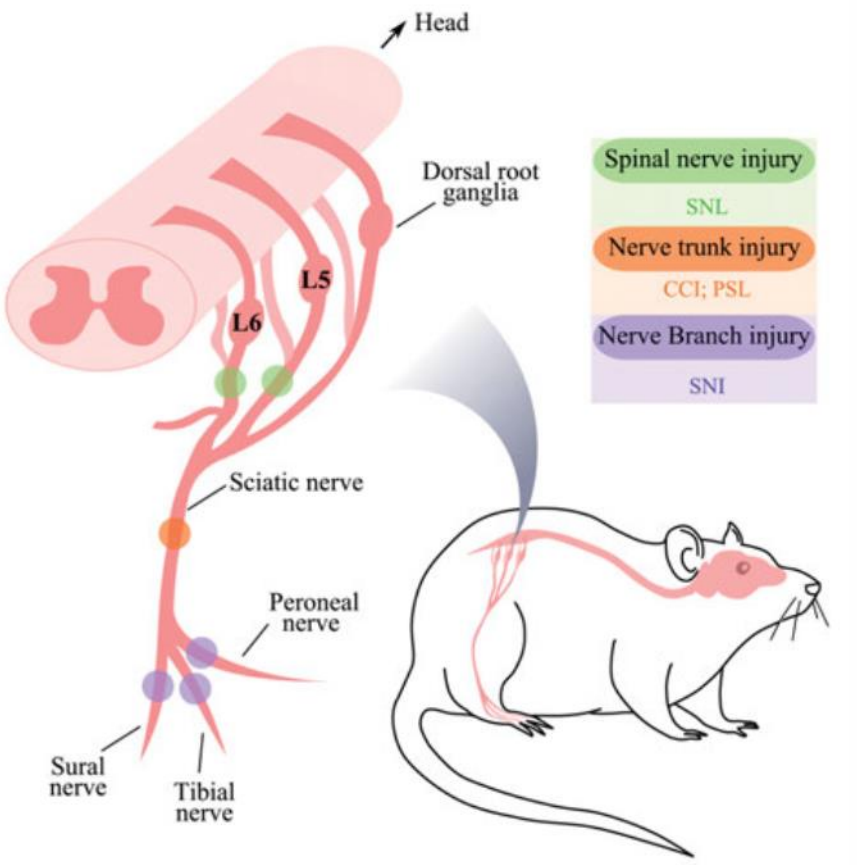


Figure 2.1: Common models of neuropathic pain evoked by peripheral nerve injury at different locations [Jaggi, AS *et al.*, 2011]. SNL: spinal nerve ligation; CCI: chronic constriction injury; PSL: partial sciatic nerve ligation; SNI: spared nerve injury.

2.3 Various pathways and targets for neuropathic pain

Neuropathic pain generation and propagation is well understood from the recent advances in pain research and now it is believed that the first synapse in pain pathway is one of the most aimed targets in producing analgesic compounds. There are several proteins involved in the neuropathic pain pathophysiology [Gangadhar *et al*., 2014].

2.3.1 Proteomics and Neuropathic Pain

The spinal cord acts as a connecting bridge between peripheral nervous system and central nervous system, which makes it essential biological system for pursuing neural transmission, pain, or drug tolerance [Monroe EB *et al.*, 2008]. Moreover NP is often mediated by neuronal

networks in the spinal cord [Campbell JN *et al.*, 2006]. Proteomic analysis of spinal cord during development or maintenance of pain could suggest the associated proteins which could serve as a diagnostic and prognostic marker, also could act as a target for treatment of NP. On the other hand, neuropeptides are known to have innumerable functions and influence physiological processes [Svensson M *et al.*, 2007]. Therefore, understanding the neuropeptides distribution in pain conditions may help to reveal the mechanism involved. Although proteomic and peptidomic methods have been frequently applied in brain research, only few studies have been focused on the spinal cord [Gil-Dones F *et al.*, 2009]. The reason for less number of studies in spinal cord may be due to its complex cellular and sub-cellular structure [Zhang CG *et al.*, 2010] and limited access to spinal cord material.

2.3.2 Sodium ion (Na+) channels

Sodium ion channels are important for the action potential generation as they are responsible for rapid depolarization of the membrane. Till date, ten transcripts encoding for the α-subunit of Na+ channels have been discovered (Nav1.1-Nav1.9) [Goldin AL *et al.*, 2000] which are expressed in different proportions in sensory neurons. Nav1.1 is mainly expressed in large neurons while Nav1.6 and Nax are highly expressed in medium and large neurons whereas Nav1.7, 1.8 and 1.9 are expressed favorably in selective neurons. Since Na+ channels are sensitive to tetrodotoxin (TTX); these channels are divided further into tetrodotoxinsensitive and tetrodotoxin resistant types.Nav1.3 and 1.7 are TTX-sensitive whereas Nav1.8 and 1.9 are considered as TTX-resistant. Since tetrodotoxin blocks the ectopic discharges that originate from injured sensory nerves involved in generation of signals in neuropathic pain, it indicates the involvement of Nav channels in the generation of ectopic discharges. This has been supported by the fact that Nav1.3 and Nax levels were increased in DRG after nerve injury [Kim CH *et al.*, 2002].On the other hand, Nav1.8, which is TTX resistant Na+ channel, is found to be downregulated in injured sensory neurons and upregulated in un-injured sensitized neurons confirming their involvement in neuropathic pain pathways [Cummins TR *et al.*, 1997].

The sodium channels Nav1.8 and 1.9 α subunits are expressed exclusively in small unmyelinated fibers, whereas the Nav1.7α subunit is expressed in sensory and sympathetic neurones.Studies have also indicated that Nav1.7, Nav1.8 and Nav1.9 play important roles in inflammatory pain

[Lolignier, S *et al.,* 2011]. Hence it could be concluded that sodium channels are implicated in neuropathic pain, though it is partly clear which channel isoforms contribute to neuropathic pain. It has been found that increased density of sodium channels in the sensory neurons after axonal injury was associated with abnormal excitability [Zhang JM *et al.,* 1997]. It has also been estimated that mRNAs encoding Nav1.1, Nav1.3and Nav1.6-1.9 were expressed in sensory neurons. Nav1.1 and 1.6 are also expressed at high levels in other neurons within CNS while the distribution of Nav1.3, Nav1.7, Nav1.8 and Nav1.9 was limited to peripheral nociceptive neurons [Cummins, T.R *et al*., 2007] and these are the sodium channels specifically targeted for neuropathic pain therapy. Na channel blocking drugs currently under research for neuropathic pain therapy are Mexiletine, carbamazepine and other anti-arrhythmic drug [Bhattacharya A et al., 2009; Kalso E. 2005]

2.3.3 Calcium (Ca^{2+}) Channels

Voltage gated Ca^{2+} channels (VGCCs) are present inalmost every cell which converts biological activity to an electrical signal. VGCCs are large proteins where α1 subunit at the center creates the pore, surrounded by auxiliary α2δ, β and γ subunits [Catterall WA *et al.,* 2000]. VGCC's can be divided in two categories: high-voltage activated (HVA) which include L-, N-, P/Q- and R-type Ca2+ channels, requiring strong depolarization for activation, and low voltage activated (LVA) T-type Ca2+ channels which can be activated by milder depolarization. L-type Ca2+ channels are mainly located in CNS whereas P/Q-, N- and R- type Ca2+ channels mostly are involved in synaptic transmission and T-type Ca2+ channels are responsible for neuronal excitability. Voltage gated calcium channels (VGCC) are another pharmacological target for pain relief. Gabapentin and pregabalin fall under the classification of gabapentinoids, which, while structurally similar to the endogenous neurotransmitter GABA, do not function as such. Instead, they bind to the $\alpha^{2-\delta}$ subunit of VGCC to reduce calcium influx into nerve terminals and thereby decrease neurotransmitter release. The $\alpha^{2-\delta}$ subunit of VGCC is highly expressed in the dorsal horn of the spinal cord, and decreasing the release of glutamate and substance P from nociceptive primary afferent neurons here is likely the main mechanism of action for drugs of this type [Tanabe M *et al.,* 2008].

2.3.4 Rho Kinase

Rho proteins comprise a subfamily of highly conserved small molecular G-proteins that belong to the Ras superfamily. In neuronal cells, RhoA is involved in the guidance and extension of axons and the development and structural plasticity of dendrites and dendritic spines [Nakayama AY *et al.*, 2000]. Several studies have suggested that RhoA regulates the stability of dendritic branches in neurons [Billuart P *et al.*, 2001]. Like PKMζ, RhoA has also been shown to play an important role in the formation of long-term potentiation in hippocampal neurons [Wang HG *et al.*, 2005]. Thus, the activation of RhoA signalling is involved in the formation of synaptic plasticity in the CNS. Injury to the adult vertebrate brain and spinal cord abnormally activated Rho kinase/ROCKs and showed inhibition of neurite growth and spouting [Mueller BK *et al.*,, 2005].Rho kinase activation causes modulation of microglial phenotype which in turn mediates mechanisms of neuroinflammation, increased ROS production and production of various cytokines [Anna-Elisa Roser *et al.*, 2017].From the literature it is understood that pharmacological inhibition of ROCK with small molecules such as fasudil and Y-27632 could be useful in neuropathic pain states [Hensel *et al.*, 2015].

2.3.5 Cytokines

Cytokines are produced by activated immune cells such as T-cells, macrophages and neutrophils upon chemical or physical stimulation. Cytokines are low molecular weight glycoproteins whose main function is intercellular communication. Cytokines are mainly produced in response to disease, inflammation of tissue damage and act as mediators regulating the functions and differentiation of neighbouring cells. They are also produced by other cell types like keratinocytes, dendritic cells of the skin, schwann cells and glial cells of the CNS. Cytokines have short half-life and act immediately as its effects are localized.

Till date many cytokines have been discovered and are categorised into five different classes such as interleukins (ILs), interferons, tumor necrosis factors, growth factors and chemokines. All the cytokines are responsible for inflammation and pain but particularly TNF-α, IL-1β and IL-6 have been associated with the development of neuropathic pain in various animal models [Leung L *et al.*, 2010].

Activated astrocytes express phosphorylated c-Jun N-terminal kinases (JNK and JNK1) phosphorylated extracellular signal-regulated kinase (ERK), endothelin receptor-B, TNF-α, basic

fibroblast factor (bFGF), neurokinin-2 receptor, IL-18 receptor and monocyte chemoattractant protein-1 (MCP-1), in response to nerve injury or inflammation. Importantly, intrathecal administration of these molecules has shown to reduce chronic pain symptoms [Gao YJ *et al.,* 2010].

IL-1 is a major pro-inflammatory cytokine and is upregulated in the spinal cord under different chronic pain conditions [Kawasaki Y *et al.,* 2008]. Studies have shown IL-1β upregulation in astrocytes after nerve injury, hind paw inflammation and masseter inflammation [Guo W *et al.,* 2007]. IL-1β was also found in neurons in the spinal cord [Fu D *et al.,* 2006]. Several reports support the important role of IL-1 in pain sensitization. Intrathecal IL-1 receptor antagonist administration had blocked the effects of IL-1 such as pain signalling and inflammation [Fu D *et al.,* 2006]. Activation of microglia produces inflammatory cytokines and glial inhibitors such as fluorocitrate and propentofylline can alter pain sensitivity [Watkins LR *et al*., 1997; Meller ST et al., 1994; Sweitzer SM *et al*., 2001]

2.3.6 Brain derived neurotropic factor (BDNF)

In addition to the above factors brain derived neurotrophic factor (BDNF) levels were found to be elevated in the spinal dorsal horn of nerve injured rat. The role of BDNF is in the central sensitization and synaptic plasticity in the spinal cord has been linked to chronic pain. It has been shown to contribute to the development and maintenance of neuropathic pain by activation of the dorsal horn NR2B-containing NMDA (NMDA-2B) receptors [Geng SJ *et al.,* 2010].Activation of certain receptors such as P2X4 was found to produce BDNF from microglia and mediated neuropathic pain. Drugs which act by inhibiting such receptors (P2X4 and CX3CR1) which produces BDNF upon activation to mediate neuropathic pain could be useful targets for pain therapy [Coull JA *et al*., 2005].

2.3.7 Tumor Necrosis Factor-Alpha (TNF-α)

TNF-α belongs to a superfamily of ligand/receptor proteins called the tumor necrosis factor/tumor necrosis factor receptor superfamily proteins (TNF/TNFR SFP). TNF-α possess a trimeric symmetry with a structural motif called the TNF homology domain (THD), which is shared with all other members of the TNF proteins. This THD binds to the cysteine-rich domains (CRDs) of the TNF receptors (TNFRs) and variations of these CRDs lead to heterogeneity of the

TNFRs. Several lines of evidence suggest that pro-inflammatory cytokines such as TNF-α released into the local milieu after injury and these may initiate the underlying cascade facilitated processing. TNFRs are either constitutively expressed (TNFR1, p55-R) or inducible (TNFR2, p75-R). TNF-α has been detected at the injury site of CCI of sciatic nerve in rats and shows temporal upregulation. TNF-α is located mainly in macrophages and Schwann cells and is detected by immuno-reactive staining. Similarly, there is local upregulation of both TNFR1 and TNFR2 as injured neurons undergo Wallerian degeneration, albeit at differential rates. TNF-α enhances the TTX - resistant Na+ current in cultured DRG cells from wild-type but not from TNFR1-knockout mice and such current is abolished by a p38-MAPK inhibitor; implying that TNF-α acts via TNFR1 and activates TTX-R Na+ channels via the p38 MAPK.

Studies have shown that after nerve injury, TNF-α expression increases in dorsal root ganglia (DRG) [Schaefer AT *et al.,* 2003] and spinal cord [Hashizume H *et al.,* 2000]. The inhibition of TNF-α reduced the hyperalgesia associated with two models of neuropathic pain: chronic constriction injury and partial nerve transection [Schäfers M *et al.,* 2003].

2.3.8 Matrix Metalloproteinases (MMPs)

Matrix metalloproteinases (MMPs) are zinc dependent endopeptidases that play essential role in a wide range of proteolytic processes. So far over twenty members have been identified in this family. The main function of MMP's is to cause neuroinflammation by cleavage of extracellular proteins. They are also involved in activation of other cytokines and chemokines. Their role is also implicated in cellular differentiation, signalling, migration, survival and apoptosis by acting on additional substrates like proteinases, chemotactic factors, growth factors, cell surface receptors and cell adhesion molecules [Page-McCaw A *et al.,* 2007].

The mode of action of MMPs is thought to be associated with non-neuronal pathways. Extensive studies were conducted on temporal and cellular profiles of MMP expression in the injured spinal cord and the most important MMPs which were thought to propagate and maintain neuropathic pain were identified as MMP-2, MMP-9 and MMP-24 [Zhang H *et al.,* 2011].

Increased levels of MMP-2 and MMP-9 immunoreactivity were found in nerve tissue in chronic inflammatory demyelinating polyneuropathy (CIDP) and non-systemic vasculitic neuropathy (NSVN), compared to non-inflammatory neuropathies (NINs) [Leppert D *et al.,* 1999].

In last few decades, much effort has been directed towards understanding the mechanisms of neuropathic pain. As illustrated above, there is now abundant evidence for various promising targets which have already shown a significant degree of efficacy in terms of chronic pain. But none of these targets have shown complete pain relief in long term, which in turn underscores the need of newer and better pain therapeutics. Hence in this thesis, an attempt was made to explore new pharmacophores in neuropathic pain and neurodegeneration therapy. Further studies would be required to make sure that these new therapeutic targets possess better efficacy with lesser side effects. From the literature it is understood that MMP-9 and MMP-2 inhibitors could be useful in the management of neuropathic pain [Shaheen E *et al*., 2012].

Neuropathic pain (NP) emerged as a huge clinical problem as available treatment options do not meet the patient compliance in providing analgesic effect. Extensive literature survey was performed to select three different chemical moieties having GABA inhibitory activity, Anti-inflammatory activity and anti-oxidant properties in common and were synthesized based upon the structures obtained from BITS in-house database.

2.4 Semicarbazone moiety

Semicarbazones are generally derived of imines by the condensation of aldehydes or ketones with semicarbazide [NH NHC (=O) NH] and classified as imine derivatives. In this thesis, advances made in the application of semicarbazones as a versatile pharmacophore model for the design of new drugs for neuropathic pain and neurodegenerative disorders are updated. Some reviews on the biological activity of semicarbazones and thiosemicarbazones [Pandeya SN *et al.,* 1993] and their metal complexes [Beraldo H *et al.,* 2004] are mentioned to focus on their useful biological properties **Fig 2.2**.

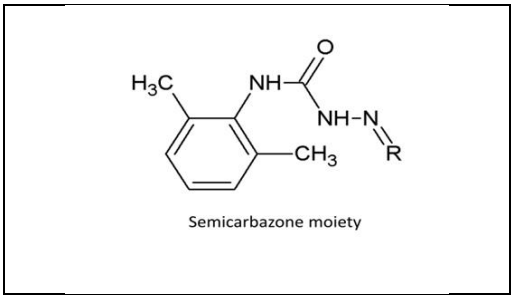


Fig 2.2: Semicarbazone moiety

Till date semicarbazone moiety containing compounds with various substitutions on proximal, distal auxiliary binding site and semicarbazono handle for H-bonding have shown anticonvulsant

action. During literature survey semicarbazone compounds were found to possess various pharmacological activities including anticonvulsant, antitumor, antioxidant, antipyretic, analgesic, anti-inflammatory, antimicrobial activity and Na+ channel blocking activities Nain S*et al.,* 2015]. Considering the multiple biological effects of semicarbazone moiety, we understood the importance of its derivatives for their potency on neuropathic pain and neurodegeneration. There were three major research groups which contributed extensively in semicarbazones, they are described as below.

2.4.1 Dimmock *et al.,* work on semicarbazones:

A major achievement of Dimmock [Dimmock JR *et al.,* 1999] was the identification of 4-(4'-fluorophenoxy) benzaldehyde semicarbazone as a lead molecule (**Fig. 2.3**) Derivatives of this molecule were highly potent and were patented [Wang Y *et al.,* 2003].He proved the anticonvulsant property of semicarbazone moiety. The majority of compounds demonstrated no neurotoxicity upto doses of $500mg.kg^{-1}$. Among the phenoxybenzaldehydeanalogs, halobenzosubstituted derivatives were the most active ones. Further, exploring the structural changes, various heterocyclic, carbocyclic and fused rings were introduced in the molecule in place of the aryloxy group [Wang Y *et al.,* 2003]. The heterocycles included thiophene, pyrrole, furan, imidazole, pyridine, pyrazine, pyrimidine, phenothiazine, quinoline, indole, benzoxazole and benzimidazole. The carbocyclic groups included cyclohexyl and cycloheptyl. 4-(3,4-methyleneoxyphenoxy) benzaldehyde semicarbazone had MES (mice, *p.o.*) ED_{50} of $5.3mg.kg^{-1}$. Several other substituents showed promising activity and ability to inhibit human skeletal muscles. The Na+ subchannel subunit stable expression in HEK-293 cells was studied [Wang Y *et al.,* 2003]. Since 4-(4-fluorophenoxy) benzaldehyde semicarbazone metabolized to the corresponding semicarbazide, some novel substituted semicarbazides were prepared and found active [Cai SX *et al.,* 2001]. Semicarbazone act as base due to presence of N-1 nitrogen though it was not a base as N-2 nitrogen was slightly acidic in nature. The structure of semicarbazone was found partly rigid due to C=N bond, while –C-N- single bond in semicarbazide makes it relatively non-rigid thereby altering its biological functions.

Acetophenone semicarbazone [Dimmock JR *et al.,*1993] and phenyl acetaldehyde semicarbazone [Dimmock J R et al.,, 1993] protected against seizures induced by scPTZ (ED_{50} 60.75 mg kg^{-1}) and subcutaneous bicuculline (scBIC) (ED_{50} 57.63mg kg^{-1})

Arylsemicarbazonescould be predominantly used to treat grandmal epilepsy because they were active against MES and have been shown to act through Na^+ channel blocking effect.

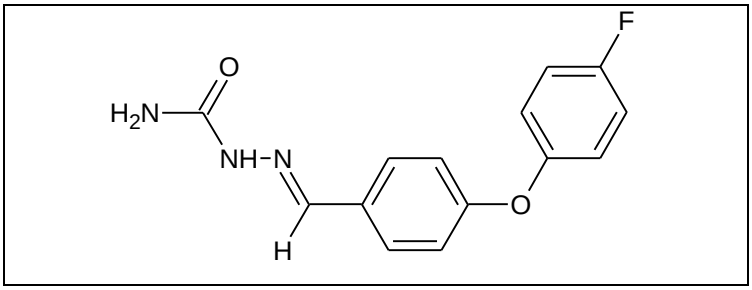
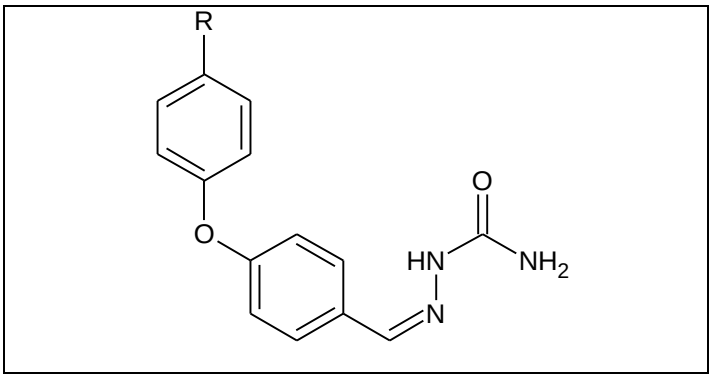


Figure 2.3 4-(4′-fluorophenoxy)benzaldehyde semicarbazone

Modifications in the distal aryl ring

The hydrazono terminal has been reported with a wide spectrum of aldehydes and ketones having aliphatic, aromatic, alicyclic and heterocyclic moieties. From these studies, three compounds emerged as the most potent anticonvulsants, which were investigated in various phases of drug development, 4-fluorophenoxy benzaldehyde semicarbazone, 4-bromobenzaldehyde semicarbazone and 3,4-dimethoxy benzaldehyde-N4-(p-chlorophenyl) semicarbazone. 4-Fluorophenoxy benzaldehyde semicarbazone displayed MES ED_{50} (*i.p*.) 12.9mg.kg^{-1}; scPTZ> 54mg.kg^{-1}, with MES, PI (protective index) of 3.09. This compound was orally active with very high PI (MES) > 315. The oral ED_{50} was 1.59mg.kg^{-1}. It was more potent compared to clinically effective drugs such as phenytoin (PI > 21.6), carbamazepine (PI 101) and Na-valproate (PI 2.17) [Dimmock JR *et al.*, 1999].Dimmocket al., [Dimmock JR*et al.*,, 1999] isolated the major inactive urinary metabolite, 4-fluorophenoxy phenylcarboxysemicarbazide, from 4-(4-fluorophenoxy) benzaldehyde semicarbazone after oral dosing to rats. On the other hand, Ramu*et al.*, [Ramu K *et al.*, 2000]studied the pharmacokinetics of this compound (C0102862) in rats. Its half-life was 14 days and renal excretion was 74%. The main metabolite was the corresponding carboxylic acid. They also developed a HPLC method for the determination of pharmacokinetics of C0102862 in mouse, rat, monkey and dog plasma [Ramu K *et al.*, 2000; Lam NC *et al.*, 2000] patented a large number of substituted phenoxybenzaldehyde semicarbazones as anticonvulsants and sodium channel blockers (**Fig. 2.4**). Micale*et al*[Micale N *et al.*, 2002].studied the anticonvulsant property of 2-[(4-alkyl semicarbazono-(4-aminophenyl) methyl-4, 5-methylenedioxy phenylacetic acid alkyl esters.



R=H, F, Cl, Br, 3,4-diF, 4-Cl, 2F, CF_3, NO_2, CH_3, OCH_3, 3-CH_3, 3-CH_3, 4-C_3H_7, 5-C_4H_9, t-C_4H_9, 3,4-CH-O-O–C_6H_{11}, C_7H_{13}, 5-indanyloxy, 6-quinolyoxy.

Fig 2.4 Substituted phenoxy/alicyclic/heteroaryl oxybenzaldehyde semicarbazones

2.4.2 Pandeya*et al.,* work on semicarbazones:

Pandeya*et al.,* [Pandeya SN *et al.,* 1998]synthesized thiouriedo derivatives of acetophenone semicarbazone. Compound 4-(N' methylthioureido) acetophenone had MES (i.p.) ED_{50} 23.5 mg kg^{-1} compared to phenytoin with ED_{50} of 23.2 mg kg^{-1}. This compound showed a better protective index (PI = TD_{50}/ED_{50}) than sodium valproate (2.55 vs. 2.17) and was also active in the subcutaneous pentylenetetrazole (scPTZ) test. A rigid analog of semicarbazone linker was prepared, a 2-[4-(4-Chlorophenyl-2-fluorophenoxy) phenyl]-pyrimidine-4-carboxamide was found to be a potent, broad spectrum state-dependent sodium channel blocker for treating pain states. This compound was approximately 1000 times more potent and had 2000-fold faster binding kinetics and 10-fold higher levels of state dependence than carbamazepine and lamotrigine. It showed ED_{50} at least 13mg.kg^{-1} (*p.o.*) in partial sciatic nerve ligation [Ilynin JD *et al.,* 2006].

The semicarbazones linker provided two essential requirements for its biological activity. The carboxamide group was implicated in hydrogen bonding. Pandeya *et al.,* [Pandeya SN *et al.,*2003] replaced the CONH-moiety with non-hydrogen bonding –O-CH2– moiety. Whereas compounds with –CONH– were active in both the MES and scPTZ tests, compounds with the –O-CH2– moiety were completely devoid of any anticonvulsant activity, clearly demonstrating

the importance of hydrogen bonding characteristic of the semicarbazono linker [S. N. Pandeyaet al.,, 2003]. Substitution of the carboxamido hydrogen with ethyl group [S. N. Pandeya*et al.,* 2003] produced inactive compounds. Continuing work based on the role of –CONH– in hydrogen bonding,substitution by a methyl group yielded active compounds, compared to diazepam with a desmethyl active metabolite.

In an another study, phenacylhydrazones lacking the hydrogen bonding characteristic also exhibited no anticonvulsant activity [Pandeya SN *et al.,* 2001]. Semicarbazono linker was replaced with a rigid pyrimidine ring to yield 2-[4-(4-chloro-2-fluorophenoxy) phenyl]-pyrimidine-4-carboxamide [Ilynin VI *et al.,*2006]. This compound was approximately 1000 times more potent, and had 2000-fold faster binding kinetics than carbamazepine and lamotrigine tested on recombinant rat Na(v) 1.2 channels and native Na(+) currents in cultured rat dorsal root ganglion neuron (ED_{50} 13mg.kg^{-1}p.o. in partial nerve ligation).

2.4.3 Yogeeswari *et al.,* work on semicarbazones:

Semicarbazones are also GABA-transaminase inhibitors. Extensive structure-activity relationship has demonstrated that F, Cl, Br and NO_2 substituents in the aryl hydrophobic pocket and a hydrogen bonding domain (HBD) are generally found in active anticonvulsant agents. Substituents on the aryl ring and substituents in the carbimino terminal was compared to derive structure-activity relationship of the synthesized derivatives [Yogeeswari p *et al*., 2006].

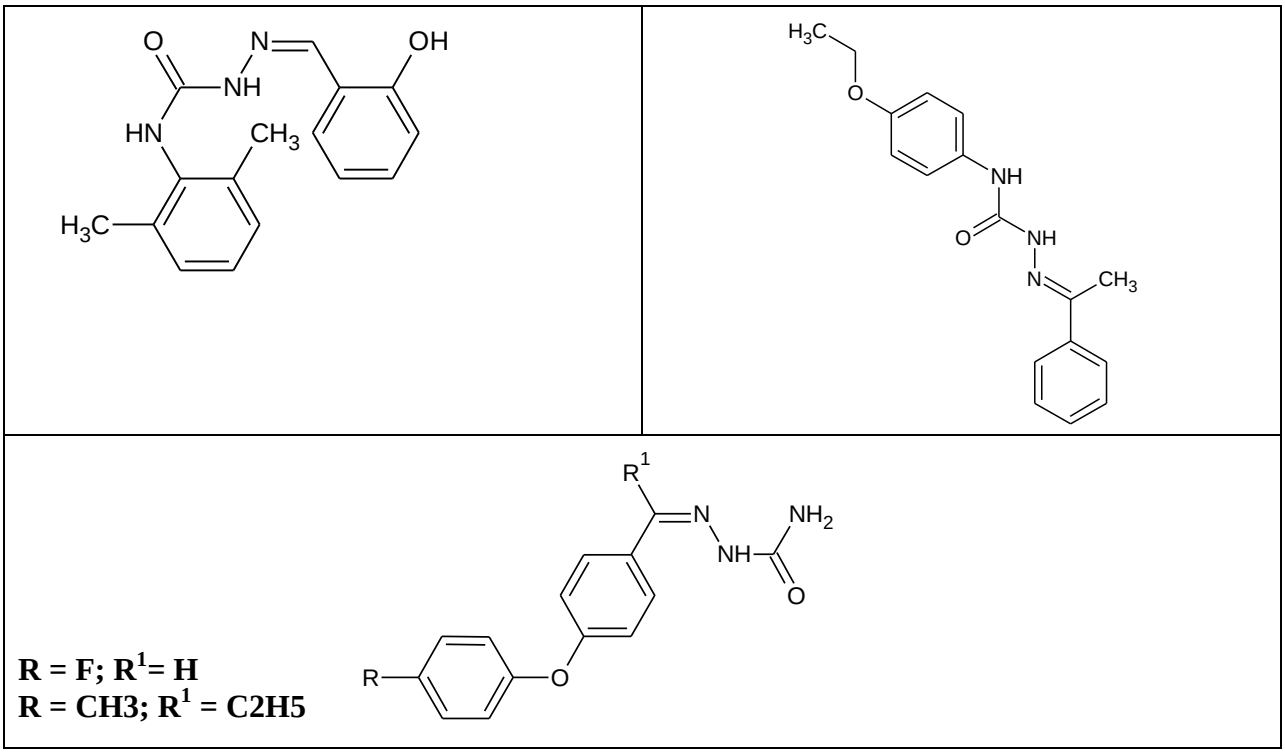


Fig 2.5: Semicarbazone derivatives having GABA transaminase inhibition properties

In the series of N-(2,6-dimethylphenyl) substituted semicarbazones, a large number of compounds were found active at a dose of 30mg.kg^{-1} in scSTY tests. Therefore, these compounds could be assumed to act through glycine receptors [Yogeeswari P *et al.,* 2005]p-nitrophenyl substitution on semicarbazone also showed activity in scSTY tests. Extensive research was performed by Yogeeswari et al., anticonvulsant activity and safety profile of anilide and hydrazine pharmaocophore[Yogeeswari P *et al.,* 2007], 4-Sulphamoylphenyl semicarbazones [Yogeeswari P *et al.,* 2004], N-(2,6-Dimethylphenyl)-Substituted Semicarbazones [Yogeeswari P *et al.,* 2005], γ-aminobutyric acid (GABA) derivatives with the combination of thiosemicarbazone [Yogeeswari P *et al.,* 2008], 4-(2-(2,6-dimethylphenylamino)-2-oxoethylamino)-N-(substituted)butanamides [Yogeeswari P et al., 2007], N4-phthalimido phenyl (thio) semicarbazides [Yogeeswari P et al., 2003], N4-(2, 5-dimethylphenyl/-2-fluoro-5-methyl phenyl) Semicarbazones [Yogeeswari P *et al.,* 2006],N4-(2,4-dimethylphenyl) semicarbazones as 4-aminobutyrate aminotransferase inhibitors [Yogeeswari P *et al.,* 2006], N-(3-Methylpyridin-2-yl)-substituted Semicarbazones as anti-convulsants [Yogeeswari P *et al.,* 2006], Na+channel blocker [4-(4-fluorophenoxy)benzaldehyde semicarbazone[Yogeeswari P *et*

al., 2007], Antiallodynic and antihyperalgesic activities of anticonvulsant gaba derivatives of thiosemicarbazones [Yogeeswari P *et al*., 2010].

2.4.4. Mechanism of action of semicarbazones

Various researchers have studied the mechanism of action of semicarbazones as anticonvulsants. Ilynin *et al.,* [Ilynin VI *et al.,* 2005] found 4-(4-fluorophenoxy) benzaldehyde semicarbazone (V102862; C0102862) a potent, broad-spectrum blocker of mammalian voltage-gated sodium channels. V102862 was found to block Na+ currents in acutely dissociated cultured rat hippocampal neurons. V102862 was a potent state dependent blocker of receptor Na 1, 1.2 channels with a KI (ligand/receptor association constant) of ~ 0.4 mmol.$L^{-1}$and KR [binding of the ligand for resting state (R) of the receptor] ~30mmol.L^{-1}. V102862 shifted the steady-state availability curve in the hyperpolarizing direction and significantly retarded recovery of Na+ channels from inactivation. Thus, inhibition of voltage-gated Na+ channels was the major mechanism underlying the anticonvulsant property of V102862. This agent also displayed ~80 fold higher affinity for inactivated Na+ channels compared to channels in the resting state. Yogeeswari *et al.,* [Yogeeswari P *et al.,* 2005]studied the effect on GABA level in different parts of the brain. N1-(2, 6-Dimethylphenyl)-N4-(2-hydroxybenzaldehyde) semicarbazone increased the GABA level greater than 100% and was found to inhibit the GABA transaminase enzyme activity both *in vitro* and *in vivo*. N-(4-Ethoxyphenyl)-N4-(2-hydroxyacetophenone) semicarbazone was reported to increase the GABA level only in the medulla oblongata region [Yogeeswari P *et al.,* 2005].

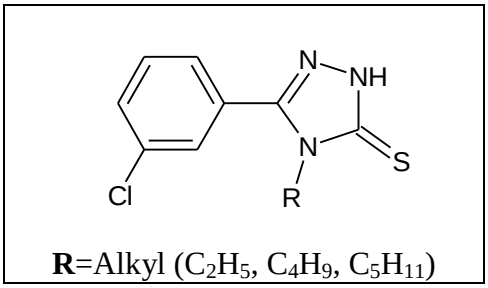
Semicarbazones have been developed as versatile pharmacophores. Two compounds, 4-(4-fluorophenoxy) benzaldehyde semicarbazone (FPBS) and N'-(4-chlorophenyl)-N4-(2-nitrobenzaldehyde) semicarbazonewere identified as lead molecules.FPBS was found to be a sodium channel blocker while the others were GABA transaminase inhibitors. Other mechanisms of action, like glycine receptors and non-competitive channel blockers, proposed by Pandeya*et al.,* [Pandeya SN *et al.,* 2012], A versatile pharmacophore model was suggested by Pandeya*et al.,* [Pandeya SN *et al.,* 2012], for future drug design and development of anticonvulsants and analgesics.

2.5 Triazole moiety

Azoles form a large group of organic substances having wide range of biological activities. These are five membered heterocyclic ring structured molecules. Triazole is a five membered azole with three nitrogen atoms in the ring and are reported to have numerous biological activities such as anti-inflammatory [ZouXJ *et al.,* 2002], antiviral [Chen H *et al.,* 2000; Holla BS*et al.,* 2002], CNS stimulant [Schenone S *et al.,* 2001], anti-HIV, anti-HBV [EL-Barbary AA *et al.,* 2004], antibacterial [EI-Sayed R *et al.,* 2006; Varvaresou A *et al.,*2000; Erol *et al.,*2001], antitumor [Pintilie O *et al.,* 2007] and antituberculosis [Heindel ND *et al.,* 1980] activities. Triazole moiety could be seen in many drug molecules for example anastrozole, ribavirin, estazolam and triazolam.

2.5.1 Triazoles as anticonvulsants

T. Plech*et al.,* [Plech T *et al.,* 2013] synthesized some 4-alkyl-1,2,4-triazole derivatives and also observed the effect of the size of the alkyl fragment on anticonvulsant activity. The compound 4-alkyl-1,2,4-triazole-3-thione derivatives (**Fig 2.6**) showed significant anticonvulsant activity when estimated in mice brain tissue using chromatography method with diode array detector (DAD). It was reported that alkyl substitution at N-4 position led to biological activity which was on par with standard anticonvulsant drug valproate. It was also reported that lack of permeability through blood brain barrier (BBB) was the reason for lack of biological activity of some compounds in this series.



R=Alkyl (C_2H_5, C_4H_9, C_5H_{11})

Fig 2.6: 4-Alkyl-1, 2, 4-triazole derivative

2.5.2 Triazoles as immunomodulators

Novel α-santonin derived 1,2,3-triazoles (**Fig 2.7**) were reported by Chinthakindi PK *et al.*,[Chinthakindi PK *et al.*,2016] through Azide-alkyne Huisgen 1,3-dipolar cycloaddition reaction between substituted aryl azide and a propargylatedadesmotrosantonin [Chinthakindi PK *et al.*, 2013]. These molecules were evaluated biologically for their duminitive effect ConA induced T-cell and LPS induced B-cell proliferation. Surprisingly most of the synthesized molecules showed better immunosuppressant activity than the parent santonin. The structure of santonin derived triazole is represented in **Fig2.7**.

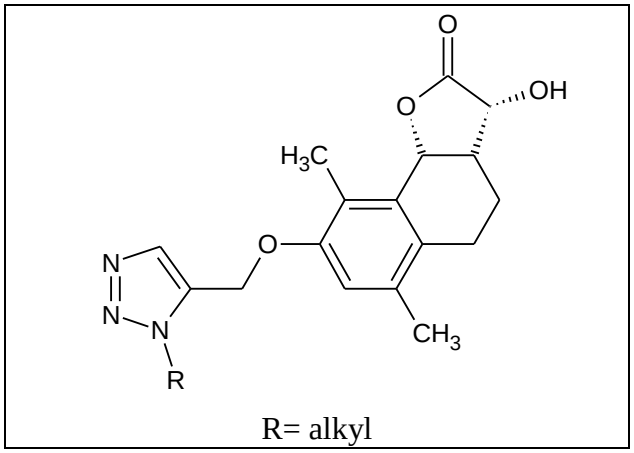


Fig 2.7: α-Santonin derived 1,2,3-triazole derivative

2.5.3 Triazoles as anti-inflammatory agents

Haider S *et al.*,[Haider S *et al.*, 2013] have synthesized benzoxazolinone-1,2,3-triazole moieties (**Fig 2.8**)by a process of methylene linkage conjugation and evaluated their anti-inflammatory activity and found to be potent anti-inflammatory agents with least side effects. These molecules were also found to possess anti-nociceptive activity.

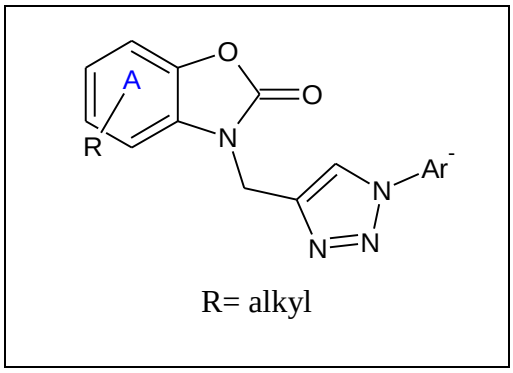


Fig 2.8: Benzoxazolinone-1,2,3-triazole moieties

Hunashal RD *et al.,* [Hunashal RD *et al.,* 2011] reported the synthesis, anti-inflammatory and analgesic activity of another triazole compounds, 2-[4-(substituted benzylideneamino)-5-(substitutedphenoxymethyl)-4H-1,2,4-triazol-3-yl thio] acetic acid derivatives.

2.5.4Triazoles as CDK5/p25 inhibitors for AD treatment

Shiradkar M*et al.,* [Shiradkar M*et al.,* 2011] reported that novel CDK5/p25 inhibitors prepared by clubbing triazole and thiophene (**Fig 2.9**) were found to be effective in Alzheimer's disease.

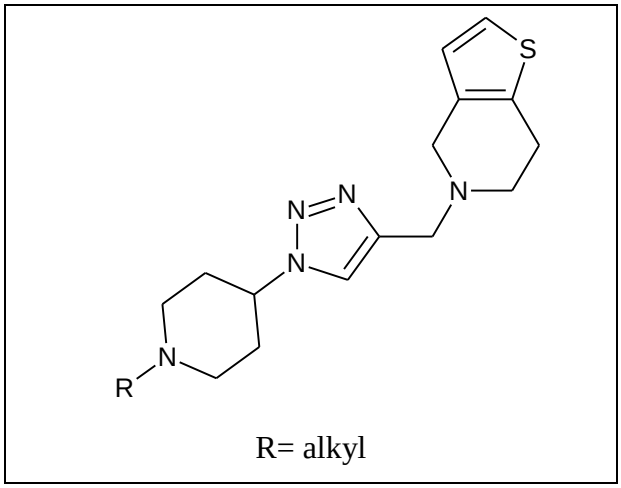


Fig 2.9: Clubbed triazolylthiophene

2.5.5 Triazoles as anti-oxidant agents

Bhalgat CM *et al.,* [Bhalgat CM *et al.,* 2011] synthesized some new dihydropyrimidinecarbonitrile and its triazole fused derivatives (**Fig 2.10**). The novel derivatives

were characterized by spectral data and elemental analysis,and were evaluated for their antioxidant and anti-inflammatory screening

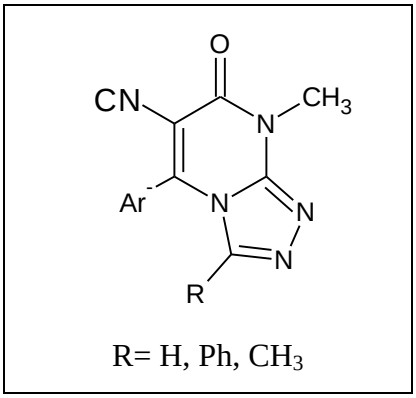


Fig 2.10: dihydropyrimidinecarbonitrile and its triazole fused derivative

Raghunath Saundane A *et al.,* [Saundane RA *et al.,* 2011] reported the synthesis, antimicrobial and antioxidant activities of 2-oxo-6-phenyl-2-yl-4-(20-phenyl-50-substituted-1H-indol-30-yl)-1,2-dihydro pyridin-3-carbonitriles and their derivatives. Gürsoy-Kol O. et al [Gürsoy-Kol Ö*et al.,* 2013] synthesized 4,5-dihydro-1H-1,2,4-triazol-5-one derivatives and also investigated by using different antioxidant methodologies such as: reducing power, 1,1-diphenyl-2-picryl-hydrazyl (DPPH) free radical scavenging and metal chelating activities.

The pharmacological potential of triazole nucleus was very clear from these literatures.These literatures revealed that triazole nucleus possessed diverse biological potential, easy synthetic routes for the production and attracted researchers for the development of new neuro therapeutic agents. From these observations the importance of the triazole nucleus is being highlighted.

2.6 Curcumin moiety:

Curcumin is an active ingredient of commonly available spice turmeric, chemically known as diferuloyl methane. Curcumin is a hydrophobic polyphenol obtained from the rhizomes (turmeric) of the herb *Curcuma longa*. The beneficial effects of turmeric used traditionally for many ailments are because of curcumin (**Fig 2.10**) which has wide spectrum of pharmacological activities (**Fig 2.11**).

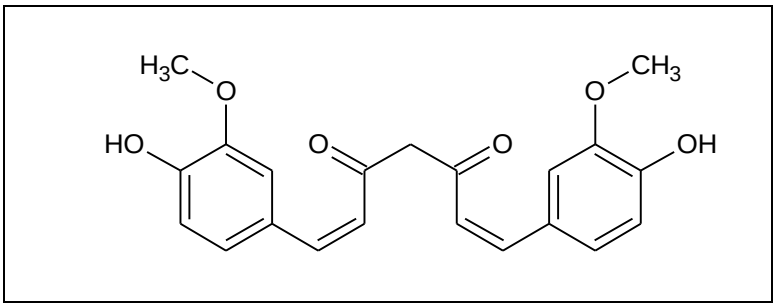


Figure 2.11: Curcumin (Diferuloyl methane)

Most of the medicinal properties of turmeric have been attributed mainly to the curcuminoids and the main component present in the rhizome include curcumin (diferuloylmethane)-(1,7-bis (4-hydroxy-3-methoxyphenyl)-1,6-hepadiene-3,5-dione).

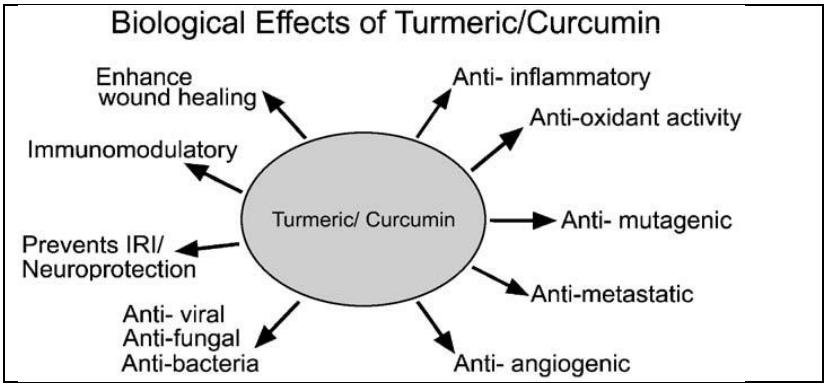


Figure 2.12: Biological effects of Curcumin

2.6.1 Anti-oxidant and anti-inflammatory activities of curcumin:

Oxidative stress is a major player in the pathogenesis of various diseases including myocardial ischemia, cerebral ischemia–reperfusion injury, haemorrhagic shock, neuronal cell injury, hypoxia and cancer. Curcumin exhibits strong antioxidant [Toda *et al.,* 1985] and anti-inflammatory properties and was shown to be a potent scavenger of a variety of reactive oxygen species including superoxide anion radicals, hydroxyl radicals [Reddy and Lokesh, 1994; Martin S *et al.,* 1997] and nitrogen dioxide radicals [Unnikrishnan and Rao, 1995; Sreejayan and Rao, 1997]. Number of studies have reported the protective effect of curcumin to oxidative cell injury of kidney cells (LLC-PK1) by inhibiting lipid degradation, lipid peroxidation and cytolysis [Cohly et al.,, 1998 models; Reddy and Lokesh, 1992]. Curcumin prevented oxidant mediated injury by increased heme oxygenase production in vascular endothelial cells [Motterlini R *et al.,*

2000]. Curcumin was found to protect rat myocardium against isoprenaline (ISO) induced myocardial ischemic damage by inhibiting free radial generation [Manikandan et al.,, 2004] and inhibition of lysosomal enzyme release [Nirmala C et al.,, 1999]. Curcumin has shown beneficial effects on renal injury by its ability to inhibit the expression of the apoptosis-related genes,Fas and Fas-L [Jones Jr HRet al.,, 2000]. Studies in our laboratory have shown that pretreatment with curcumin resulted in significant restoration of the liver cytokines IL-1alpha, IL-1beta, IL-2, IL-6, and IL-10 to normal levels that were increased by hemorrhage/resuscitation regimen in rats. In fact, IL-1beta levels were lower than sham levels. NF-kappaB and AP-1 were differentially activated at 2 and 24 h post-hemorrhage and were inhibited by curcumin pretreatment [Gaddipati *et al.,* 2003].

Curcumin an antioxidant containing two electrophilic β-unsaturated carbonyl groups, which react with nucleophiles such as glutathione [Awasthi S *et al.,* 2000].Dinkova-Kostova AT *et al.,[*[Dinkova-Kostova AT *et al.,,* 2001]and Ramos-Gomez M *et al.,*[Ramos-Gomez M *et al.,,* 2001] had demonstrated that curcumin and several other polyphenolic compounds induced activities of Phase I and Phase II detox systems e.g., inhibition of COX-1 and COX-2 enzymes and stimulated glutathione-S-transferase [Dinkova-Kostova A T et al.,, 2001; Ramos-Gomez M et al.,, 2001; Venkatesan N., 1998]. A part from its ability to scavenge carcinogenic free radicals [Masuda T et al.,, 1999; Abe Y et al.,, 1999], curcumin also interfere with cell growth through inhibition of protein kinases. Several times more potent than vitamin E in reducing oxidative stress [Zha0 B l et al.,, 1989]. Dietary supplementation of curcumin showed beneficial effects in neurodegenerative diseases such as Alzheimer's disease [Calabrese *et al.,* 2003; Yang *et al.,* 2005]. In a focal cerebral ischemia model of rats, curcumin offered significant neuroprotection [Thiyagarajan and Sharma, 2004].The anti-inflammatory effect of curcumin was most likely mediated through its ability to inhibit cyclooxygenase-2 (COX-2), lipoxygenase (LOX), and inducible nitric oxide synthase (iNOS). These enzyme mediates various inflammatory processes. Because inflammation is closely linked to tumor promotion, curcumin with its potent anti-inflammatory property is anticipated to exert chemo-preventive effects on carcinogenesis.A standardized preparation of curcuminoids (NCB-02) have been tested against Type-2 Diabetes Mellitus, and the outcomes advocates improved endothelial function accompanied by reduced oxidative stress, inflammatory and pro-inflammatory markers for diabetes [Usharani P *et al.,* 2008].

2.6.2 Role of curcumin in neurological disorders

Curcumin has been reported to have positive effect on various neurodegenerative disorders viz. Alzheimer's and Parkinson's disease. Reports suggest that curcumin was able to cross blood brain barrier (BBB) [Yang F *et al.,* 2005] and reduced plaque formation and amyloid beta accumulation in mouse model of Alzheimer's disease [Garcia-Alloza M *et al.,* 2007]. Studies had also revealed that curcumin was a good candidature against Parkinson's disease in rodent models which can be justified by improved behavioral deficits, increased dopamine level and enhanced neuron survival in the substantia nigra, and also along with its anti-oxidant activity and ability to cross BBB [Zbarsky V etal., 2005; Yu S *et al.,* 2010, Jagatha B *et al.,* 2008; Rajeswari A *et al.,* 2008].

Epilepsy is one of the chronic neurological disorder in which curcumin showed promising preclinical effects. The animals treated with curcumin displayed decreased seizures and less cognitive impairment on epilepsy induced by pentylenetetrazole and kainic acid [Mehta J*et al.,* 2010, Gupta YK *et al.,* 2009]. Mounting evidence over the past several years has indicated curcumin's efficacy in various animal models of psychiatric disorders. For example, curcumin exhibited anti-depressant activity potentiated by the concomitant administration of fluoxetine, venlafaxine, or bupropion [Kulkarni SK *et al.,* 2008]. Curcumin is also identified to abrogate olfactory bulbectomy-induced major depression in a rat model [Xu Y *et al.,* 2005]. Curcumin was also investigated for its potential against diabetic encephalopathy [Kuhad A *et al.,* 2007], encephalomyelitis [Verbeek R *et al.,* 2005], intracerebral hemorrhage [King MD *et al.,*2011], spinal cord injury [Sahin KH *et al.,* 2011], cerebral malaria [Waknine-Grinberg JH *et al.,* 2010], convulsions [Bharal N *et al.,* 2008], brain ischemia [Shukla PK *et al.,* 2008] and neuropathic pain [Sharma S et al.,, 2006].

The comprehensive activity of curcumin is associated with a marked decrease in serum levels cytokines like interleukin-β, interleukin-6, tumor necrosis factor-α [Gupta A *et al.,* 2009], and an increase in expression of brain derived neurotrophic factor (BDNF) [Wang R *et al.,* 2008]. A study has concluded that the neuroprotective effects of curcumin might be mediated through the BDNF signalling pathway [Wang R *et al.,* 2008].

2.6.3 Curcumin-problem of poor bioavailability

Although curcumin showed therapeutic efficacy against many human ailments, one of the major problems with curcumin is its poor bioavailability [Anand P *et al.*, 2007], which appear to be primarily due to poor absorption, rapid metabolism, and rapid systemic elimination. Therefore, efforts have been made to improve its bioavailability by improving various features. Piperine, when administered along with curcumin was found to block the metabolic pathway of curcumin consequently increasing the bioavailability of curcumin by 2000% [Shoba G *et al.,* 1998]. However, the effect of piperine on curcumin's bioavailability was witnessed greater in humans as compared to rodents [Anand P *et al.,* 2007]. Other reliable approaches to increase the bioavailability of curcumin include use of nanoparticles [Tiyaboonchai W *et al.,* 2007], liposomes [Kurzrock *et a.* 2005], micelles [Suresh D *et al.,* 2007] , phospholipid complexes [Liu A *et al.,* 2006] , and structural modifications [Preetha A *et al.,* 2007; Ohori H *et al.,* 2006].

Curcumin has been shown to target multiple signalling molecules and has shown activities at the cellular and organism levels that provide a basis for its use against multifactorial human diseases. Although most of the currently available mono-targeted therapies are associated with numerous side effects, curcumin has been reported to be safe in clinical studies even at dosing levels in grams. In spite of ample evidences supporting therapeutic efficacy of curcumin against various diseases *in vitro* and *in vivo*, it has yet not been approved for treatment clinically. Therefore, more extensive and well-controlled clinical studies are required to validate the safety and efficacy of curcumin. Since these three moieties possess numerous biological properties and specifically properties such as anti-inflammatory, anti-oxidant, anti-cancer and anti-convulsant which are useful in neuropathic pain states and neuroinflammatory diseases, we have chosen these core moieties as derivatives to evaluate their efficacy against neuropathic pain and neurodegenerative candidate rodent models.

Current research in neuropharmacology has provided in-depth information on various targets such as proteins, receptors, ion channels, small peptide molecules (pro inflammatory mediators) that play a pivotal role in neurodegenerative disease generation and propagation. A brief description of these factors which influence the disease states is necessary to understand the disease modalities.

CHAPTER-3

Objectives & plan of work

3.1 Objectives

After thorough review of the literature, it was strongly felt that compounds having GABA inhibitory properties could be further screened to evaluate their potency in alleviating neuropathic pain symptoms and neurodegeneration. Neuropharmacological screening studies were considered important and hence the thesis work was focused on the following objectives.

The objective of the proposed work was to identify novel potential target modulators for the treatment of CNS disorders like neuropathic pain, inflammation and neurodegeneration by utilizing the knowledge from the fields of behavior pharmacology and drug design, bioinformatics, biotechnology and receptor pharmacology. We utilized extensive pre-clinical studies using various animal models followed by *in vitro* cell based assays to understand the molecular mechanisms by which compounds elicited their antinociceptive properties.

3.2 Plan of work

Extensive literature survey was performed to select three different chemical moieties having GABA inhibitory activity and were synthesized based upon the structures obtained from BITS in-house database [15 Semicarbazone (MG1 to MG15), 6 Triazole (MG16 to MG21) and 4 Curcumin derivatives (MG22 to MG25)].

3.2.1 Phase-1 (MG1 to MG25)

a. Neurotoxicity and cytotoxicity studies to evaluate compounds safety profile.

b. Anti-neuropathic pain screening in three rodent models (CCI and PSNL).

3.2.2 Phase-II (MG2, MG17 and MG24)

a.Anti-neuropathic pain screening of potential candidates in diabetic neuropathy model (DPN).

b. Quantification studies (ED_{50}) for potential compounds.

c. Protective effect of potential compounds evaluated against methyl mercury (MeHg) induced neurodegeneration in rodents.

d. Anti-inflammatory screening in carrageenan induced rat paw edema model.

3.2.3 Phase-III (MG1 to MG25)

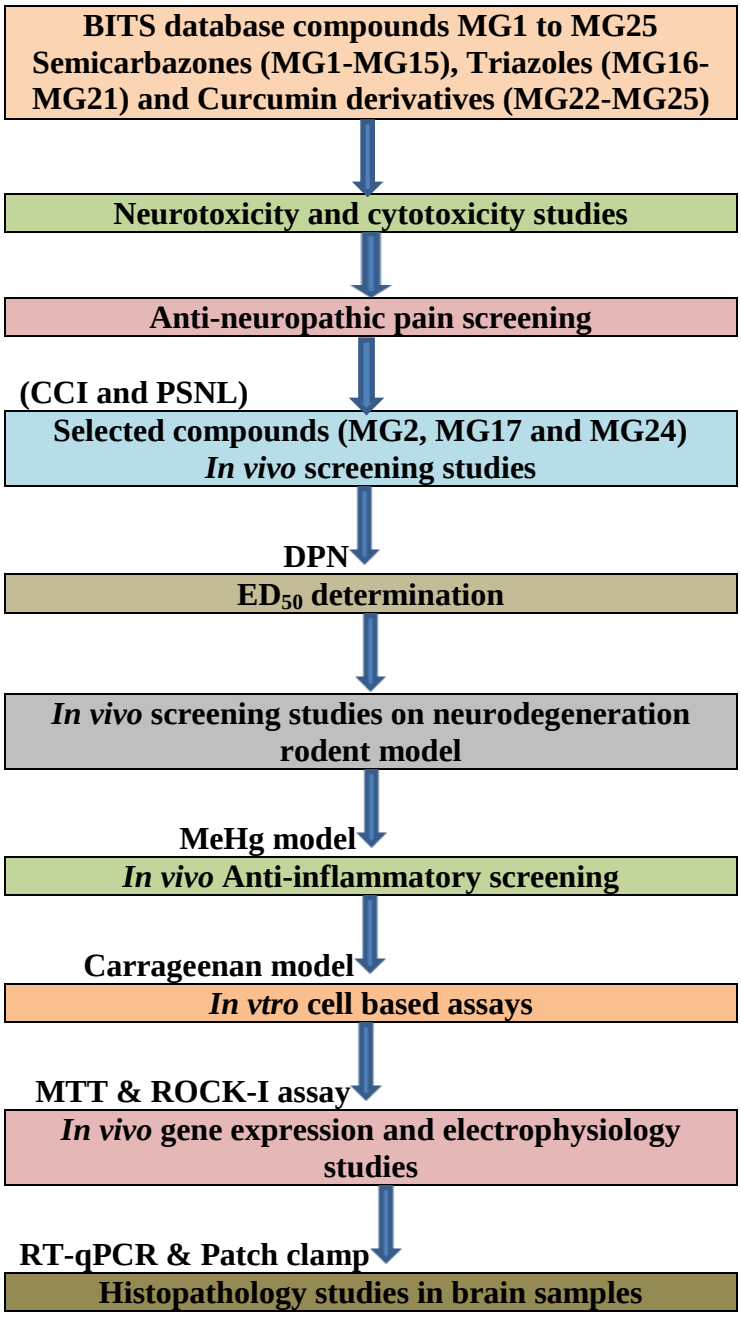
a. *In vitro* cell based ROCK-I enzyme inhibition assay (ADP glow assay).

b. *In vivo* gene expression studies using RT-qPCR.

c. Electrophysiology studies on selected compounds.

d. Histopathology studies on brain samples for selected compounds.

3.2.4 Work plan flowchart



CHAPTER-4

Materials and methods

4.1 Biological Assessments

4.1.1 Cell culture

The human cell lines IMR-32 (neuroblastoma) and HEK-293 (Human embryonic kidney cells) were used for the growth inhibition screening studies to screen novel chemical entities (NCE) to assess their selectivity towards cancer cells (IMR-32) and the normal cell (HEK-293) cytotoxicity. Cells were obtained from National Center for Cell Science (NCCS, Pune, India) and IMR-32 was cultured in minimum essential medium with 2 mM L-glutamine, 1.5 g/l sodium bicarbonate (Himedia) supplemented with 10% fetal bovine serum (FBS) (NBCS, Himedia) and 100 μg/ml streptomycin, and 100 U/ml penicillin. While the HEK-293 cells were cultured in RPMI-1640 with L-glutamine (Himedia) supplemented with 10% fetal bovine serum (FBS, Himedia) and 100 μg/ml streptomycin, and 100 U/ml penicillin. Cell culture treated culture flasks (Corning) and 96 well assay plates (Corning) were used for the entire studies in humid atmosphere containing 5% CO2 at 37°C temperature.

4.1.2. Cytotoxicity studies:

MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)] reduction assay was used to measure cytotoxicity [Van Meerloo J *et al*., 2011]. 10,000 cells/well HEK-293 cells were seeded in a 96-well plate in MEM medium containing 1% FBS and were treated with different concentrations of compounds from 100 μM–10 nM for 48 h. 10mg.mL^{-1} of MTT solution (dissolved in 1X PBS) was added to the wells and incubated for 3 h. The violet crystals formed were dissolved in 100% DMSO and absorbance was measured at 595 nm in spectrophotometer (Spectra Max, Molecular Devices, LLC). The experiment was done in triplicates, and % cytotoxicity was calculated to draw CC_{50}.

4.1.3 Growth inhibition and cell viability assay

HEK-293 cells were grown in 96-well plates at an initial concentration of approximately 3500 cells followed by proper treatment. Morphological changes were examined under the upright type phase-contrast microscopy (Olympus). Triplicate of wells were treated with six logarithmic concentrations of the each test compounds and DMSO concentration was constantly maintained at 1% in final dilution. Initial reading at non-treated state and the final reading after 72h drug incubation were measured with Vector X3 2030 multi label reader (Perkin Elmer). 10 microliters of 5mg.ml^{-1} 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrasolium bromide (MTT) (Sigma-Aldrich Co. LLC. USA) in growth medium was added to each well. After incubation for 4h at 37

°C with MTT, cell medium was removed. The precipitated formazan, a product of MTT tetrazolium ring by the action of mitochondrial dehydrogenases, was solubilized with DMSO and quantified spectrophotometrically at 540 nm.

4.2 ROCK-I inhibitory assay

4.2.1. Expression and purification of human ROCK-I

pET21(+)-ROCK recombinant obtained from NII, New Delhi was transformed into *E.coli* BL21 (DE3) for ROCK1 production based on IPTG induction [Yogeeswari P *et al*., 2012]. Transformed cells with recombinant BL21 (DE3) were grown in LB medium supplemented with 50μg.ml^{-1} of kanamycin and incubated at 37°C in a shaking incubator with 150 rpm to an A600 ≈0.6. Protein synthesis was induced with 0.1mM IPTG at 18°C overnight. Induced cells from culture of BL21 (DE3)/pET21(+)-ROCK recombinant (300mg of cells) were suspended in lysis buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 1 mM DTT, protease inhibitor cocktail and 5% glycerol). The cell suspension was sonicated for 12-15 cycles (20s pulse and 45s halt) and centrifuged at 8000 rpm at 4°C for 10 min. Repeated the centrifugation of supernatant with 10000 rpm at 4°C for 35 min consecutively to get a clear lysate. Pre-equilibrated the clarified lysate with Ni-NTA resin for 3.30 hrs at 4°C and centrifuge whole content at 500 rpm for 5min at 4°C and removed the supernatant as much as possible and loaded the pre-equilibrated beads on to column. The column was equilibrated with lysis buffer and wash buffer(500 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 1 mM DTT, 1 mM PMSF and 5% glycerol), each for 10min and centrifuged for 5 min at 4°C. Washed the protein bound beads with elution buffer (140 mM NaCl, 25 mM Tris-Cl (pH 8.0), 1 mM DTT, 1 mM PMSF, 5% glycerol and 200 mM imidazole). The eluted protein was concentrated upto 3mg.ml^{-1}.

4.2.2 *In vitro* ROCK-I enzyme assay

The human ROCK-I enzymatic studies were performed as per the earlier reported literature [Doran JD *et al*., 2008]. A reaction mix was prepared with all components of the coupled assay except for ATP, which was added to initiate the assay. The final volume of each reaction was 100μL, of which 10μL was the ATP stock solution. The reagents were mixed such that they were at the final concentrations listed below after the addition of ATP- 0.1 M HEPES, pH 7.6, 10 mM $MgCl_2$, 2.5mM PEP, 0.2mM NADH, 0.03 mg.mL^{-1} PK, 0.01 mg.mL^{-1} LDH, 2mM DTT, 100-150 nM ROCK protein and 100 μM ROCK substrate (MBP). 90 μl of the reaction mix was

added to each well in a 96-well plate. The reaction was then initiated by the addition of 10 µL ATP, giving a final volume of 100 µL. Reagents weremixed well using a plate shaker for 1 min. The ROCK Km for ATP was 30µM when using the ROCK peptide, so a final ATP concentration of 300 µM yielded a high signal-to-noise ratio.

4.3 Gene expression analyses (RT-qPCR studies)

4.3.1 RNA extraction and cDNA synthesis

Total RNA was extracted from the *in vivo*sample tissues of brain, spinal cord and sciatic nerve of vehicle treated, naïve, **GBP** treated and most active compound (**MG2)** treated groups each comprised of three animals. Tissue samples were collected between 60 to 120 minutes of post injection, where both **GBP** and **MG2** were given in a dose of 30mg.kg^{-1}. Total RNA was extracted using TRI reagent according to the protocol of the manufacturer (Sigma-Aldrich Co. LLC. USA). Concentrations and purity of RNA were quantified with Perkin Elmer VICTOR X3 (Perkin Elmer, Shelton, CT, USA) according to optical density. Purity of RNA was determined with A260/280 ratio (1.8–2.0 was considered pure).

cDNA synthesis of respective samples were acquired according to the Verso cDNA synthesis kit (Thermo Fisher Scientific Inc. USA) protocol. Equal amount of RNA (about 1µg) from the samples was suspended in 5 µl of RNAse-free water. RNA sample was further added to reaction mixture containing final volumes of 1 cDNA synthesis buffer, 500 µM of dNTP mix, 500 ng of anchored Oligo-dT primers, 1µl of RT enhancer (to remove contaminating DNA), Verso enzyme mix (include Reverse transcriptase) and the final volume was adjusted with water (PCR grade). Reverse transcription cycling program was set at the temperature 42ºC in 1 cycle of 30 min and at 95°C for 2 min for reaction termination using MJ Mini Thermal Cycler (Bio-Rad Laboratories, Inc. USA).

4.3.2 Real-time quantitative polymerase chain reaction

Real-time quantitative polymerase chain reaction (PCR) was performed with CFX CONNECT Real-Time System (Bio-Rad Laboratories, Inc.) in a 25 µl reaction mixture containing 10µl KAPA SYBR FAST qPCR MasterMix Universal (Kapa Biosystems, Inc.), 1µl of cDNA solution of all samples diluted to attain 20ng of final concentration, 200 nM final concentration of primer pairs, and the volume was adjusted with water (PCR grade). All samples were run in triplicates. Rat forward (F) and reverse (R) primers were used for NFκB (gene accession number

NM_199267.2) forward: 5'-CCTCTACACATAGCGGCTGG-3'; reverse: 5'-GCACCTTGGGATGCGTTTTT-3', for ERK1 (gene accession number NM_017347.2) forward: 5'-CACTGGCTTTCTGACCGAGT-3'; reverse: 5'-GTGATGCGCTTGTTTGGGTT-3', for IL-1β [Hagiwara N *et al*., 2005; Visnagri A *et al*., 2014] forward: 5'-ATAGCAGCTTTCGACAGTGAG-3'; reverse: 5'-GTCAACTATGTCCCGACCATT-3', for IL-6 [Huang Y*et al*., 2014] forward: 5'-GACTGATGTTGTTGACAGCCACTGC-3'; reverse: 5'-TAGCCACTCCTTCTGTGACTCTAACT-3', for TNF-α [Hagiwara Net al., 2005; Visnagri A *et al*., 2014] forward: 5'-CCACGTCGTAGCAAACCACCAAG-3'; reverse: 5'-CAGGTACATGGGTCATACC-3' and for GAPDH [Rantama T *et al*., 2013] forward: 5'-GGTGAAGGTCGGTGTGAACGG-3'; reverse: 5'-CATGTAGTTGAGGTCAATGAAGGG-3'. Primers for NFκB and ERK1 were designed using Primer Blast online tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast) and remaining primers for IL-1β, IL-6, TNF-α and GAPDH were taken from recent published works [Hagiwara N *et al*., 2005; Visnagri A *et al*., 2014; Huang Y *et al*., 2014]. The thermal cycling conditions to run real time reaction was carried out with initial enzyme activation at 94ºC for 20 sec and 35 cycles of amplification with denaturation at 94°C for 15 sec, and with 30 sec annealing at respective nearby temperatures (61-69°C) with 1 min extension at 72°C. All the PCR products from every sample were subjected to melting curve analysis (60-95°C) for specificity. The amount of mRNA for all the target genes was normalized against the housekeeping gene GAPDH in the corresponding samples. Quantification of the samples was carried out with Sequence Detection CFX Manager 3.0 analysis software (Bio-Rad Laboratories, Inc.).

Table 4.1 Oligonucleotide sequences used for the RT-PCR in rat tissue samples

Primer	Oligonucleotide sequence	Tm (°C)	Reference
Rat-TNF-α-FP	CCACGTCGTAGCAAACCACCAAG	61	[Visnagri A*et al*., 2014]
Rat -TNF-α-RP	CAGGTACATGGGTCATACC		
Rat -IL-1β-FP	CTGGTGTGTGACGTTCCCATTA	64	[Yue Wang *et al*., 2015]
Rat -IL-1β-RP	CCGACAGCACGAGGCTTT		
Rat -NFκB-FP	CCTCTACACATAGCGGCTGG	66.7	[Hagiwara N*et al*.,2005]
Rat -NFκB-RP	GCACCTTGGGATGCGTTTTT		
Rat -IL-6-FP	GACTGATGTTGTTGACAGCCACTGC	65	[Haung Y *et al*., 2014]
Rat -IL-6-RP	TAGCCACTCCTTCTGTGACTCTAACT		
Rat -GAPDH-FP	GGTGAAGGTCGGTGTGAACGG	68.9	[Hagiwara N*et al*., 2005]
Rat -GAPDH-RP	CATGTAGTTGAGGTCAATGAAGGG		

4.4 MeHg induced neurodegeneration

4.4.1 Administration and dosage

Methyl mercury (MeHg) was procured from Sigma Chemical Co. Male wistar rats were given free access to water and food, and were maintained on a 12h light-dark cycle. Rats were randomly divided into four experimental groups: MeHg induced control (n=5), MG2+MeHg induced (30 $mg.kg^{-1}$, n=5), GBP+MeHg induced (30 $mg.kg^{-1}$, n=5) and naïve (n=5). MeHg was dissolved in water and all animals were given a daily dose of 20 ppm (approximately 1.0 $mg.kg^{-1}$) orally except naïve group [Jin X *et al*., 2007]. The duration of exposure for MeHg was 3 weeks.

4.4.2 Observation of clasping and body weight

Rats were examined for deaths and also were tested for the degree of hind limb clasping phenomenon. Hind limb clasping is a marker of disease progression in a number of rodent models of neurodegeneration. To achieve dyskinetic posture, rats were suspended by holding them at the tail position, whereby rats clasp their hind limbs tightly into their abdomen. Observe the hind limb position for 10seconds. If the hind limb is consistently splayed outward, away from the abdomen, it is assigned a score of 0. If the hind limb is retracted toward the abdomen for more than 50% of the time suspended, it receives a sore of 1. If both the hind limbs are partially retracted toward the abdomen for more than 50% of the time suspended, it receives a score of 2. If its hind limbs are completely retracted and touching the abdomen for more than 50% of the time, it receives a score of 3. Body weight was measured daily before the observation [Whishaw IQ *et al*., 1985].

4.5. Western blotting studies

The lumbosacral spinal cord of the rats were extracted and stored in liquid nitrogen. Tissue samples were homogenized in TRI reagent (1 ml per 50–100 mg of tissue). Allowed the samples to stand for 5 min at room temperature and added 0.2 ml of chloroform at room temperature and later centrifuged to separate the mixture into 3 phases: a red organic phase (containing protein), an interphase (containing DNA), and a colorless upper aqueous phase (containing RNA). After separation of upper aqueous phase (RNA), remaining organic and interphases were mixed with 0.3 ml of 100% ethanol and centrifuged to get phenol-ethanol supernatant containing proteins (this pellet contained DNA) and later separated supernatant was added with 1.5 ml of 2-propanol and centrifuged to get protein pellet. All protein samples were dissolved in 1% SDS and concentrations were determined using the Bradford method and stored at -20°C until use. Protein samples were dissolved in 5X sample buffer [Tris-HCl 0.2µM, glycerol 20% v/v, DDT 10 mmol, 0.05% w/v bromo phenol blue, and 10% w/v sodium dodecyl sulfate (SDS), pH 6.8], and denatured at 95 °C for 5 min, then the equivalent amounts of proteins were separated by using 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto a nitrocellulose membrane. The membranes were incubated overnight at 4 °C with mouse polyclonal anti-pERK primary antibody. The membranes were extensively washed with Tris-buffered saline Tween-20

(TBST) and incubated for 1h with the secondary antibody conjugated with horse radish peroxidase (HRP) at room temperature. The immune complexes were detected by using an entry-level peroxidase substrate for enhanced chemiluminescence (ECL) kit based assay (Thermo Scientific Pierce ECL Western Blotting substrate). The scanned images were imported into Image J software (http://imagej.nih.gov/ij/index.html) for band density and was used for semiquantitative analyses to compare the test samples.

4.6 Reactive oxygen species (ROS) assay

Reactive oxygen species (ROS) in cells were estimated using a fluorescent dye, DCFDA (2', 7'-Dichlorodihydrofluorescein diacetate) [Eruslanov E *et al*., 2010]. The cell permeant DCFDA was chemically reduced to fluorescein in presence of ROS upon cleavage of acetate groups by intracellular esterases and oxidation. Briefly, 100,000 cells were plated in a 6-well plate and incubated with compounds (MG2, MG17 and MG24) for 24h. Cells were trypsinized and DCFDA was added at 1 μM concentration and incubated at 37^0C for 15 min followed by a wash with PBS to remove the excess of dye. The fluorescent intensity of cells was obtained using the Amnis flow cytometer (Millipore, USA).

4.7 Pharmacology

4.7.1 Animals

Wistar rats of either sex (200–250g) were utilized for the neuropathic pain models were kept under a 12h/12h light-dark cycle regimen, with free access to food and water. All experiments were approved by the Institutional Animal Ethics Committee. All of the animals were acclimatized to the housing conditions for a period of one week before the start of experiments. For all experiments, animal groups were divided into vehicle control, naïve, standard drug control and test drug groups and were included for optimal comparison analysis, where each group comprised of five animals (n=5).

4.7.2 Drug administration

All the test compounds including synthesized compounds and standard drug gabapentin (GBP), indomethacin (10 $mg.kg^{-1}$ body weight) were administered through intra peritoneal (*i.p*) route to rats. Test compounds injected were given in a volume of 0.5ml per each animal.For the neurotoxicity screening studies, rats were administered with 100 $mg.kg^{-1}$ test drug dose intraperitoneally (*i.p*). All the test compounds are administered in 30, 10 and 3 $mg.kg^{-1}$ doses

dissolved in 30% PEG 400, whereas control group animals received only 30% PEG 400 (Vehicle).

4.7.3 *In vivo* pharmacological neuropathic pain rodent screening protocols

All the synthesized NCE's were evaluated for their antinociceptive and anti-inflammatory properties in neuropathic pain, neurodegeneration and diabetic peripheral neuropathy states using respective animal models (CCI, PSNL, DPN, Carrageenan induced paw edema).

4.7.3.1 Peripheral nerve injury models

Since long it has been known that the perception of pain towards different stimuli such as chemical, thermal and mechanical can be identified and analysed by acute behavioral and electrophysiological response. In earlier time the tail flick and the hot plate test were the only available tests that were vigorously utilized by researchers for carrying out the preclinical assessment for potent analgesics [D'Amour FE *et al*., 1941]. The major clinical manifestations of pain were characterized by the episodes of spontaneous pain and hypersensitivity to any noxious stimuli. However, the previously established models shared their contribution only for the assessment of the anti-nociceptive property and were of limited importance in assessing the hypersensitivity changes associated with chronic pain situations. In 1970s Wall and his co-workers developed a newer chronic pain model, which was aimed at producing pain by peripheral nerve injury. This model had significantly contributed in understanding of the major pathophysiological mechanisms involved in chronic pain, which were significantly different from the mechanisms which were linked with the acute noxious pain [Wall PD *et al.*, 1974]. Hence, this had led to the attempts for developing newer and convenient animal models for nerve injury as surrogates for neuropathic pain.

4.7.3.2 Chronic constriction injury (CCI) method

Chronic constriction nerve injury induced neuropathic pain is one of the tremendously utilized method to produce unilateral mononeuropathy in rats. CCI was induced as per the method described by Bennett & Xiao [Bennett GJ *et al*., 1988]. An *i.p*. dose of ketamine ($55mg.kg^{-1}$) and xylazine ($5mg.kg^{-1}$) was administered to rats for anesthetization. Surgery was made under aseptic conditions, on the left hind limb by a 2 cm incision made on its lateral aspect at the mid-thigh level. The surgery was further followed by the spinous processes to separate left paraspinal muscles and to expose the common left sciatic nerve immediately above the trifurcation point.

Sciatic nerve was ligated by tying four loose knots about 1 mm spacing with the help of 4-0 braided silk suture. The wound was then closed by suturing the muscle using chromic catgut with a continuous suture pattern. Finally, the skin was closed using silk thread with horizontal mattress suture pattern.

4.7.3.3 Partial sciatic nerve ligation (PSL/Seltzer model)

PSL was performed as per the method described by Seltzer *et al*. [Seltzer Z *et al*., 1990]. PSL is one of the most frequently used models for producing neuropathy. Basically, before the surgery, the left hind limb of the rat was shaved and an incision was made to expose the sciatic nerve at the upper-thigh level. The dorsal one-third to half of the sciatic nerve was tightly ligated with an 8-0 silk suture just distal to the point at which posterior biceps semitendinosus nerve branches off [Seltzer Z *et al*., 1990; Kim KJ *et al*., 1997; Xu M *et al*., 2007]. Paw guarding and licking at the site of injury were the most common behavioral signs that are observed and reported for the spontaneous pain. Further, series of behavioral changes such as chemical hyper-reactivity, and mechanical hyperalgesia were known to occur within 1 week after the surgery, and these changes were reported to last for approximately 6 weeks [Dowdall T *et al*., 2005; Seltzer Z *et al*., 1990; Mitchell VA *et al*., 1999]. After one week of surgery sympathetically independent pain (SIP) was observed in rats, which in later weeks frequently transformed to sympathetic dependent pain [Kim KJ *et al*., 1997]. Partial sciatic nerve injury could also be performed in mice to achieve comparable allodynia and neurochemical plasticity which was reported in rat models [Malmberg AB *et al*., 1998]. The magnitude and duration of pain responses could vary depending upon the suture material and strains employed [Yoon YW *et al*., 1999].

4.7.3.4 Sensory Testing Using Nociceptive Assays

For determining the severity of behavioral neuropathic responses, different nociceptive assays such as spontaneous pain, (dynamic and cold) allodynia and hyperalgesia were performed on day 9 post CCI/PSNL surgery. Pre-drug measurement was done for measuring the degree of spontaneous (ongoing) pain and the paw withdrawal latency of hind limb to cold and mechanical stimuli (dynamic allodynia, cold allodynia and mechanical hyperalgesia). Tests were carried out at an interval of 30 min, 60 min and 120 min post drug administration. In between each test a time gap of minimum of 10 min was kept to reduce the influence of the previous test. The order of testing was as follows: spontaneous pain, dynamic allodynia, cold allodynia and lastly

mechanical hyperalgesia.Compounds (3, 10, 30 $mg.kg^{-1}$, *i.p*.) were administered at t=0, in 30% v/v PEG 400. The control group received only the vehicle. Animals in the positive control were treated with gabapentin ($30mg.kg^{-1}$, *i.p*.). Following compound or vehicle administration, behavioral changes were observed at an interval of 30, 60 and 120 min of post injection of test compounds. Paw withdrawal duration (PWD) was observed in spontaneous pain and cold allodynia, where as paw withdrawal threshold (PWT) was assessed in dynamic allodynia and mechanical hyperalgesia. Percentage reversal in spontaneous pain, allodynia or hyperalgesia was calculated for each animalas per the formula defined below [Whiteside *et al*., 2004].

$$\% \text{ Reversal} = \frac{\text{(post dose value – pre dose value)}}{\text{(contralateral paw value – pre dose value)}} \text{ X 100}$$

4.7.3.4.1 Spontaneous Pain

Spontaneous pain was assessed for a total time period of 5 min as described previously by Yoon *et. al* [Yoon C *et al*., 1994]. A total of four rats (n=5) were randomly allocated to each treatment group. Each rat was placed individually inside an observation cage which was kept 5 cm above the ground level. Rats were allowed to acclimatize individually for a period of 10 min before the start of measurement period. Cumulative duration for which the rat held its ipsilateral paw off the floor was noted as a measure of the test. The paw lifts associated with locomotion or body repositioning was not considered in the observations. Reports suggest that the paw lifts in the absence of any overt external stimuli were often correlated with spontaneous pain, and represented the condition of ongoing pain.

4.7.3.4.2 Tactile allodynia behavioral assessment

Tactile allodynia (TA) was assessed for the examination of neuropathic pain state in rats by understanding PWT in response to mechanical stimuli as measured using calibrated Von Frey filaments [Eliav E *et al*., 1999]. Animals were placed on a metallic grid floor covered with a plastic box giving access to the underside of their paws and allowed to acclimatize prior to the start of the experiment. A set of Von Frey monofilaments (0.4-15g), with intensities of mechanical stimulation increasing in graded manner with successively greater diameter filaments was applied to the plantar surface of the hind paw five times at intervals of 1-2 s in the immediate vicinity. The lowest amount of force required to elicit a response was recorded as the

PWT (in grams) and pattern of responses were tabulated using updown method of Dixon [Dixon WJ *et al*., 1980].

Chaplan pain assessment statistical table was utilized to determine the 50% paw withdrawal threshold and was interpolated using the formula [Chaplan SR *et al*., 1994]:

50 g threshold (10 Xf κδ)/10,000

Where Xf was the value (in log units) of the final Vonfrey hair used; k was tabular value [Chaplan SR *et al*., 1994] for the pattern of positive/negative responses; and δ was mean difference (in log units) between stimuli. 50% g threshold of initial reading (before administering the drug dose) was subtracted from the final reading (1h post administration) and finally compared with vehicle control (each group of animals n=5). Assessments were done after 30, 60 and 120 min of post administration of test compounds.

4.7.3.4.3 Cold allodynia behavioral assessment

Unilateral mononeuropathy induced rats were assessed for acute cold allodynia (CA) sensitivity using the acetone drop application technique as described by Caudle *et al.* [Caudle RM *et al*., 2001]. The operated rat was placed inside an observation cage that was kept 5 cm above the ground level and was allowed to acclimatize for 10 min or until the cessation of exploratory behavior five rats were randomly assigned to each group. One to two drops (100-200 μL) of acetone was sprayed onto the mid plantar region of the affected paw. Duration of paw-withdrawal response was noted as a measure of cold allodynia. Three readings were taken for each measurement with a time gap of 3 min inbetween the readings. Paw withdrawal duration (PWD) of CCI rats were measured on both the ipsilateral (ligated) and contralateral (non-ligated as basal reading) paw prior to (pre-dose) and at several time points following compound or vehicle administration. Assessments/ observations were carried out after 30, 60 and 120 min of post administration of test compounds.

4.7.3.4.4 Mechanical hyperalgesia behavioral assessment

Mechanical paw withdrawal thresholds (PWT) were assessed with a slightly modified version of the Randall-Selitto method [Randall LO *et al*., 1957] using analgesymeter (UGO Basile, Italy).

The instrument exerted a force that increased at a constant rate. This force was applied to the hind paw of the rat, which was placed on a small plinth under a cone-shaped pusher with a rounded tip (1.5 mm in diameter) until the animal withdrew its paw. A cut-off of 250g was used to avoid injury. Mechanical paw withdrawal thresholds were calculated as the average of two consecutive measurements. PWD of CCI rats were measured on both the ipsilateral (ligated) and contralateral (non-ligated as basal reading) paw prior to (pre-dose) and at several time points

4.8 Diabetic peripheral neuropathy (DPN-Rat model of peripheral neuropathic pain)

Wistar rats (200–300g) were fasted overnight and then injected with 30mg.kg^{-1}*i.p*. streptozotocin (STZ) purchased from Sigma-Aldrich (St Louis, MO, USA) in 0.2 ml of 10 mmol/l citrate buffer (pH 5.5). Diabetes was defined as a no fasting plasma glucose level >200 mg/dL in tail vein blood (Accu chek; Performa, Roche) 72h after STZ injection. The blood glucose levels before (0h) and after (72h) STZ injection confirmed the induction of diabetes. Uninjected nondiabetic (ND) and STZ-injected diabetic (STZ-D) rats were maintained in individual air filtered metabolic cages with *ad libitum* access to water, and they were fed a standard pellet diet (Sainath agencies, Hyderabad). STZ-D rats were randomly assigned to receive no treatment (Negative control) or, after 4 weeks, to begin receiving 30mg.kg^{-1} once *p.o* route, vehicle control group received 30% PEG solution and standard control group received gabapentin (Sigma Aldrich, INDIA) 30mg.kg^{-1} by *i.p*. injection. All end point measurements were performed on 21st day, after reconfirmation of diabetes. Nociceptive assays were performed on 21st day of the induction of diabetes. Tactile allodynia, tail dip, hot plate and nerve conduction velocity determination were used to evaluate the compounds [Murakami T *et al*., 2013].

4.8.1 Hot plate study (Heat hyperalgesia)

This test was performed according to the method described previously by [Eddy and Leimbach, 1953], with some minor modifications. Different treatment groups of rat (n = 5) were treated with test compounds (30mg.kg^{-1}, *i.p*), indomethacin (10mg.kg^{-1}*i.p*.) as positive control) and normal saline (normal control, 0.9%). After 30 min of treatment, the animals were placed individually on hot plate maintained at 55 ± 0.5˚C and latency time (time for which mouse remains on the hot plate) was recorded in seconds by observing either jumping from hot surface or the first licking of the hind paws. The readings were taken at time interval of 0, 30, 60, 90 and

120 min after administration of the test compounds. A cut-off time of 20 sec was kept as standard time for all animals, in order to prevent any damage or injury to the animal's paw.

4.8.2 Cold water tail dip test (Cold allodynia)

This test was performed according to the method described previously by Sanchez-Mateo *et al*., [Sanchez-Mateo *et al*., 2006], with some modifications Different treatment groups of rat (n = 5) were treated with test compounds at a dose (30mg.kg^{-1}, *i.p*), 10 mg.kg^{-1} of indomethacin *i.p*. (positive control), and normal saline (normal control) respectively as mentioned earlier. Thereafter, the terminal 2 cm portion of the rat tail was immersed in the water bath containing hot water maintained at 55 ± 0.5°C. Pain response was taken as the time interval between immersion and withdrawal of the tail by the mice and these were taken at 30, 60, 90 and 120 min after treatment. A cut-off time of 10 sec was maintained. After each reading the tail was dried carefully.

4.8.3 Nerve conduction velocity (NCV) determination

Tail nerve conduction velocity in diabetic animals was determined using PowerLab (AD Instruments, Australia). In brief, rats were restrained and the core body temperature was maintained at 37±1°C. Stimulating electrodes were kept at the base of the tail. Recording electrodes were placed 10cm towards distal end, near tail tip. Stimulating current of 5V was applied and the latency registered by recording electrodes was measured.

4.9 Carrageenan-induced mice paw edema

Paw edema was induced by intra-plantar injection of 100 µl of 1% carrageenan (λ-carrageenan, type IV, Sigma) diluted in saline into the hind paw of each rat [Solanki HK *et al*., 2015]. The volume of the injected paw of these rats was measured at 0, 30, 60 and 120 min using a plethysmometer (Ugo Basile, Italy) as previously described by Harris JM *et al*., [Harris JM *et al*., 1962] with slight modifications. Compounds (100mg.kg^{-1}, *i.p*.) were administered at t=0, in 30% v/v PEG 400. The vehicle control group of rats received only the solvent. Indomethacin (10 mg.kg-1, i.p.) was used as positive control. Paw edema was calculated as follows,

Paw edema = inflamed hind paw volume –basal hind paw volume

4.10 Histopathology studies

Histopathology studies were performed on rat brain sections. Rats were treated with 20 mM MeHg solution (MeHg dissolved distilled water) for four weeks until neurodegenerative symptoms were observed through behavioral signs such as clasping. In our study we isolated brain tissue on 30^{th} day based on clasping score (clasping score maximum was 3). Brain samples were sliced into 5μm thin section following general procedure of tissue processing and paraffin embedding. Brain tissues were then stained with Hematoxylin-Eosin (H&E) staining and images were captured with image J software under bright field microscope at magnification of 20X. Histopathology reports were obtained from Center for Cellular and Molecular Biology (CCMB), Hyderabad, India.

4.11 Toxicity assessment (Neurotoxicity screening)

In order to assess the possible toxic effects of synthetic compounds (semicarbazones,triazoles and curcumin derivatives) at the maximum dose ($300mg.kg^{-1}$), we observed the animals for 15 days after treating them with 25 different compounds (from three series) and animal groups survived without any noticeable signs of toxicity or complications and which did not display any abnormality in their gait and posture within this period those compounds were selected for further neuropathic pain activity assessment studies. Additionally, we assessed the possible effects of these NCEs on the motor coordination and/or fatigue resistance on mice by using rotarod test. NCEs treated animal groups which did not showany alteration in motor performance were only considered for further studies.

4.11.1 Rotarod test

Minimal motor impairment was measured in rats (n=3) by the rotarod test [Rao *et al*., 2005]. The rats were trained to stay on an accelerating rotarod that rotates at 25 revolutions per minute. The rod diameter was 3.2 cm. Trained animals were given an *ip*/*sc* injection of the test compounds in doses of 300 and 100 mg.kg-1 and were subjected to rotating rod before and after dosing (60 min and 120 min) respectively. Neurotoxicity was indicated by the inability of the animal to maintain equilibrium on the rod for at least 2 min in each of the three trials. The animals were subjected to rotating rod before dosing and 1 and 2h after dosing respectively.

4.11.2 Behavioural test using actophotometer

The test compounds (300 and 100 mg.kg-1) were screened for their behavioural effects using a photoactometer (Dolphin, India) at 30 min and 60 min after *i.p* injection to rats. The behaviour of the animals inside the photocell was recorded as a digital score [Boissier JR *et al*., 1965]. Increased scores suggested good behavioural activity. The control animals were administered 30% PEG alone. The photoactometer was placed in a soundproof box, and rats were placed inside. The duration of the experimental observation was up to 10 min (2+8). After an initial period of 2 min during which the animals became accustomed to the new environment, the counter was reset and the remaining 8 min reading was noted. After each trial, the base was cleaned with 20% v/v ethyl alcohol [Asakura W *et al*., 1993].

4.12 Electrophysiology studies

4.12.1 Rat hippocampal slice preparation

All experiments were performed in our collaborator lab at Ukraine in accordance with the Guiding Principles for Care and Use of Animals in the Field of Physiological Sciences of the Physiological Society of Ukraine and approved by the local animal care Committee of the Bogomoletz Institute of Physiology. Wistar rats (14-16 days old) were anaesthetized by diethyl ether and then decapitated. Hippocampi were gently removed and cut into transverse slices (400 μM) with a vibrating slice cutter (Campden Instruments) in ice-cold artificial cerebrospinal fluid (ACSF). The cutting procedure was performed in gassed (95% O_2 / 5% CO_2) ACSF that contained (in mM): 127 NaCl; 2.5 KCl; 5 $MgSO_4$; 1.25 NaH_2PO_4; 26 $NaHCO_3$; 0.1 $CaCl_2$; 11 glucose (320 mOsm/L; pH 7.4 when saturated with the gas mixture).The slices were incubated in the standard ACSF (in mM: 127 NaCl; 2.5 KCl; 1.3 $MgSO_4$; 1.25 NaH_2PO_4; 26 $NaHCO_3$; 2 $CaCl_2$; 11 glucose) for 40-60 min at temperature 32°C.

4.12.2 Patch-clamp recordings

Just before recording, the slice was transferred to the recording chamber that was continuously perfused at 32 °C at a rate 2.5-3 ml/min with gassed ACSF, which contained (in mM): 127 NaCl; 5.5 KCl; 1.25 $MgSO_4$; 1.25 NaH_2PO_4; 26 $NaHCO_3$; 2 $CaCl_2$; 11 glucose (310 mOsm/L; pH 7.4). The CA1 pyramidal cells were visually identified with the infrared-differential interference contrast (IR-DIC) video microscope (Olympus); IR-1000 (DAGE-MTI, Michigan City) and captured with CoolSNAP ES2 (CCD ICX285).

sIPSCs were recorded from CA1 pyramidal cells using patch-clamp technique in a whole-cell configuration. Patch electrodes were fabricated from borosilicate glass capillaries of 1.5 mm outer diameter (Sutter Instruments, USA) using programmable puller (P-97; Sutter Instruments, USA). The recording pipettes were filled with (mM): 117.5 Cs-gluconate, 17.5 CsCl, 8 NaCl, 10 HEPES, 10 EGTA, 2 MgATP (310 mOsm/L, pH 7.3). When filled with intracellular solution, recording pipettes typically had resistances of 5-7 MΩ.

4.12.3 Electrophysiological recordings and data acquisition

All recordings were performed using an RK-400 amplifier (BioLogic, France). Recordings and pre-processing of data were made by custom software written in Labview 8.0. The signals were typically low-pass filtered with a corner frequency (−3 dB) of 3 kHz, and sampled at 12 kHz.

4.12.4 General data analyses

Data analysis was performed with p-CLAMP (Molecular Devices, USA) and Origin 7. Spontaneous PSCs were detected with a threshold of 5-7 pA, depending on the noise level (p-Clamp, Molecular Devices, USA) and verified by eye.

4.13 Statistical analyses

All the statistically compared pharmacological data were expressed as means ± standard error of mean (SEM). The data were analyzed by one-way ANOVA in the case of neuropathic pain studies, statistical significance was determined for drug effects by Dunnett's multiple comparison post hoc test for individual comparisons with vehicle values. For gene expression studies, analyzed by a one-way ANOVA followed by post hoc Tukey’s multiple range post hoc test was used. Significance was assigned to a p value of less than 0.05. The statistical software package PRISM 6 (Graphpad Software Inc., San Diego, CA) was used for the analyses.

CHAPTER-5

Results and discussion: Neuropharmacological and molecular interventions on semicarbazone derivatives

5.1 Series-I molecules

The compounds selected for screening studies were categorized into three groups based on the common central moiety present in them. First group was named as Series-I molecules containing the central core moiety 'Semicarbazone'. Fifteen derivatives containing different substituents on semicarbazone moiety were present in this group. Some of these compounds are synthesized and reported for their potent anti-convulsant properties by Yogeeswari *et al.* The following table contains the structures and molecular formula of these compounds.

5.1.1 Structure of Semicarbazone moiety

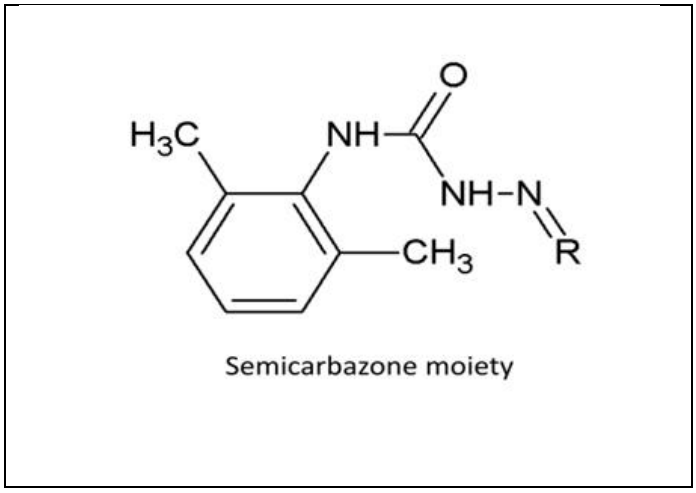
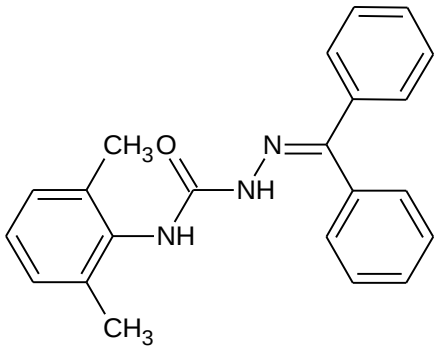
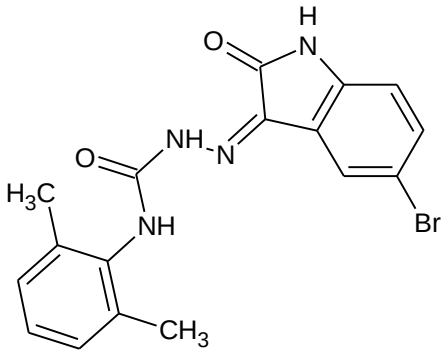
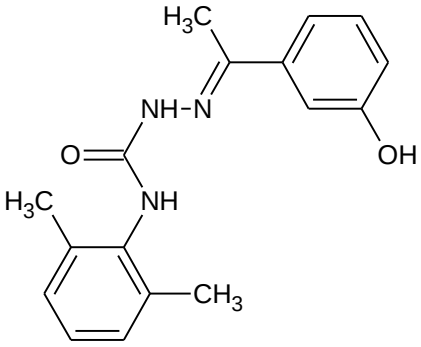
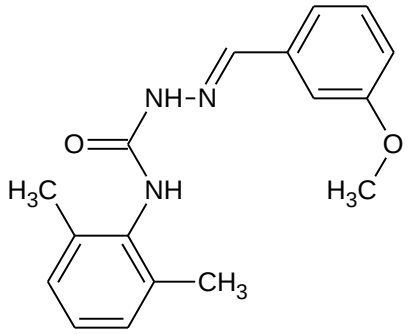
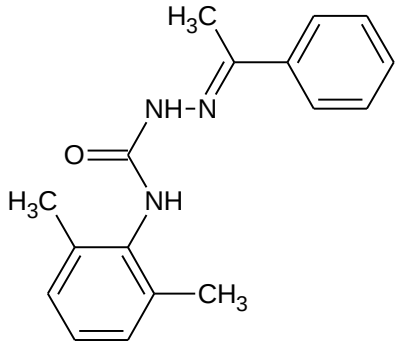
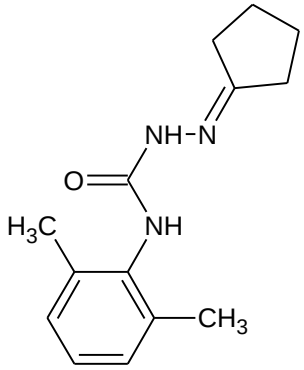
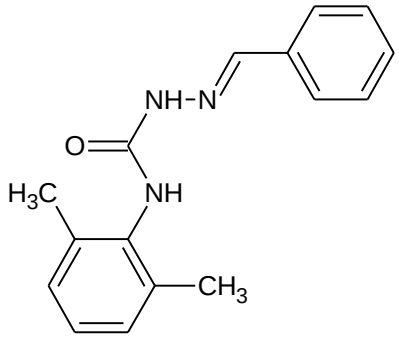


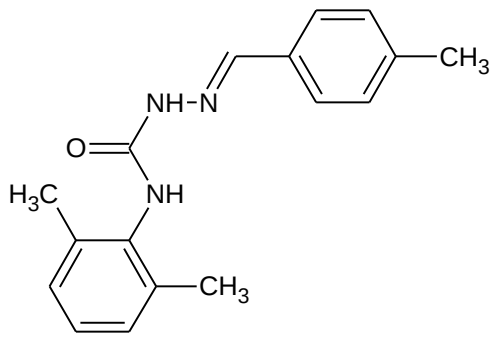
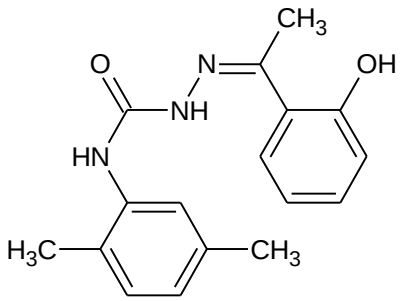
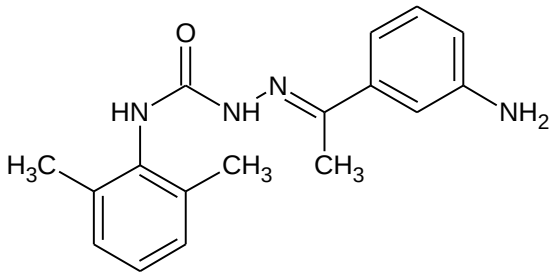
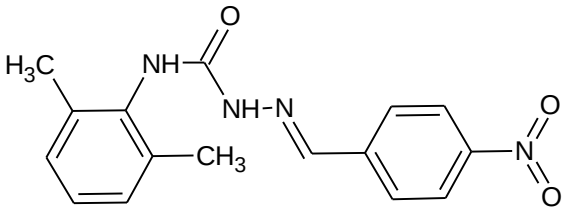
Figure 5.1: Structure of semicarbazone moiety

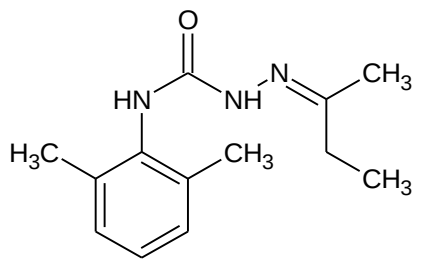
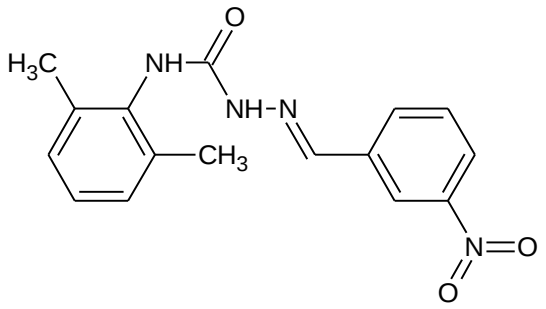
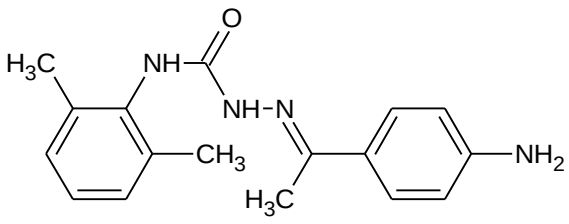
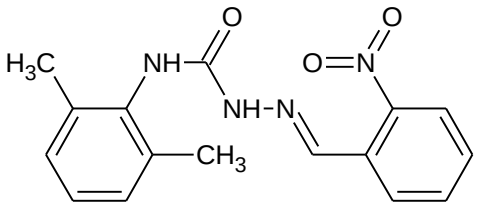
5.1.2 Structures of Series-I compounds

Table 5.1: Structures of Series-I compounds

Compound Code	Structure	Molecular weight
MG1		343.42
MG2		387.23
MG3		297.35

MG4		297.35
MG5		281.35
MG6		245.32
MG7		267.32

MG8		281.35
MG9		297.35
MG10		296.36
MG11		312.32

MG12		233.30
MG13		312.32
MG14		296.36
MG15		312.32

5.2 Pharmacological evaluation

Semicarbazones are versatile pharmacophores and are reported to potentiate GABA in the medulla of rat brain and also have GABA T inhibitory properties [Yogeeswari *et al*., 2005], hence could be further screened to evaluate their potency in alleviating neuropathic pain symptoms and neurodegeneration. Neuropharmacological screening studies were considered important and hence the thesis work was focused on exploring the biological activities of the compounds employing animal models and cell based assays.

5.2.1 Neurotoxicity screening

We studied the effect of NCEs on neuro-motor coordination using the natural fear of falling motivation (Rota rod test). In the present study, neurotoxicity was assessed using two animal models *viz.* rota rod and actophotometer based screening. Compounds were administered in three doses (300,100 and 30mg.kg^{-1}). Minimal motor impairment was measured by rota rod test and neurotoxicity was indicated by the inability of the animal to maintain balance on the rotating rod for at least 2 min. In these acute neurotoxicity assays, all the test compounds except MG1 and MG8 exhibited motor deficit at the highest tested dose (300 mg.kg^{-1}) up to 2h post administration (**Table 5.2**). But at a dose of 100mg.kg^{-1} most of the compounds except MG1, MG2, MG8 and MG9 were found to be toxic in rota rod test and also in actophotometer observations. However, none of the compound was found to be toxic at lower dose (30mg.kg^{-1}). Thus, we decided to proceed with a lower dose (30mg.kg^{-1}) for further *in vivo* experiments. The results of neurotoxicity assays clearly indicated that all the thirteen compounds showed neurotoxicity at high doses (300 and 100mg.kg^{-1}) except MG1 and MG8. Thus, we further carried out our *in vivo* experiments with a sub toxic dose of 30mg.kg^{-1}.

Table 5.2: Neurotoxic activity of test compounds of Series-I

Test Group	Neurotoxicity[a]			
	Rotarod		Actophotometer	
	1h	2h	1h	2h
MG1	-	-	-	-
MG2	300	300	300	300
MG3	100	300	100	100
MG4	100	300	100	300
MG5	300	100	300	100
MG6	100	100	100	100
MG7	100	100	100	100
MG8	-	-	-	-
MG9	300	300	300	300
MG10	100	100	100	100
MG11	300	100	300	100
MG12	100	100	100	100
MG13	100	100	100	100
MG14	100	100	100	100
MG15	100	100	100	100

[a] Neurotoxicity screening of Series-I compounds. Dose of 30, 100 and 300mg.kg^{-1} were administered. The figures in the table indicate the dose (mg.kg^{-1}) whereby bioactivity (neurotoxicity) was demonstrated in half or more of the mice. '-' indicates non-neurotoxic at the maximum dose tested (300mg.kg^{-1}).

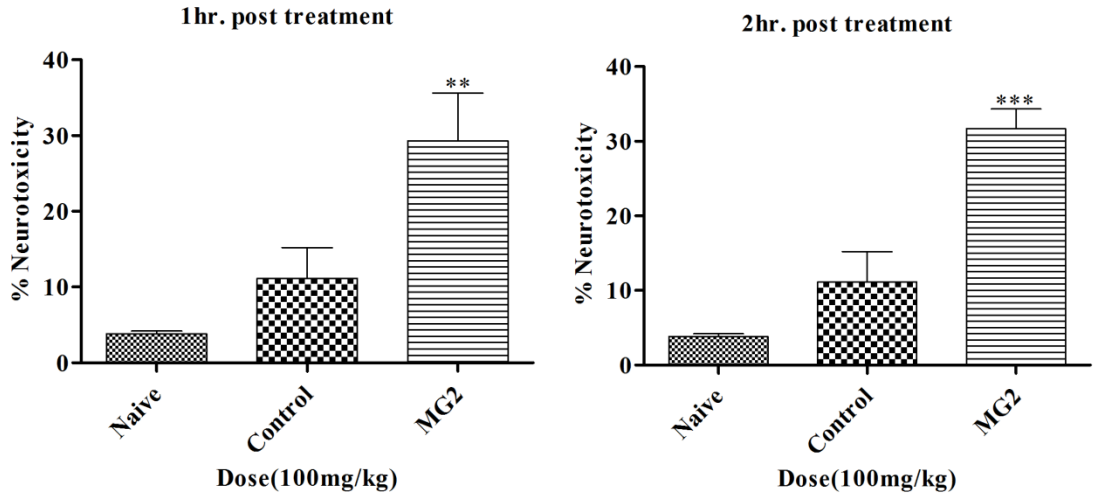


Figure 5.2: MG2 % neurotoxicity assessment

Neurotoxic effects of MG2 administered intraperitoneally (*i.p*. route) on actophotometer is presented in **Fig 5.2**. Each bar represents the mean±SEM of 3 rodents; *p<0.5 statistically significant compared to their respective control. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; One-way ANOVA followed by a Dunnett's post hoc test was used).

Compound was considered as neurotoxic if % neurotoxicity was greater than 50. In the above graph % neurotoxicity observed was less than 50 at a dose of 100mg.kg^{-1} at both 1h and 2h post administration. All the studies were performed at a much lower dose i.e., 30mg.kg^{-1} with which significant pharmacological activity was observed. Since 100mg.kg^{-1} did not show neurotoxic effects, 30mg.kg^{-1} is considered as safe dose to proceed for further studies.

5.2.2 Screening studies on animal models of neuropathic pain

Compounds selected were screened for their preliminary pharmacological activities employing various behavioral screening modules. Compounds which are pharmacologically active at 30mg.kg^{-1} dose were selected for median effective dose (ED_{50}) determination and further screened on animal models at 10 and 3mg.kg^{-1}doses.

Animal models employed in the screening studies included:

- Chronic Constriction Injury (CCI)
- Partial Sciatic Nerve Ligation (PNL)
- Diabetic Peripheral Neuropathy (DPN)
- Methyl mercury induced neurodegeneration (MeHg model)

5.2.3 Chronic constriction injury (CCI): Rat model of mononeuropathy

Four modules of behavioral analysis were employed to identify the preliminary biological effect of Series-I compounds on peripheral mononeuropathy viz. Spontaneous pain, Dynamic allodynia, Cold allodynia and Mechanical hyperalgesia.

Table 5.3 Effect of semicarbazone derivatives on spontaneous pain in CCI rats

Spontaneous Pain_MG1 to MG15			
Compound	**30 min**	**60 min**	**120 min**
Vehicle	6.97 ± 2.88	8.90 ± 2.22	11.83 ± 2.50
Gabapentin	48.34 ± 2.20	68.50 ± 3.04*	68.31 ± 5.00*
Dose 30mg.kg^{-1} *i.p.* route			
MG1	21.84 ± 7.02	33.91 ± 8.16	32.68 ± 10.35
MG2	57.71 ± 7.67*	81.22 ± 3.42*	70.63 ± 4.23*
MG3	42.53 ± 5.01	44.91 ± 7.17	49.54 ± 5.62
MG4	27.83 ± 10.70	37.99 ± 6.58	42.65 ± 10.66
MG5	28.22 ± 6.53	37.26 ± 10.24	54.17 ± 4.00*
MG6	40.02 ± 11.65	53.15 ± 11.80*	52.37 ± 6.22*
MG7	39.04 ± 8.36	45.43 ± 9.26	52.14 ± 6.13*
MG8	31.49 ± 7.44	40.36 ± 13.76	45.11 ± 5.94
MG9	58.40 ± 4.52*	80.22 ± 2.83*	78.55 ± 3.20*
MG10	27.20 ± 9.64	51.76 ± 3.40*	56.09 ± 6.09*
MG11	48.46 ± 4.76	61.75 ± 7.86*	66.87 ± 8.38*
MG12	16.30 ± 3.50	26.90 ± 2.60	40.50 ± 3.20
MG13	37.00 ± 3.70	55.50 ± 3.40*	46.60 ± 2.70
MG14	53.30 ± 10.70*	64.90 ± 10.90*	59.50 ± 5.80*
MG15	42.70 ± 6.80	52.00 ± 14.9*	51.60 ± 17.10*
Dose 10mg.kg^{-1} *i.p.* route			
MG2	31.15 ± 8.27	56.33 ± 6.45*	66.75 ± 6.69*
MG9	52.84 ± 5.07*	72.29 ± 3.67*	68.42 ± 3.62*
MG10	29.03 ± 2.52	41.25 ± 5.86	57.72 ± 5.91*
MG11	49.06 ± 3.04	63.58 ± 2.57*	67.99 ± 3.62*
Dose 3mg.kg^{-1} *i.p.* route			
MG2	28.25 ± 4.01	51.19 ± 5.71*	65.51 ± 7.33*
MG9	41.80 ± 5.22	52.13 ± 7.35*	50.57 ± 5.39*
MG11	37.87 ± 3.94	45.02 ± 5.54	47.86 ± 4.35

Each value represents the % reversal of spontaneous pain (mean±SEM) of five rats; * denotes significant reversal of spontaneous pain at tested dose in comparison to their respective vehicle control at $p<0.05$ (One-way ANOVA, followed by post-hoc Dunnet's test).

Behavioral screening observations for spontaneous pain response were reported in **Table 5.3**. Observations were recorded at 0.5h, 1h and 2h post administration of compounds. Compounds **MG2**, **MG9** and **MG14** were effective in reversal of spontaneous pain from 0.5h post administration and were active till 2h. Out of fifteen compounds screened, 8 compounds were active at 1h post administration at 30mg.kg^{-1} but based on consistent behavioral score, compounds **MG2**, **MG9**, **MG10** and **MG11** were considered for 10mg.kg^{-1} dose, where except MG10 all the three were active at 1h and all the four were active at 2h post administration. Compounds **MG2**, **MG9** and **MG11** were further screened at 3mg.kg^{-1} dose and found that **MG2** and **MG9** were active at 1h and 2h post administration. Gabapentin at 10mg.kg^{-1} dose reversed spontaneous pain effectively at 1h and 2h post administration through *i.p* route. The efficacy of compounds **MG2**, **MG9** and **MG11** was comparable to standard drug gabapentin at same dose (10mg.kg^{-1}).

Table 5.4 Effect of semicarbazone derivatives on dynamic allodynia in CCI rats

Dynamic Allodynia_MG1 to MG15			
Compound	**30 min**	**60 min**	**120 min**
Vehicle	1.40 ± 1.74	2.79 ± 2.01	4.61 ± 1.98
Gabapentin	15.30 ± 6.04	48.48 ± 7.98	60.15 ± 7.95*
Dose 30mg.kg^{-1} *i.p.* route			
MG1	13.06 ± 5.90	18.06 ± 6.37	18.06 ± 6.37
MG2	27.61 ± 7.05	74.64 ± 11.41*	55.24 ± 5.62*
MG3	16.67 ± 7.22	23.33 ± 7.22	16.67 ± 7.22
MG4	20.30 ± 6.04	31.36 ± 6.57	32.42 ± 12.83
MG5	9.70 ± 9.34	21.36 ± 9.45	8.64 ± 2.62
MG6	1.82 ± 2.27	8.48 ± 7.87	15.15 ± 7.87
MG7	15.15 ± 7.87	27.88 ± 8.95	34.55 ± 1.52
MG8	36.70 ± 10.95	56.46 ± 6.83*	56.46 ± 6.83*
MG9	29.70 ± 6.08	45.76 ± 2.65	52.88 ± 9.16*
MG10	16.67 ± 7.22	16.67 ± 7.22	23.48 ± 5.07
MG11	40.00 ± 5.89	46.67 ± 10.21	61.67 ± 6.25*
MG12	6.80 ± 5.90	24.50 ± 3.50	24.50 ± 3.50
MG13	40.00 ± 9.20	45.00 ± 9.20	45.00 ± 6.80
MG14	1.80 ± 2.30	18.50 ± 6.20	18.50 ± 6.20
MG15	8.20 ± 13.90	21.90 ± 19.40	29.20 ± 14.30
Dose 10mg.kg^{-1} *i.p.* route			
MG2	35.91 ± 11.76	53.03 ± 8.33*	53.03 ± 8.33*
MG9	33.48 ± 9.24	51.21 ± 7.98*	50.61 ± 7.98*
MG10	18.48 ± 5.07	18.48 ± 5.07	19.55 ± 9.75
MG11	28.33 ± 2.41	40.00 ± 9.92	50.00 ± 6.80*
Dose 3mg.kg^{-1} *i.p.* route			
MG2	9.70 ± 9.34	19.70 ± 8.71	25.76 ± 9.29
MG9	1.82 ± 2.27	13.48 ± 7.54	20.15 ± 7.54
MG11	18.64 ± 4.59	24.70 ± 6.19	24.70 ± 6.19

Each value represents the % reversal of dynamic allodynia (mean ± SEM) of five rats; * denotes significant reversal at tested dose in comparison to their respective vehicle control at $p<0.05$ (One-way ANOVA, followed by post-hoc Dunnet's test)

Behavioral screening observations for dynamic allodynia response were reported in **Table 5.4**. Observations were recorded at 0.5h, 1h and 2h post administration of compounds. Compounds **MG2** and **MG8** were effective in reversal of tactile allodynia from 1h post administration and were active till 2h. Out of fifteen compounds screened, 2 compounds were active at 1h and 4 compounds were active at 2h post administration at 30mg.kg^{-1} but based on consistent behavioral score, compounds **MG2**, **MG9**, **MG10** and **MG11** were considered for 10mg.kg^{-1} dose, where only **MG2** and **MG9** were active at 1h and **MG2**, **MG9** and **MG11** were active at 2h post administration. Compounds **MG2**, **MG9** and **MG11** were further screened at 3mg.kg^{-1} dose and found that none was active at 1h and 2h post administration. Gabapentin at 10mg.kg^{-1} dose reversed tactile allodynia effectively at 2h post administration through *i.p* route. The efficacy of compounds **MG2** and **MG9** was comparable to standard drug gabapentin at same dose (10mg.kg^{-1}).

Table 5.5 Effect of semicarbazone derivatives on cold allodynia in CCI rats

Cold Allodynia_MG1 to MG15			
Compound	**30 min**	**60 min**	**120 min**
Vehicle	11.06 ± 4.96	11.93 ± 2.85	10.26 ± 4.62
Gabapentin	33.59 ± 3.66	50.48 ± 3.42*	58.07 ± 7.17*
Dose 30mg.kg^{-1} *i.p.* route			
MG1	18.41 ± 9.51	21.23 ± 9.07	22.08 ± 8.37
MG2	36.95 ± 2.71	49.55 ± 5.36	64.31 ± 4.83*
MG3	15.27 ± 10.22	19.24 ± 5.44	25.31 ± 9.64
MG4	26.03 ± 4.03	44.33 ± 8.14	55.04 ± 4.29*
MG5	36.68 ± 7.26	45.26 ± 5.62	39.28 ± 7.02
MG6	24.09 ± 7.30	40.33 ± 8.56	46.88 ± 3.29
MG7	14.07 ± 12.21	30.86 ± 7.44	17.48 ± 9.68
MG8	29.02 ± 10.78	34.53 ± 4.80	44.83 ± 4.90
MG9	39.50 ± 9.27	74.77 ± 4.17*	62.53 ± 3.97*
MG10	23.01 ± 11.62	49.55 ± 4.80	54.25 ± 4.46*
MG11	41.73 ± 3.45	53.51 ± 7.74*	63.17 ± 7.31*
MG12	14.30 ± 2.60	47.10 ± 4.50	45.70 ± 6.30
MG13	30.70 ± 6.20	40.10 ± 5.10	41.80 ± 4.80
MG14	34.40 ± 1.30	59.90 ± 9.50*	57.40 ± 13.40*
MG15	25.20 ± 7.20	30.20 ± 6.10	36.10 ± 11.70
Dose 10 mg.kg^{-1} *i.p.* route			
MG2	32.28 ± 5.96	54.59 ± 8.20*	45.97 ± 5.34
MG9	30.55 ± 5.80	63.85 ± 2.65*	57.36 ± 4.34*
MG10	30.49 ± 9.61	50.92 ± 4.61*	48.17 ± 0.84
MG11	37.51 ± 3.19	57.57 ± 8.43*	57.07 ± 2.34*
Dose 3 mg.kg^{-1} *i.p.* route			
MG2	30.96 ± 8.14	45.01 ± 3.26	50.48 ± 8.09*
MG9	34.51 ± 10.99	36.65 ± 3.32	47.87 ± 4.85
MG11	25.82 ± 4.73	43.99 ± 7.96	46.00 ± 4.04

Each value represents the % reversal of cold allodynia (mean ± SEM) of five rats; * denotes significant reversal at tested dose in comparison to their respective vehicle control at $p<0.05$ (One-way ANOVA, followed by post-hoc Dunnet's test)

Behavioral screening observations for cold allodynia response were reported in **Table 5.5**. Observations were recorded at 0.5h, 1h and 2h post administration of compounds. Compounds **MG9**, **MG11** and **MG14** were effective in reversal of allodynia from 1h post administration and were active till 2h. Out of fifteen compounds screened, 3 compounds were active at 1h post administration at 30mg.kg^{-1} and 4 compounds were active at 1h post administration at 10mg.kg^{-1}. At 2h post administration 6 compounds were found to have antiallodynic property. Based on consistent behavioral score, compounds **MG2**, **MG9**, **MG10** and **MG11** were considered for 3mg.kg^{-1} dose, where except **MG2** all the other three were inactive at 1h and 2h post administration. Gabapentin at 10mg.kg^{-1} dose reversed allodynia effectively at 1h and 2h post administration through *i.p* route. The efficacy of compounds **MG2**, **MG9** and **MG11** was comparable to standard drug gabapentin at same dose (10mg.kg^{-1}).

Table 5.6 Effect of semicarbazone derivatives on mechanical hyperalgesia in CCI rats

Mechanical Hyperalgesia_MG1 to MG15			
Compound	**30 min**	**60 min**	**120 min**
Vehicle	5.71 ± 4.12	9.05 ± 3.81	5.36 ± 3.57
Gabapentin	26.19 ± 4.52	39.52 ± 8.50	56.90 ± 5.05*
Dose 30 mg.kg^{-1} *i.p.* route			
MG1	20.24 ± 7.53	22.74 ± 7.53	25.24 ± 5.17
MG2	25.61 ± 3.56	49.67 ± 6.91	51.39 ± 3.82*
MG3	12.50 ± 5.98	24.17 ± 11.07	29.17 ± 5.89
MG4	22.38 ± 4.62	32.26 ± 9.07	49.52 ± 5.62
MG5	21.14 ± 3.38	30.86 ± 6.49	37.71 ± 9.79
MG6	12.86 ± 4.49	19.52 ± 8.10	21.90 ± 8.86
MG7	23.71 ± 6.29	29.24 ± 7.94	40.10 ± 7.30
MG8	28.00 ± 5.27	40.57 ± 9.48	39.43 ± 0.71
MG9	25.91 ± 5.20	43.02 ± 2.89	50.32 ± 3.01*
MG10	16.44 ± 7.25	31.27 ± 9.47	34.54 ± 10.03
MG11	18.72 ± 7.80	45.83 ± 5.98	52.22 ± 5.29*
MG12	31.30 ± 7.00	31.30 ± 10.40	48.70 ± 10.40
MG13	35.70 ± 11.50	48.00 ± 8.50	45.50 ± 7.50
MG14	13.50 ± 4.40	29.70 ± 3.10	36.40 ± 6.30
MG15	20.60 ± 8.30	25.00 ± 11.30	36.50 ± 10.20
Dose 10mg.kg^{-1} *i.p.* route			
MG2	28.48 ± 4.76	42.00 ± 9.82	37.52 ± 2.25
MG9	23.88 ± 2.82	46.68 ± 3.70	45.60 ± 11.27
MG10	26.03 ± 8.75	40.00 ± 1.19	41.11 ± 6.02
MG11	19.72 ± 7.86	48.33 ± 6.91	47.78 ± 6.59
Dose 3mg.kg^{-1} *i.p.* route			
MG2	17.74 ± 3.22	29.29 ± 5.44	33.10 ± 7.74
MG9	12.86 ± 4.49	16.86 ± 6.90	22.57 ± 3.12
MG11	29.43 ± 6.23	37.33 ± 7.51	49.43 ± 4.57

Each value represents the % reversal of mechanical hyperalgesia (mean ± SEM) of five rats; * denotes significant reversal at tested dose in comparison to their respective vehicle control at $p<0.05$ (One-way ANOVA, followed by post-hoc Dunnet's test).

Behavioral screening observations for mechanical hyperalgesia response were reported in **Table 5.6**. Observations were recorded at 0.5h, 1h and 2h post administration of compounds. Compounds **MG2**, **MG9** and **MG11** were effective in reversal of hyperalgesia at 2h post administration. Out of fifteen compounds screened only 3 compounds were active at 2h post administration at 30mg.kg^{-1} and none of compounds were active at 0.5h and 1h post administration at 30, 10 and 3mg.kg^{-1}. Gabapentin at 10mg.kg^{-1} dose reversed hyperalgesia effectively at 2h post administration through *i.p* route. The efficacy of compounds **MG2**, **MG9** and **MG11** was found to be less compared to standard drug gabapentin at same dose (10mg.kg^{-1}).

The behavioral scores obtained for **MG2**, **MG9** and **MG11** were impressive and median effective dose was calculated considering three doses viz. 30, 10 and 3mg.kg^{-1} and reported along with time of peak effect (**Table 5.7**). ED_{50} values for all the three compounds was found to be very less (<10mg.kg^{-1}) were as it was >28mg.kg^{-1} for anti-hyperalgesic effect.

Table 5.7: ED_{50} values of Semicarbazone derivatives in CCI animal model

ED_{50} (mg.kg^{-1}) values of Series-I compounds in CCI animal model				
Treatment	**TPE (min)[a]**			
	SP	**TA**	**CA**	**MH**
MG2	3.5 (60)	9.91 (60)	10.99 (60)	28.47 (60)
MG9	2.44 (60)	24.90 (60)	5.68 (60)	34.37 (60)
MG11	3.89 (60)	36.03 (60)	6.44 (60)	>30 (60)

Table **5.7** represents median effective dose ED_{50} in mg.kg^{-1} body weight of CCI rats with time of peak effect i.e., **60** mins post treatment. **SP** is spontaneous pain; **TA** is tactile allodynia; **CA** is cold allodynia; **MH** is mechanical hyperalgesia. ED_{50} values are calculated from dose response curve with three different doses viz., 30, 10 and 3mg.kg^{-1}.

Note: 'a' is time of peak effect observed in minutes post MG2 administration.

5.2.4 Partial sciatic nerve ligation (PSNL): rat model of mononeuropathy

Three behavioral modules of neuropathic pain assessment were studied using this model. Spontaneous pain responses were not clear and hence were not reported as quantification was difficult. Antiallodynic and anti-hyperalgesic effect of Series-I compounds were investigated in this model as represented in tables **5.8**, **5.9** and **5.10**.

Table 5.8 Effect of semicarbazone derivatives on dynamic allodynia in PSNL rats

PSNL_Dynamic Allodynia_MG1 to MG15			
Compound	**30 min**	**60 min**	**120 min**
Vehicle	11.82 ± 7.22	18.64 ± 10.95	8.64 ± 5.90
Gabapentin	26.36 ± 5.07	45.15 ± 9.82	55.15 ± 5.64*
Dose 30mg.kg^{-1} *i.p.* route			
MG1	3.21 ± 2.36	25.94 ± 7.05	15.94 ± 9.68
MG2	28.48 ± 6.06	67.88 ± 12.40*	67.27 ± 8.11*
MG3	1.82 ± 2.27	14.55 ± 10.57	14.55 ± 10.57
MG4	0.00 ± 0.00	18.48 ± 6.19	19.55 ± 9.75
MG5	1.82 ± 2.27	24.85 ± 7.97	23.64 ± 9.85
MG6	15.03 ± 4.18	42.86 ± 9.99	63.58 ± 5.23*
MG7	6.82 ± 5.90	15.15 ± 7.87	15.15 ± 7.87
MG8	6.67 ± 0.00	10.00 ± 7.22	11.67 ± 6.25
MG9	16.56 ± 12.92	44.38 ± 9.56	59.03 ± 10.95*
MG10	19.73 ± 5.90	54.03 ± 14.63*	52.36 ± 16.85*
MG11	12.48 ± 4.75	47.76 ± 5.83	53.82 ± 8.55*
MG12	1.80 ± 2.30	14.50 ± 10.60	1.80 ± 2.30
MG13	0.00 ± 0.00	8.30 ± 8.30	8.30 ± 8.30
MG14	3.20 ± 2.30	17.90 ± 6.20	13.20 ± 6.20
MG15	9.30 ± 9.40	13.90 ± 10.20	14.00 ± 4.50
Dose 10mg.kg^{-1} *i.p.* route			
MG2	23.33 ± 7.22	53.33 ± 6.80*	51.67 ± 9.85*
MG6	11.98 ± 9.02	49.55 ± 8.78	44.55 ± 9.82
MG9	25.00 ± 7.89	56.67 ± 7.98*	50.00 ± 7.98*
MG11	3.00 ± 2.50	30.00 ± 2.08	36.67 ± 9.24
Dose 3mg.kg^{-1} *i.p.* route			
MG2	6.67 ± 8.33	33.33 ± 0.00	40.00 ± 8.33
MG9	16.67 ± 7.22	23.33 ± 7.22	28.33 ± 14.58

Each value represents the % reversal of dynamic allodynia (mean±SEM) of five rats; * denotes significant reversal at tested dose in comparison to their respective vehicle control at $p<0.05$ (One-way ANOVA, followed by post-hoc Dunnet's test).

Behavioral screening observations for dynamic allodynia response were reported in **Table 5.8**. Observations were recorded at 0.5h, 1h and 2h post administration of compounds. Compounds **MG2**, **MG6**, **MG9**, **MG10** and **MG11** were effective in reversal of tactile allodynia at 2h post administration, **MG2** and **MG10** were active from 1h. Out of fifteen compounds screened, 2 compounds were active at 1h and 5 compounds were active at 2h post administration at $30mg.kg^{-1}$ but based on consistent behavioral score, compounds **MG2**, **MG6**, **MG9** and **MG11** were considered for $10mg.kg^{-1}$ dose, where only **MG2** and **MG9** were active at 1h and 2h post administration. Compounds **MG2** and **MG9** were further screened at $3mg.kg^{-1}$ dose and found that none was active at 1h and 2h post administration. Gabapentin at $10mg.kg^{-1}$ dose reversed tactile allodynia effectively at 2h post administration through *i.p* route. The efficacy of compounds **MG2** and **MG9** was comparable to standard drug gabapentin at same dose ($10mg.kg^{-1}$).

Table 5.9 Effect of semicarbazone derivatives on cold allodynia in PSNL rats

PSNL_Cold Allodynia_MG1 to MG15			
Compound	**30 min**	**60 min**	**120 min**
Vehicle	14.93 ± 7.21	15.75 ± 10.01	22.24 ± 6.54
Gabapentin	46.53 ± 5.62	67.68 ± 1.40*	63.81 ± 4.45*
Dose 30mg.kg^{-1} *i.p.* route			
MG1	31.11 ± 11.29	34.90 ± 14.27	35.73 ± 5.13
MG2	21.05 ± 12.13	56.13 ± 4.62*	60.85 ± 3.76*
MG3	28.27 ± 6.42	36.08 ± 5.10	50.63 ± 6.60*
MG4	37.86 ± 9.67	52.55 ± 7.58*	37.90 ± 4.68
MG5	0.67 ± 5.03	22.57 ± 6.49	44.48 ± 6.84
MG6	41.30 ± 3.49	59.01 ± 2.68*	57.67 ± 4.31*
MG7	11.87 ± 16.56	21.16 ± 16.18	11.58 ± 16.83
MG8	31.01 ± 6.54	41.65 ± 9.61	39.76 ± 4.16
MG9	45.31 ± 6.98	65.30 ± 6.43*	61.59 ± 11.65*
MG10	36.74 ± 4.33	60.41 ± 3.46*	48.34 ± 4.52
MG11	28.63 ± 8.00	58.72 ± 2.29*	38.12 ± 3.02
MG12	14.30 ± 2.60	53.50 ± 5.10*	52.10 ± 6.00*
MG13	24.10 ± 5.70	60.10 ± 1.20*	45.70 ± 14.10
MG14	35.00 ± 1.30	50.80 ± 4.70*	50.90 ± 10.60*
MG15	32.70 ± 11.40	50.30 ± 9.50*	40.40 ± 9.90
Dose 10mg.kg^{-1} *i.p.* route			
MG2	38.72 ± 3.83	59.14 ± 2.71*	46.43 ± 8.88
MG6	41.41 ± 5.67	49.75 ± 11.55	42.78 ± 9.42
MG9	34.31 ± 6.02	53.98 ± 3.71*	49.55 ± 4.53
MG11	36.43 ± 1.39	50.13 ± 7.27*	42.09 ± 7.44
Dose 3 mg.kg^{-1} *i.p.* route			
MG2	33.14 ± 4.32	44.41 ± 2.25	40.08 ± 5.13
MG9	23.29 ± 4.77	47.73 ± 4.42	46.97 ± 8.66

Each value represents the % reversal of cold allodynia (mean ± SEM) of five rats; * denotes significant reversal at tested dose in comparison to their respective vehicle control at $p<0.05$ (One-way ANOVA, followed by post-hoc Dunnet's test).

Behavioral screening observations for cold allodynia response were reported in **Table 5.9**. Observations were recorded at 0.5h, 1h and 2h post administration of compounds. Compounds **MG2**, **MG4**, **MG6** and **MG9-MG15** were effective in reversal of allodynia at 1h post administration and compounds **MG2**, **MG3**, **MG6**, **MG9**, **and MG12** and were active at 2h post administration at 30mg.kg^{-1}. Out of fifteen compounds screened, 10 compounds were active at 1h post administration at 30mg.kg^{-1} and 3 compounds were active at 1h post administration at 10mg.kg^{-1}. At 2h post administration 5 compounds were found to have antiallodynic property. Based on consistent behavioral score, compounds **MG2** and **MG9** were considered for 3mg.kg^{-1} dose, where none of the compounds were active at 1h and 2h post administration. Gabapentin at 10mg.kg^{-1} dose reversed allodynia effectively at 1h and 2h post administration through *i.p* route. The efficacy of compounds **MG2**, and **MG9** was found to be less compared to standard drug gabapentin at same dose (10mg.kg^{-1}).

Table 5.10 Effect of semicarbazone derivatives on mechanical hyperalgesia in PSNL rats

PSNL_Mechanical Hyperalgesia_MG1 to MG15			
Compound	**30 min**	**60 min**	**120 min**
Vehicle	7.50 ± 9.24	5.36 ± 3.88	7.86 ± 7.83
Gabapentin	24.64 ± 3.81	43.57 ± 1.55	52.14 ± 5.67*
Dose 30mg.kg^{-1} *i.p.* route			
MG1	8.33 ± 4.29	13.69 ± 5.20	19.52 ± 7.20
MG2	17.58 ± 3.37	47.74 ± 6.54	52.82 ± 5.94*
MG3	23.10 ± 4.05	20.60 ± 5.17	31.67 ± 9.11
MG4	8.57 ± 4.12	11.43 ± 3.57	22.86 ± 7.14
MG5	8.57 ± 4.12	14.29 ± 3.57	21.14 ± 6.70
MG6	24.44 ± 3.39	43.17 ± 5.85	51.11 ± 9.13*
MG7	20.00 ± 6.84	29.52 ± 7.14	20.48 ± 8.93
MG8	14.29 ± 6.84	16.79 ± 3.74	22.14 ± 8.91
MG9	20.07 ± 3.62	52.36 ± 5.19*	39.79 ± 4.46
MG10	13.80 ± 4.60	39.35 ± 6.56	40.10 ± 4.62
MG11	18.88 ± 2.81	44.95 ± 11.10	52.76 ± 5.64*
MG12	31.30 ± 7.00	38.00 ± 12.00	45.30 ± 7.90
MG13	39.60 ± 11.50	43.80 ± 7.70	42.70 ± 10.50
MG14	12.10 ± 4.30	27.60 ± 2.80	31.20 ± 4.80
MG15	20.80 ± 8.30	26.20 ± 11.30	35.70 ± 10.20
Dose 10mg.kg^{-1} *i.p.* route			
MG2	32.14 ± 5.90	52.38 ± 11.29*	49.05 ± 5.09
MG6	22.14 ± 3.68	36.07 ± 3.37	27.50 ± 2.23
MG9	10.28 ± 5.73	28.41 ± 7.71	26.55 ± 4.94
MG11	16.21 ± 1.94	36.29 ± 6.43	36.29 ± 6.43
Dose 3mg.kg^{-1} *i.p.* route			
MG2	17.62 ± 3.42	35.71 ± 5.36	20.95 ± 4.91
MG9	11.55 ± 6.97	28.57 ± 5.61	25.24 ± 6.96

Each value represents the % reversal of mechanical hyperalgesia (mean ± SEM) of five rats; * denotes significant reversal at tested dose in comparison to their respective vehicle control at $p<0.05$ (One-way ANOVA, followed by post-hoc Dunnet's test).

Behavioral screening observations for mechanical hyperalgesia response were reported **in Table 5.10**. Observations were recorded at 0.5h, 1h and 2h post administration of compounds. Compounds **MG2**, **MG6** and **MG11** were effective in reversal of hyperalgesia at 2h post

administration and only **MG6** was active at 1h. Out of fifteen compounds screened only 3 compounds were active at 2h post administration at 30mg.kg^{-1} and **MG2** was active at 1h post administration at 10mg.kg^{-1}. None of compounds were active at 0.5h, 1h and 2h post administration at 3mg.kg^{-1}. Gabapentin at 10mg.kg^{-1} dose reversed hyperalgesia effectively at 2h post administration through *i.p* route. The efficacy of compound **MG2** was found to be comparable to standard drug gabapentin at same dose (10mg.kg^{-1}).

Based on behavioral screening scores, two compounds MG2 and MG9 were selected for median effective dose calculation (ED_{50}) Out of fifteen compounds screened two compounds (**MG2** and **MG9**) were found pharmacologically active and hence ED_{50} was calculated employing three different doses (30, 10 and 3mg.kg^{-1}) of the active molecules.

Table 5.11: ED_{50} values of Semicarbazone derivatives in PSNL animal model

ED_{50} (mg.kg^{-1}) values of Series-I compounds in PSNL animal model			
Treatment	**TPE (min)[a]**		
	TA	**CA**	**MH**
MG2	4.08 (60)	5.18 (60)	23.40 (60)
MG9	24.28 (60)	4.56 (60)	33.48 (60)

Table **5.11** represents median effective dose ED_{50} in mg.kg^{-1} body weight of PSNL rats at time of peak effect i.e. 60 mins post treatment. TA is tactile allodynia; CA is cold allodynia; MH is mechanical hyperalgesia. ED_{50} values are calculated from dose response curve with three different doses viz., 30, 10 and 3mg.kg^{-1}.

The compound **MG2** showed anti-allodynic activity at <10mg.kg^{-1}and anti-hyperalgesic effect at 23.4mg.kg^{-1} where as **MG9** ED_{50} was <5mg.kg^{-1} for cold allodynia and 24.28 for tactile and 33.4mg.kg^{-1} for anti-hyperalgesic effect.

5.2.5 Diabetic peripheral neuropathy

Streptozotocin at a dose of 30mg.kg^{-1} was given through i.p route at pH 4.5 in ice cold citrate buffer as detailed protocol presented in chapter-4 (Materials and Methods). Hyperglycaemia for prolonged period will bring changes in microenvironment surrounding neurons. The damage to microvascular system affects neuronal health and led to peripheral neuropathy. Table **5.12** represents effect of **MG2** on diabetic peripheral neuropathy.

Table 5.12: Effect of MG2 in rat DPN model

Behavioral screening scores of MG2 in DPN rat model

	Behavioral module	Group	0hr	1hr
a	Dynamic allodynia (50% paw withdrawal threshold)	Naïve	7.24±0.63	7.43±0.52
		Control	1.85±0.22	2.05±0.13
		Gabapentin	1.63±0.12	3.63±0.30
		MG2	1.78±0.21	6.13±0.30*
b	Nerve Conduction Velocity (Meters/second)	Naïve	66.40±3.72	65.3±2.42
		Control	49.00±1.34	50.8±1.30
		Gabapentin	51.70±1.56	51.8±0.79
		MG2	53.3±0.7	58.6±2.2*
c	Heat Hyperalgesia (52±0.5^{0}C) (Reflex time)	Naïve	13.4±0.81	13.6±0.87
		Control	6.80±0.86	7.20±0.86
		Gabapentin	5.60±0.42	5.80±0.62
		MG2	6.20±0.86	7.40±1.08*
d	Tail flick (0±0.5^{0}C) (Reflex time)	Naïve	4.00±0.45	3.80±0.20
		Control	7.00±0.55	7.20±0.37
		Gabapentin	8.60±0.32	8.30±0.44
		MG2	6.40±0.51	10.4±1.12*

Note: * represents values significant ($p<0.05$) at 60 min post treatment compared to control (vehicle-20% PEG 400) group (n=5). Values compared with t test between treatment group and control group.

MG2 was selected based on behavioral scores obtained in CCI and PSNL models. MG2 was effective in reducing tactile allodynia and improving nerve conduction velocity at an ED_{50} and hence was considered for chronic dose study. Below table represents the behavioral scores of DPN rats at 30mg.kg^{-1} dose of MG2. MG2 was further considered for chronic dosing study, where **MG2** was administered every alternate day for ten days and the behavioral responses were monitored closely. The following **table 5.13** represents the effect of MG2 on allodynia and sensory nerve conduction.

Table 5.13: Effect of MG2 chronic dosing on DPN rats

Effect of MG2 chronic dosing on diabetic peripheral neuropathy

Treatment day	TA	NCV	HH	CWTF
0	1.74	52.50±0.90	6.8±0.86	7.0±0.55
1	**5.43^{s}**	55.60±2.49	8.60±0.68	7.80±1.07
3	**6.26^{s}**	**59.40±6.16^{s}**	7.4±1.08	8.0±0.55
5	**6.83^{s}**	57.10±1.91	**10.2±1.24^{s}**	9.80±1.36
7	**4.25^{s}**	**68.60±1.75^{s}**	8.2±1.83	11.20±1.11
9	**6.26^{s}**	**65.40±1.79^{s}**	**13.4±1.40^{s}**	10.03±0.21

TA is tactile allodynia measured as 50% paw withdrawal threshold;
NCV is motor nerve conduction velocity represented in meter.sec-1;
HH is heat hyperalgesia represented as response time (seconds);
CWTF is cold water tail flick represented as response time (seconds);
Note: 'S' represents values significant at 60 min post treatment compared to pre-treatment response (n=5).

From the behavioral screening studies it was found that compound MG2 is having potent anti-allodynic and anti-hyperalgesic properties as MG2 significantly improved paw withdrawal threshold value from 1.78 to 6.13 (generally >4 value is considered to be significant according to modified Dixon method), nerve conduction in tail from 53 to 58 m/s, delayed thermal hyperalgesia but was not effective in cold water tail flick test. In chronic dose MG2 treatment for

10 days significantly improved paw withdrawal threshold, tail nerve conduction velocity and reduced or delayed thermal hyperalgesia but chronic dosing did not show any significant effect in cold water tail flick test.

5.2.6 MeHg induced neurodegeneration

Detailed protocol is presented in chapter-3 (Materials and Methods). Out of fifteen compounds screened, MG2 compound was identified to have consistent anti-allodynic and anti-hyperalgesic effect in all the three peripheral neuropathic pain rat models (CCI, PSNL and DPN), we decided to further screen this compound on methyl mercury induced neurodegeneration to evaluate its neuroprotective activity against environmental pollutants. Below table represents the behavioral scores of MeHg treated rats at 30mg.kg^{-1} dose of MG2.

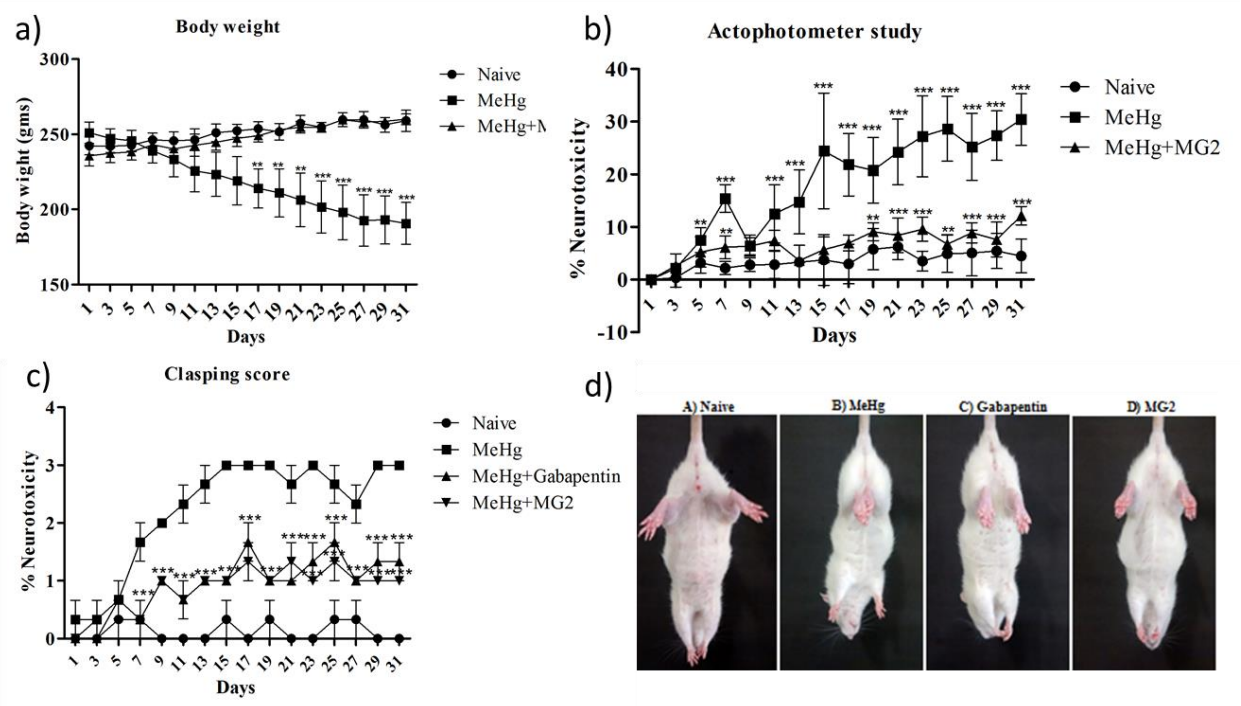


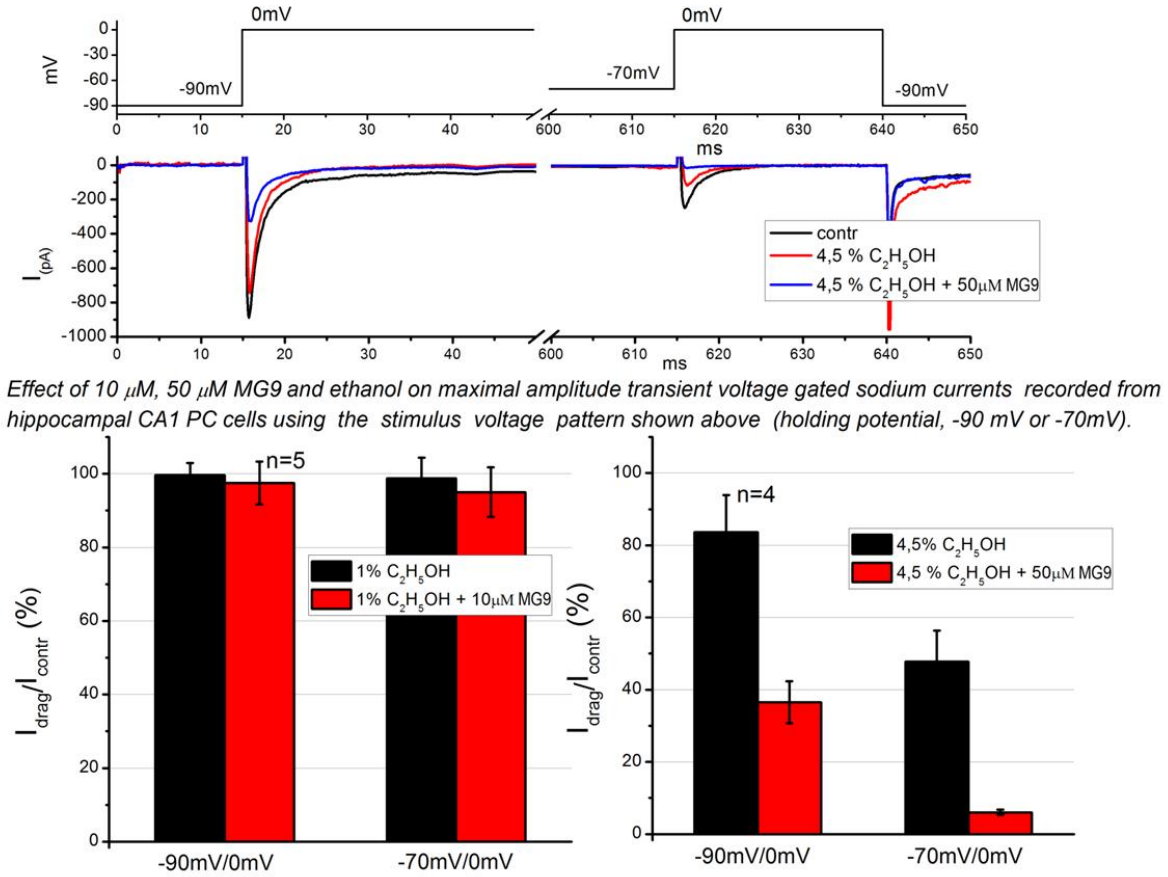
Figure 5.3: Effect of MG2 on behavioral responses of MeHg induced neurodegenerative rats

Behavioral analysis of MeHg treated animals. a) Bodyweight analysis of animals from Day 1 to Day 31. b) Actophotometer graph represents the % neurotoxicity in animals from Day 1 to Day 31. c) Clasping score of animals on each day was represented. d) Representative animals from each group clasping were shown.

MG2 was taken up for *in vivo* studies to assess the neuroprotective activity in MeHg induced animal model using a cascade of behavioral assays including clasping behavior and locomotor test as it was having anti-allodynic and anti-hyperalgesic activities in mononeuropathy models and improved NCV, reduced thermal hyperalgesia in DPN model. MG2 was found to reduce MeHg induced neurodegeneration in rats more efficiently than gabapentin as observed in behavioral screening studies. MeHg treated control rats drastically reduced weight within 31days of treatment due to toxic effects of the chemical, MG2 treated group did not show such drastic drop in body weight which can be considered as its protective effect against environmental pollutants. Both MG2 and gabapentin treatments were significantly effective in decreasing clasping score throughout the treatment period of 31days. Activity score was also improved with MG2 treatment as it was found that MeHg toxic effects reduced exploratory behaviour of rats making them more sedentary. Further the activity was also reascertained using histopathological studies and expression levels of inflammatory mediators.

5.3 Electrophysiology Studies

The sIPSCs were recorded from CA1 pyramidal cells using patch-clamp technique in a whole-cell configuration. Patch electrodes were fabricated from borosilicate glass capillaries of 1.5 mm outer diameter (Sutter Instruments, USA) using programmable puller (P-97; Sutter Instruments, USA). The recording pipettes were willed with (mM): 117.5 Cs-gluconate, 17.5 CsCl, 8 NaCl, 10 HEPES, 10 EGTA, 2 MgATP (310 mOsm/L, pH 7.3). When filled with intracellular solution, recording pipettes typically had resistances of 5-7 MΩ.



Effect of 10 μM, 50 μM MG9 and ethanol on maximal amplitude transient voltage gated sodium currents recorded from hippocampal CA1 PC cells using the stimulus voltage pattern shown above (holding potential, -90 mV or -70mV).

Figure 5.4: Effect of MG9 on voltage gated sodium channels

Pain following the nerve damage occurs due to a cascade of neurobiological events triggered in afferent conduction pathways that together result in neural hyperexcitability. The cascade includes up-regulation of the expression of certain Na+ and Ca2+ channels in the injured primary sensory neurons, down-regulation of certain K+ channels, increased levels of cytokines and other hyperalgesic substances in the spinal gray matter and suppression of γ- aminobutyric acid (GABA)ergic neurotransmission in the spinal cord .

Whole cell patch clamp studies were performed to assess Na channel blockade property of MG9. Whole cell patch clamp studies were performed on pyramidal cells in CA1 zone of rat brain. The reason for selecting CA1 zone is that pyramidal cells of CA1 zone express abundant sodium channels. Neuropathic pain is a condition which is due consequence of overexcitation or hyperactivity of sodium channels. The activated sodium channels causes ectopic firing, which is responsible for neuopathic pain symptoms. So, If we control over excitation of sodium channels, we can prevent further damage to neurons and symptoms of neuropathic pain. Here, sodium channel over excitation causes repeated spontaneous post synaptic potential, by measuring spontaneous post synaptic potential we can know whether the drug is having neuropathic pain alleviating property. Our compound MG9 was screened for its effect on post synaptic potential currents of sodium channels present in rat pyramidal CA1 cells assessed by whole cell patch clamp technique. In our study we reported that MG9 has inhibitory property on sodium channels (spontaneous inhibitory post synaptic potential) so finally we conclude that sIPSC property of MG9 is one of the possible mechanisms for its amelioration of neuropathic pain.

5.3.1 General data analysis

Data analysis was performed with p-CLAMP (Molecular Devices, USA) and Origin 7. Spontaneous PSCs were detected with a threshold of 5–7 pA, depending on the noise level (p-Clamp, Molecular Devices, USA) and verified by eye.

Differences were considered significant at the $P < 0.05$ level. Values are the means ± SEM. for n=5 slices. Two-tailed Student's t-test was used for statistical comparison.

5.3.2 Electrophysiological recording and data acquisition

All recordings were performed using an RK-400 amplifier (BioLogic, France). Recordings and pre-processing of data were made by custom software written in Labview 8.0. The signals were typically low-pass filtered with a corner frequency (−3 dB) of 3 kHz, and sampled at 12 kHz.

5.4 Gene expression studies-RT-PCR

The mRNA expressions of pro-inflammatory mediators was estimated in nervous tissues of CCI induced and MeHg treated rats and compared with the levels post MG2 treatment.

5. 4. 1 Gene expression studies in CCI rats

The gene expression studies were carried out to estimate the effect of MG2 on altered expression of inflammatory mediators in brain, spinal cord and sciatic nerve tissues of neuropathic rats.

MG2 treatment normalized the elevated mRNA expressions of IL-1β in brain, IL-6 in sciatic nerve, TNF-α in spinal cord sciatic nerve, BDNF and ERK in brain and spinal cord tissues.

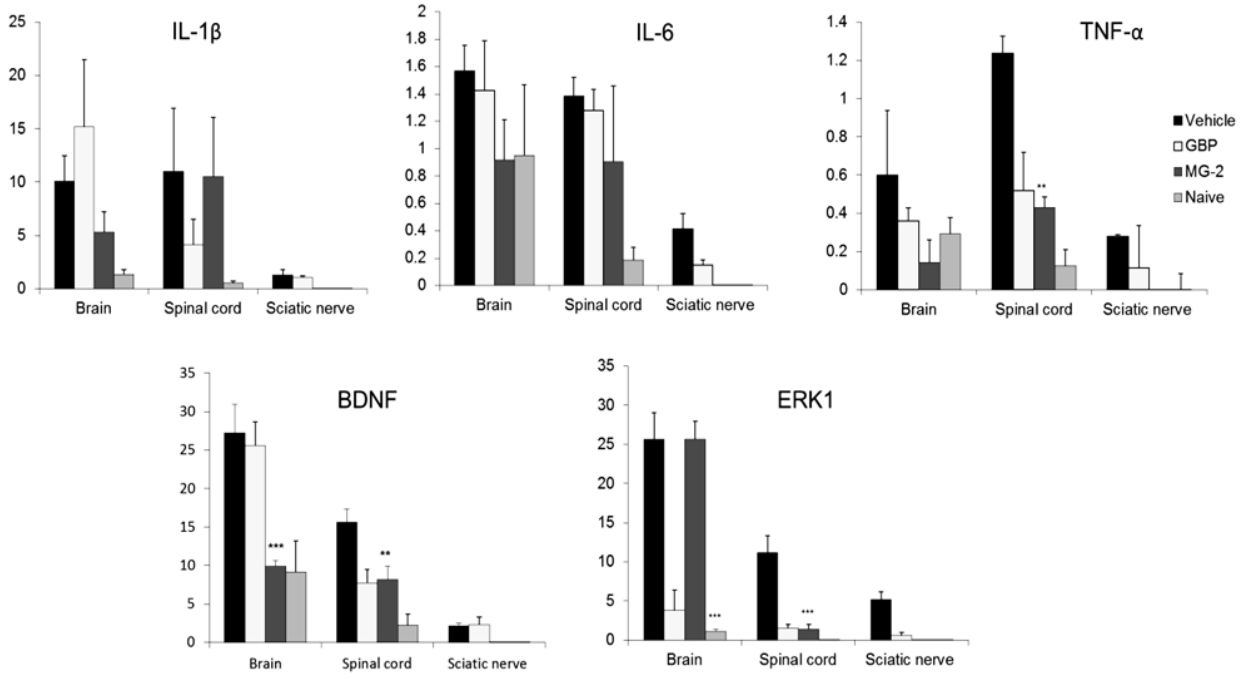


Figure 5.5: Effect of MG2 on mRNA expression of various inflammatory mediators in CCI rats. Statistical significance was obtained by Two Way ANOVA with Dunnet's post-test at *p<0.05, **p<0.001 and ***p<0.0001.

The compound MG2 was selected for *in vivo* gene expression studies as it showed consistent and very good pharmacological activity (anti allodynic, anti-hyperalgesic and neuroprotective effects) in all the four rodent models -CCI, PSNL, DPN and MeHg induced neurodegeneration. MG2 was also found to be potent ROCK-I inhibitor and it showed least cytotoxic effect on HEK-293 cell lines, hence we were interested to explore the mechanism by which MG2 is showing all these activities. We have done thorough literature search to make a list of inflammatory genes that are upregulated during neuropathic pain states. IL-1β, IL-6 and TNF-α [Austin and Moalem Taylor, 2010; Yogeeswari P *et al*., 2012] are the most commonly released proinflammatory mediators upon injury and well known mediators of inflammation and pain which can act directly on nociceptors to induce pain hypersensitivity. In addition to above mediators we have also selected BDNF and ERK1 as the expression of these mediators were found to be elevated in neuroinflammatory disease states [Smith T *et al*., 2015]. ERK pathway gets activated after CCI and p-ERK-IR neurons are overexpressed which play major role in induction and maintenance of neuropathic pain [Song XS *et al*., 2005].

5. 4. 2 Gene expression studies in MeHg treated rats

The gene expression studies were carried out to estimate the effect of MG2 on altered expressions of inflammatory mediators in spinal nervous tissue of methyl mercury induced neurodegeneration rats. MG2 treatment normalized the elevated expressions of Il-6, TNF-α, BDNF and NF-kB but the levels of IL-1β expression remained unaltered. The mRNA expression levels were determined in spinal nervous tissue collected from rats post four week (31days) treatment with 20ppm MeHg and after confirming neurodegenerative symptoms by clasping analysis.

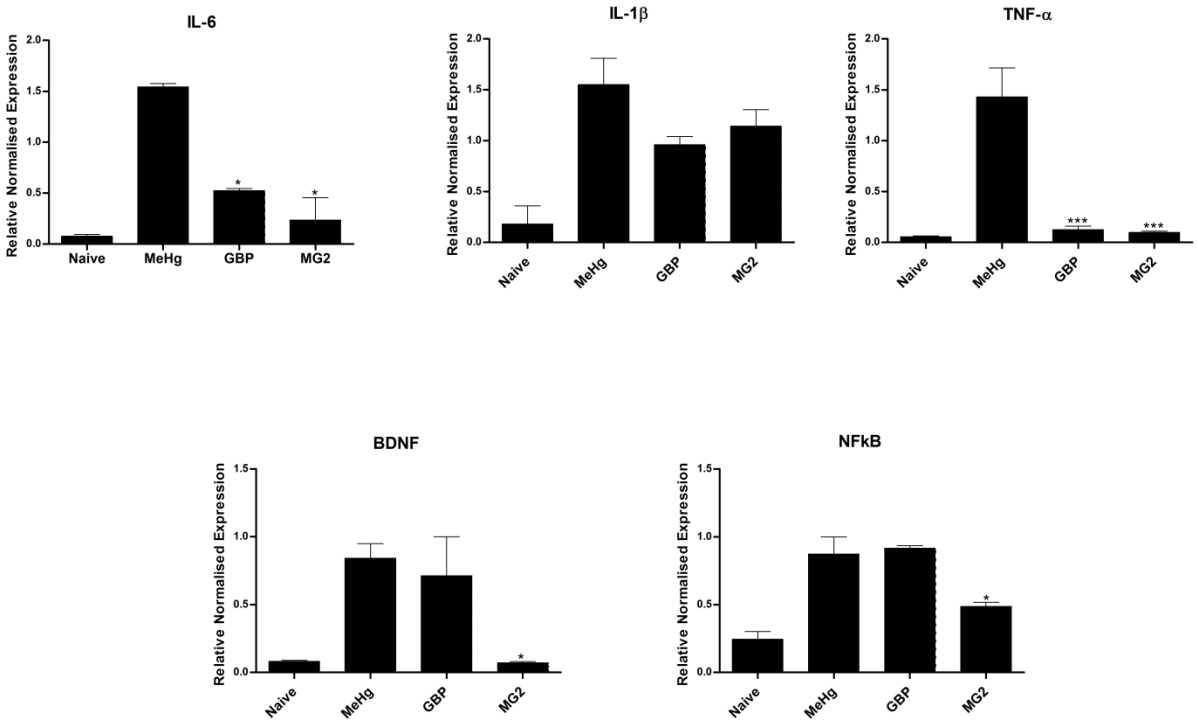


Figure 5.6: Effect of MG2 on mRNA expression of various inflammatory mediators in MeHg treated rats.

Statistical significance was obtained by One Way ANOVA with Dunnett's post-test at *p<0.05, **p<0.001 and ***p<0.000.

MeHg treated rats were sacrificed on day 31 after behavioral assessment post MG2 treatment and spinal tissues and brain tissues were collected for RT-qPCR and western blot studies. When compared to normal group, the levels of TNF-α, IL-1β, IL-6 and NFκB were increased significantly in the positive control group (MeHg treated group). The increased TNF-α levels were decreased by treatment with MG2 (30mg.kg^{-1}) and gabapentin (10mg.kg^{-1}) (p<0.01-0.001). The increased IL-6 levels were decreased by treatment with MG2 (30mg.kg^{-1}) and gabapentin at 10mg.kg^{-1} (p<0.01). However, there are no significant changes on the IL-1β levels by treatment with the test compound MG2 (p<0.05). The effects of test compounds MG2 on TNF-α, IL-1β, IL-6 BDNF, ERK and NFκB levels are shown in **Figures 5.5** and **5.6.** Further studies will be carried out to understand the reason why MG2 inhibits the levels of TNF-α, IL-6 and NFκB but not IL-1β. To conclude, MG2 at the dose of 30mg.kg^{-1} produced a marked anti-inflammatory activity by inhibiting the mediators of chronic neuro-inflammation in neurodegenerative rat models.

5.5 Acute paw edema model

The effect of compound MG2 on acute inflammation was studied to evaluate its efficacy on inflamed paw in carrageenan induced rat paw edema model **Fig. 5.7**. This study was performed to correlate our *in vivo* inflammatory gene expression results with *in vivo* pharmacological effect on acute paw edema which can be quantified with plethysmometer. The compound MG2 ($30mg.kg^{-1}$) was compared with standard anti-inflammatory drug Indomethacin ($10mg.kg^{-1}$) and disease control group.

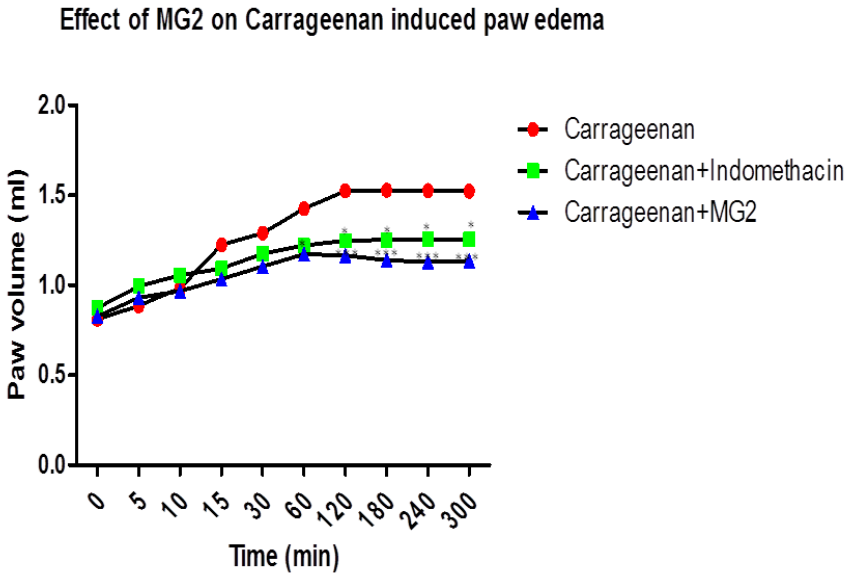


Figure 5.7: Effect of MG2 on carrageenan induced acute paw edema in rats

Carrageenan Vs Carrageenan + Indomethacin = * significance

Carrageenan Vs Carrageenan + MG2 = *** significance

Effect of treatment of compound Indomethacin, MG2 on Carrageenan induced paw edema in rat. Data is represented as mean ± SEM of n=5 rats and analysed by one way ANOVA followed by Dunnett's test. Significance values are represented as ***$p<0.001$ and *$p<0.05$.

These test compounds were further evaluated for its anti-inflammatory activity by carrageenan induced mice paw edema. These results suggested that **MG2** was found to possess promising anti-nociceptive and anti-inflammatory properties. Finally, we could conclude that **MG2** emerged as a potent anti-inflammatory agent and it may be further developed into a therapeutic agent for the treatment or prevention of acute pain and inflammatory diseases.

It was observed that test compound **MG2** ($30mg.kg^{-1}$) and indomethacin ($10mg.kg^{-1}$) significantly inhibited the development of carrageenan induced paw edema after three, four and five hours of treatment ($p<0.001$).

5.6 Brain histopathology studies (H &E staining)

The brain tissue from rats treated with methyl mercury (20ppm/day *P.0*) for over four weeks were collected by sacrificing them, tissues stored in formaldehyde and histopathological analyses were performed on 2-4 μm thin sections using hematoxylin and eosin stain. During the analysis, the extent of neurodegeneration in MeHg group was compared with naïve group and MeHg+MG2 (30mg.kg^{-1} *i.p*) group. The histopathology images were obtained at a magnification of 20X with electron microscope under bright field **Fig. 5.8**.

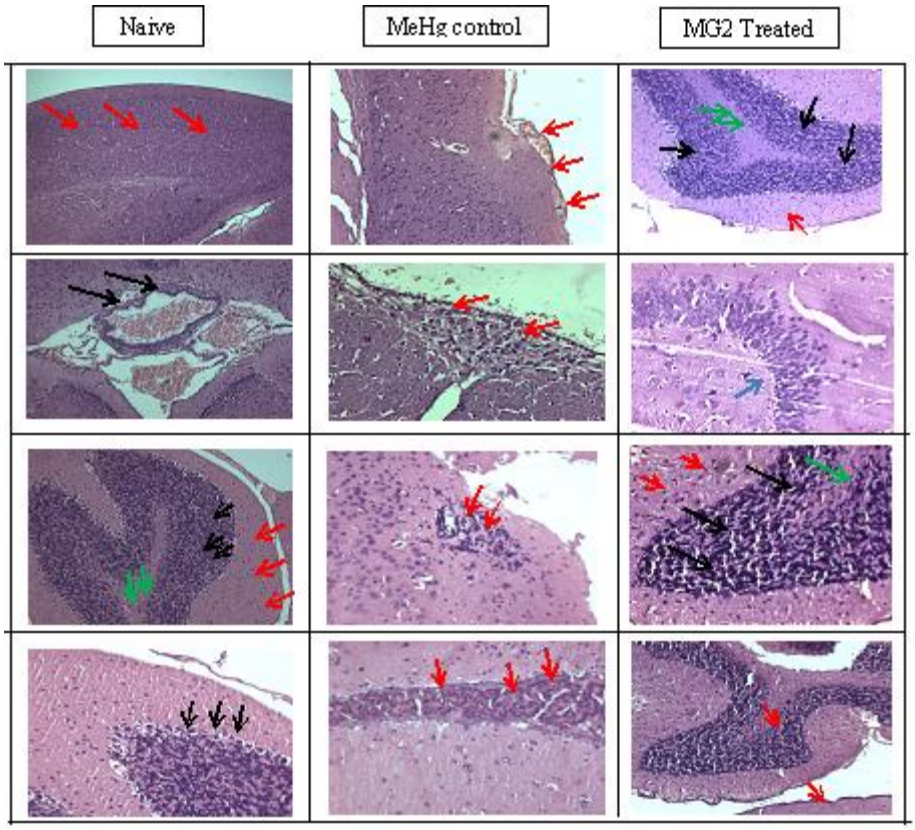


Figure 5.8: Effect of MG2 on MeHg treated rat Brain tissue

Brain sections of study rats upon hematoxylin and eosin stain revealed the extent of degeneration that took place upon treatment with MeHg for three weeks. Arrows indicate different zones of healthy tissues observed in naïve group. Arrows in MeHg control group showed degradation,

meningeal haemorrhage, microglial and ependymal cell infiltration. Arrows in MG2 treated group showed no signs of degradation except small number of inflammatory cells and slight thickening of meningeal layer. Based on histopathological analyses it was confirmed that MG2 is having protective effect on neuronal tissue against environmental pollutants like MeHg.

5.7 MTT assay on HEK-293 cells

MTT assay was performed to test compounds cytotoxic concentration with six different concentrations ranging from 1nM to 100μM. From the CC_{50} values, it is clear that compound MG2 is having CC_{50}>100 indicating that it is more safe to use at high doses.

Table 5.14. Effect of Semicarbazone derivatives on cytotoxicity

Compound	CC_{50}(Mean±SD)(μM)
MG1	98.26±0.20
MG2	>100
MG3	30.15±0.14
MG4	40.35±0.26
MG5	32.36±0.34
MG6	24.67±0.22
MG7	18.17±0.13
MG8	03.53±0.04
MG9	06.93±0.08
MG10	07.97±0.10
MG11	05.81±0.21
MG12	27.58±0.28
MG13	05.79±0.11
MG14	16.08±0.12
MG15	11.52±0.11

All data in triplicates was used for deriving and represented in Mean ± SEM (n=3).

Semicarbazone derivatives were screened to evaluate their cytotoxic effect on human embryonic kidney cell lines (HEK-293) and most of the compounds are found to be non-toxic below 10μM concentration except MG8, MG9, MG10, MG11 and MG13. Among the fifteen compounds compound **MG2** was found to be least toxic (**CC_{50}>100**). We performed MTT assay to select compounds for further *in vitro* cell based enzyme inhibition assays.

5.8 ROCK-I inhibition assay

Compounds were screened to evaluate their effect on ROCK-I inhibition using different concentrations and inhibitory concentration (IC_{50}) was determined. Rho kinase plays an important role in neurodegeneration and development of neuropathic pain. Recent literature revealed that ROCK inhibition promotes neuroprotection (Van de Velde S *et al*., 2015; Yogeeswari P *et al*., 2012). Table **5.15** shows that except 2 compounds MG1 and MG5 all the compounds showed ROCK-I IC_{50}<10μM. Compounds MG2, MG3, MG6, MG7, MG8, MG9, MG11and MG15 were found to be most potent inhibitors with an IC_{50}<1 μM.

Table 5.15 Effect of Semicarbazone derivatives on ROCK-I inhibition (ADP-glow assay)

Compound	ROCK IC_{50} (µM)
MG1	689.5±2.200
MG2	2.935±0.022
MG3	0.002±0.001
MG4	5.210±0.810
MG5	223±6.160
MG6	≤0.001
MG7	0.057±0.001
MG8	0.008±0.001
MG9	0.005±0.001
MG10	18.05±2.120
MG11	≤0.001
MG12	4.96±0.620
MG13	2.599±0.120
MG14	11.59±0.520
MG15	≤0.001

All data in triplicates was used for deriving and represented in Mean ± SEM (n=3).

As discussed earlier ROCK-I inhibition could yield beneficial therapeutic effects in neuropathic pain treatment, from the results it is clear that most of the semicarbazone derivatives showed excellent ROCK-I inhibition (except MG1 and MG5) and proved that they can be further explored. One of the reason for performing ROCK-I inhibition assay is that Rho kinase activation is reported in diabetic conditions and there also exists direct relationship between hyperglycaemia, Rho kinase activation and peripheral neural sensitization.

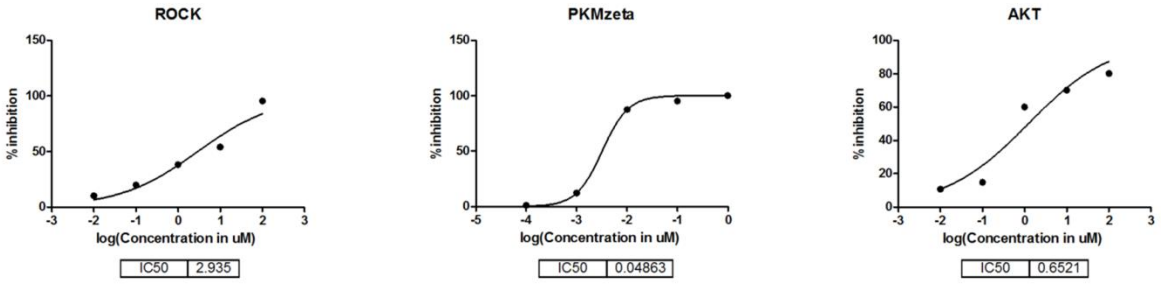


Figure 5.9: Effect of MG2 on various kinases

All the compounds except MG1, MG4, MG5, MG10, MG12, MG13 and MG14 exhibited ROCK-I enzyme inhibition with IC_{50}<1 μM (**Table 5.15**). Among these 15 compounds, **MG2** compound was found to be in nano-molar range emerging as more potent hits when compared to Fasudil, the standard ROCK-I inhibitor. Compounds that showed potency in sub-micromolar range <10 nM. All the above three kinases were reported to be activated in neuroinflammatory conditions. PKMζ is an atypical protein kinase C (aPKC) which acts as a key molecule for the maintenance of long term potentiation (LTP). [Yogeeswari P *et al*., 2012; Theodore J Price *et al*., 2013]. Protein kinase B PKB/Akt is found to be upregulated in CCI rat's ipsilateral dorsal horn and contributes to the generation and maintenance of neuropathic pain [Shuang-Shuang Li *et al*., 2013].

5.9 Western blot studies

Akt levels are reported to be upregulated in neuropathic pain conditions in recent literature which states that upon CCI surgery the Akt levels in ipsilateral dorsal horn were considerably improved which then activates and distribute α_{2A}-AR in the primary afferents and dorsal horn neurons and might contribute to neuropathic pain. Hence phosphorylated Akt is assumed as a key mediator in neuropathic pain generation [Shuang-Shuang Li *et al*., 2013]. We thought of finding pAkt protein levels in spinal tissues of MeHg induced neurodegenerative rats with western blotting.

MG2 treated rat spinal tissue samples showed significantly less phosphorylated Akt protein levels compared to MeHg treated rats in MeHg induced neurodegenerative rats. The pAkt inhibitory effect of MG2 was comparable to standard Akt inhibitor mk2206 **Fig. 5.18**.

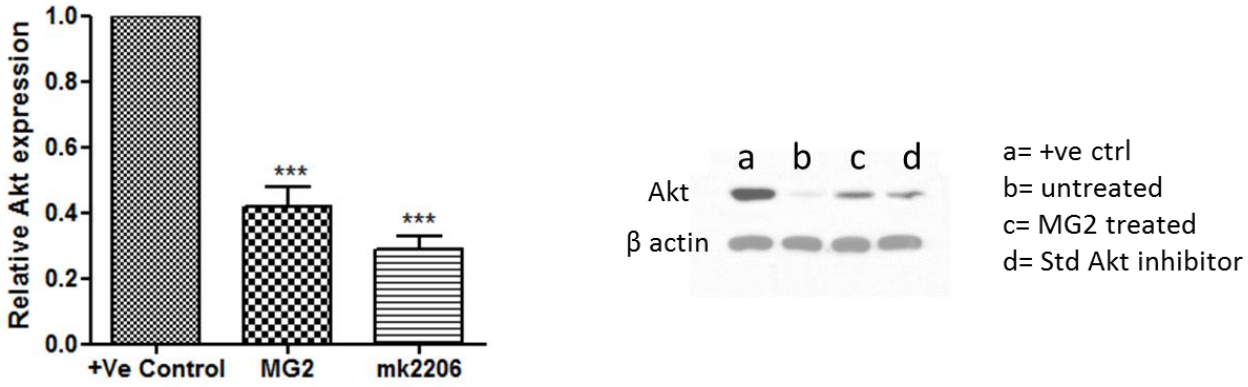


Figure 5.10: Inhibitory effect of MG2 on Akt protein

The compound MG2 significantly inhibited the production of phosphorylated Akt ($p<0.001$) compared to MeHg group and the effect was close to standard Akt inhibitor **mk2206**. Comparison was done using One Way ANOVA with Dunnet's post-test (*$p<0.05$; **$p<0.01$; ***$p<0.001$)

MeHg treatment (20ppm/day) for 31 days has significantly raised Akt protein levels in spinal dorsal horn, ten day treatment with **MG2** has significantly reduced Akt expression and the effect was comparable to that of standard Akt inhibitor **mk2206.**

5.10 ROS inhibition studies (Flow cytometer)

Effect of MG2 on reactive oxygen species in RAW 264.7 cells treated with LPS (0.1µg/ml) in different concentrations (5, 1 and 0.2 µM) was studied. The percentage of ROS positive cells at various concentrations of MG2 after 3h was found to be 66.33% at 5 µM, 32.33% at 1µM and 6% at 0.2µM which were significantly less compared to LPS treated control cells **Fig 5.19**.

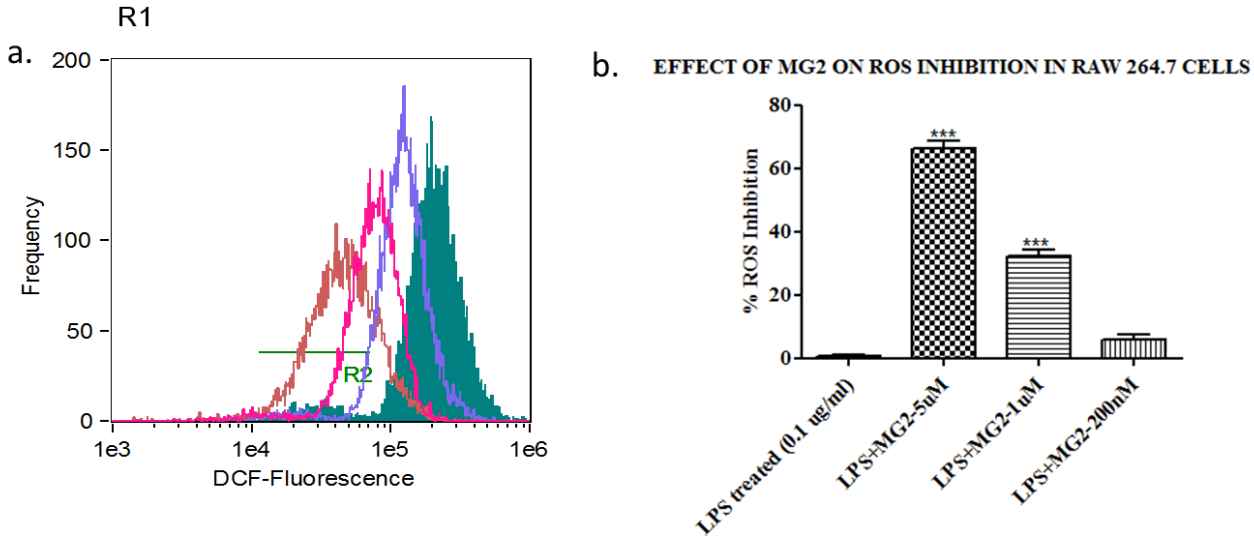


Figure 5.11: Effect of MG2 on ROS in RAW 264.7 cells. **a)** Cells were treated with LPS (0.1 µg/ml) and with different concentrations of MG2 (5, 1 and 0.2 µM) for 3h and incubated with DCFDA for 30 min. The intracellular ROS was measured by flow cytometry. **b)** Histogram depicting the percentage of ROS positive cells of indicated concentrations of MG2 after 3h. Data were expressed as mean ± SEM of three independent experiments. ***p<0.001 versus LPS treated group.

This study was performed to evaluate MG2 compound for its potency to abrogate intracellular ROS production. We considered this study to be important as ROS production is one of the major cause of neuronal apoptosis. ROS is also produced by activation of Rho kinase and in hyperglycaemic states as in case of diabetes. We were trying to identify possible mechanisms of action of MG2 and the DCFDA (Dichloro-flourescein diacetate) assay results showed that MG2 is having potent ROS inhibitory effect at a concentration of 5µM and the effect is also concentration dependant.

5.11 Summary and conclusions on Series-I

In the present study, we made an attempt to identify CNS effects of selected semicarbazone derivatives synthesized in our medicinal chemistry drug discovery laboratory (In-house database-BITS compounds). Fifteen derivatives were selected from BITS database, based on their efficacy against decreasing seizure duration and mortality rate in all rodent models. These compounds had shown GABA agonistic action in previous studies and hence were selected to screen further against neuropathic pain condition where ectopic neuronal firing could be controlled with compounds showing GABA agonistic actions [Yogeeswari P *et al*., 2005].

In the present thesis work, systematic pharmacological screening studies were performed to reach our hypothesis. Initially we screened selected compounds for neurotoxic effects and found that compounds MG1 to MG15 to be non-neurotoxic ($30mg.kg^{-1}$) in two rodent models namely rota rod and actophotometer. Followed by neurotoxicity screening studies, we checked compounds efficacy on rat models of neuropathic pain and neurodegeneration, and found that MG2 compound is having very good anti-allodynic and anti-hyperalgesic effect. Compounds were then screened using *in vitro* techniques to estimate their potency against various enzymes which are thought to be involved in neuroinflammatory disorders. Most active compound in the series MG2 was further screened to identify its efficacy to reduce acute inflammation. A representative compound MG9 showed voltage gated sodium channel blocking properties in patch clamp studies. MG2 exhibited neuroprotective effects on brain tissue as revealed from histopathology analyses.

MG2 being a potent inhibitor of neuropathic pain, its anti-inflammatory effects were found to be due to inhibition of ROS production and regulation of key inflammatory mediators that were overexpressed. MG2 also showed PKMζ, Akt and Rho kinase inhibition. A proof of concept for the mechanism of its action was thus established. However, the optimal dose and maximum tolerated dose along with pharmacokinetics have to be determined. Further, the activity of MG2 could be tested in clinical scenario. Results obtained thus confirms the potential of MG2 on neuropathic pain but still there seems to be a need to further explore this molecule on all clinical aspects to get a clearer picture. In a nutshell we would like to consider MG2 as potential antinociceptive and anti-inflammatory agent having centralized effects in treating neurodegenerative disorders. As MG2 also found to play a key role in regulating the elevated

levels of TNF-α, IL-1β, NFκB, IL-6, BDNF and other neuroinflammatory mediators which further validate its clinical use and we could expect this molecule's fate as NCE in CNS related disorders.

Finally, we conclude that MG2 emerged as a novel semicarbazone derivative possessing multiple effects with therapeutic benefits against neuropathic pain and neurodegenerative disorders and it may be further developed into a therapeutic agent for the treatment or prevention of acute inflammation, neuropathic pain and other related neurodegenerative diseases. However, this is a first investigation on semicarbazone moiety to identify its multiple central role as well as peripheral pharmacological activities.

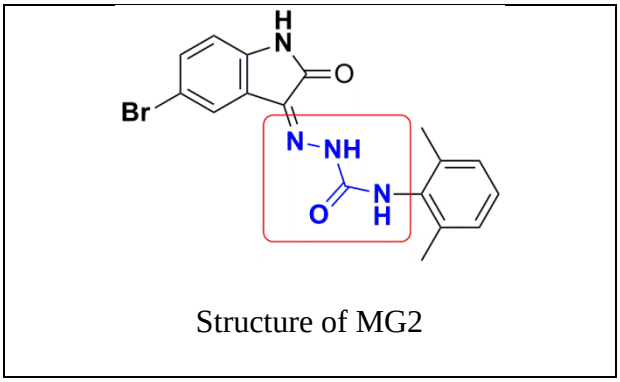


Figure 5.12 Structure of MG2

CHAPTER-6

Results and discussion:
Neuropharmacological and molecular interventions on Triazole derivatives

6.1 Series-II molecules

The compounds selected for screening studies were categorized into three groups based on the common central moiety present in them. The second series-II molecules containing the central core moiety ‘Triazole’ was selected to derive its analogues for screening studies. Six derivatives containing different substituents on triazole moiety presented in this group. The following table contains the structures and molecular formula of these compounds.

6.1.1 Structure of Triazole moiety

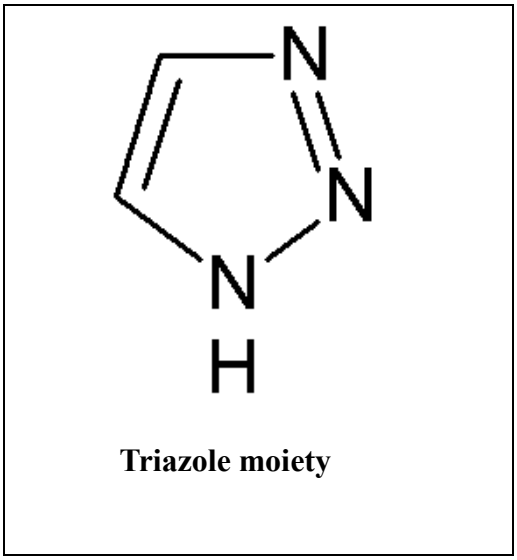
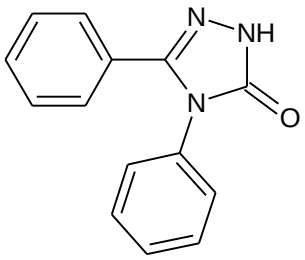
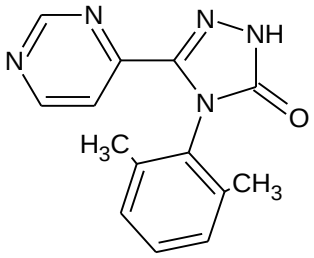
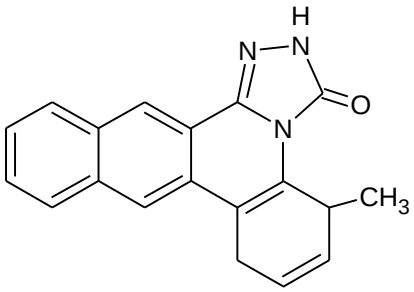
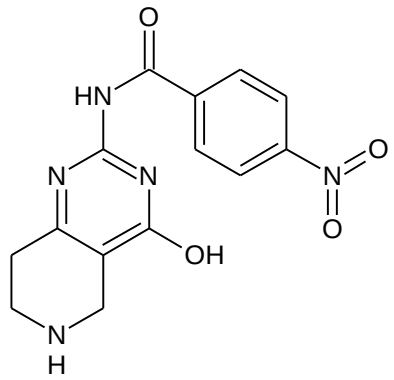
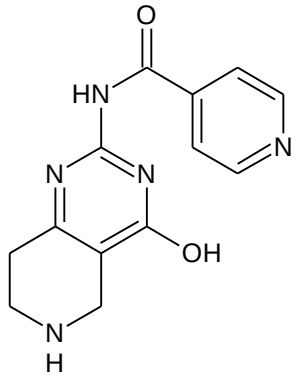
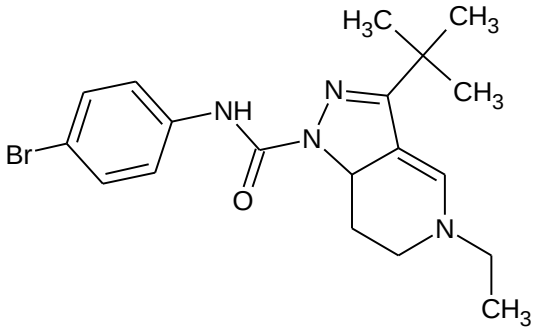


Figure 6.1: Structure of Triazole moiety

6.1.2 Structures of Series-II compounds

Table 6.1 Structures of Series-II compounds

Compound Code	Structure	Molecular weight
MG16	 Stefanska Joanna *et al.*, 2008	237.25
MG17		267.28
MG18		301.34

MG19		315.28
MG20		271.27
MG21		405.33

6.2 Pharmacological evaluation

Triazole moiety has been identified to have multiple biological activities such as anti-fungal, antiviral, antibacterial, anticancer and anti-inflammatory [Gupta D *et al*., 2015]. Our group at BITS-Pilani Hyderabad reported triazole moieties role in nociception and pain alleviation [Yogeeswari *et al*., 2013]. As an extension to our earlier finding, we have further screened for their potency in alleviating neuropathic pain symptoms and neurodegeneration in this work. Neuropharmacological screening studies were considered important and hence the thesis work was focused on exploring the biological activities of the compounds employing animal models and cell based assays.

6.2.1 Neurotoxicity screening

Neurotoxicity screening was carried out to study effect of NCEs on motor coordination using the natural fear of falling motivation (Rota rod test).In the present study, neurotoxicity was assessed using two animal models *viz* . rotarod and actophotometer based screening. Compounds were administered at three dose levels (300,100 and 30mg.kg^{-1}). Minimal motor impairment was measured by rotarod test and neurotoxicity was indicated by the inability of the animal to maintain balance on the rotating rod for at least 2 min. In these acute neurotoxicity assays, only one compound out of six exhibited motor deficit at the highest tested dose (300mg.kg^{-1}) up to 2h post administration (**Table 6.2**). But at a dose of 30 and 100mg.kg^{-1} none of compounds (MG16 to MG21) were found to be toxic in rotarod test and actophotometer assay. Thus, we decided to proceed with a lower dose (30mg.kg^{-1}) for further *in vivo* experiments. The results of neurotoxicity assays clearly indicated that one compound MG16 showed neurotoxicity at high doses (300mg.kg^{-1}). Thus, we further carried out *in vivo* experiments with a sub neurotoxic dose (30mg.kg^{-1}).

Table 6.2 Neurotoxic activity of test compounds.

Test Group	Neurotoxicity [a]			
	Rotarod		Actophotometer	
	1h	2h	1h	2h
MG16	300	300	300	300
MG17	-	-	-	-
MG18	-	-	-	-
MG19	-	-	-	-
MG20	-	-	-	-
MG21	-	-	-	-

[a] Neurotoxicity screening of Series-II compounds. Dose of 30, 100 and 300mg.kg^{-1} were administered. The figures in the table indicate the dose (mg.kg^{-1}) whereby bioactivity (neurotoxicity) was demonstrated in half or more of the mice. '-' indicates no neurotoxicity signs observed till the highest dose (300mg.kg^{-1}) tested.

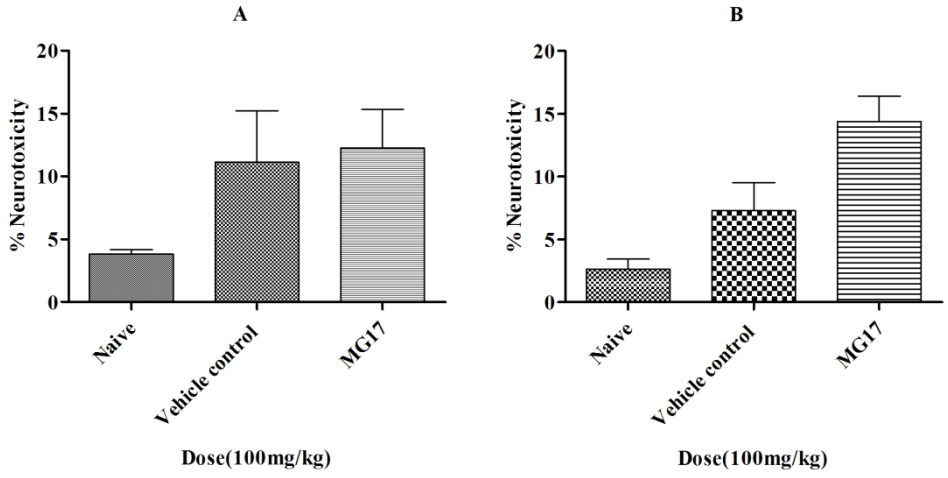


Figure 6.2: MG17 % neurotoxicity assessment

Neurotoxic effects of MG17 given intraperitoneally (*i.p*. route) on actophotometer. Each bar represents the mean ± SEM of 5 animals; *p<0.5 statistically significant compared to their respective control. (*p<0.05; **p<0.01; ***p<0.001; One-way ANOVA followed by a Dunnet's post hoc test was used).

Compound was considered as neurotoxic if % neurotoxicity is greater than 50. In our study % neurotoxicity observed was less than 50 at a higher dose of 100mg.kg^{-1}. All the studies were performed at a much lower dose i.e., 30mg.kg^{-1} with which significant pharmacological activity was observed. Since 100mg.kg^{-1} did not show neurotoxic effects, 30mg.kg^{-1} is considered as safe dose to proceed for further studies.

6.2.2 Screening studies on animal models of neuropathic pain

All the six compounds (MG16-MG21) were taken for behavioral screening studies in two rat models of neuropathic pain viz., Chronic constriction injury (CCI) and partial sciatic nerve ligation (PSNL). Compounds selected were screened for their preliminary pharmacological activities employing various behavioural screening modules (Spontaneous pain, Dynamic allodynia, Cold allodynia and Mechanical hyperalgesia). For PSNL model spontaneous pain symptoms were not clearly observed, hence SP test was not considered. Compounds which are pharmacologically active at 30mg.kg^{-1}dose were selected for median effective dose (ED_{50}) determination and further screened on animal models at 10 and 3mg.kg^{-1}doses.

6.2.3 Chronic constriction injury (CCI): Rat model of mononeuropathy

Four modules of behavioral analysis were employed to identify the preliminary biological effect of Series-II compounds on peripheral mononeuropathy viz. Spontaneous pain, Dynamic allodynia, Cold allodynia and Mechanical hyperalgesia.

Table 6.3 Effect of triazole derivatives on spontaneous pain in CCI rats

Spontaneous pain_MG16 to MG21			
Compound	**30 min**	**60 min**	**120 min**
Vehicle	6.97±2.88	8.90 ± 2.22	11.83± 2.50
Gabapentin	48.34±2.20	68.50 ± 3.04	68.31±5.00
Dose 30mg.kg^{-1} i.p. route			
MG16	27.0±1.08	19.5 ± 0.29	16.3±1.03
MG17	39.7±5.8	76.9 ± 3.2*	66.7±3.5*
MG18	35.0±4.14	21.5 ± 1.5	15.5±1.32
MG19	34.8±2.5	23.5 ± 2.18	11.0±1.29
MG20	31.3±1.8	20.3 ± 2.02	13.8±1.6
MG21	29.8±0.85	24.3 ± 1.11	21.5±1.19
Dose 10mg.kg^{-1} i.p. route			
MG17	38.3±9.4	48.6 ± 5.9	45.2±6.4
Dose 3mg.kg^{-1} i.p. route			
MG17	36.3±9.8	46.7 ± 9.3	44.6± 2.4

Each value represents the % reversal of spontaneous pain (Mean ± SEM) of five rats (N=5); '*' highlighted values denotes significant at dose tested in comparison to their respective vehicle control at $p<0.05$ (One-way ANOVA, followed by post-hoc Dunnet's test) Spontaneous pain was significantly reduced with MG17 treatment at a dose of 30mg.kg^{-1}, and the time of peak effect was observed at 60 minutes (77%) and effect was continued till 120 minutes with percentage

reversal of pain 66.7. It did not show any significant effect at 10 and 3mg.kg^{-1}. Compounds MG16, MG18, MG19, MG20 and MG21 did not show any significant effect at 30, 10 and 3mg.kg^{-1}.

Table 6.4 Effect of triazole derivatives on dynamic allodynia in CCI rats

Dynamic allodynia_MG16 to MG21			
Compound	**30 min**	**60 min**	**120 min**
Vehicle	1.40 ± 1.74	2.79 ± 2.01	4.61 ± 1.98
Gabapentin	15.30 ± 6.04	48.48 ± 7.98	60.15 ± 7.95*
Dose 30mg.kg^{-1} *i.p.* route			
MG16	5.1 ± 0.12	20.3 ± 0.34	20.1 ± 0.4
MG17	5.5 ± 2.6	55.8 ± 9.3*	55.2 ± 10.5*
MG18	4.3 ± 1.4	19.7 ± 0.54	20.09 ± 0.1
MG19	5.7 ± 0.13	18.9 ± 0.12	19.02 ± 0.2
MG20	6.5 ± 0.56	19.0 ± 0.8	19.23 ± 0.59
MG21	4.1 ± 0.45	11.7 ± 0.2	14.32 ± 0.34
Dose 10mg.kg^{-1} *i.p.* route			
MG17	38.3 ± 2.1	45.0 ± 9.2	38.3 ± 2.1
Dose 3mg.kg^{-1} *i.p.* route			
MG17	37.7 ± 9.2	40.7 ± 4.2	34.0 ± 10.5

Each value represents the % reversal of dynamic allodynia (Mean ± SEM) of five rats (N=5); '*' highlighted values denotes significant at dose tested in comparison to their respective vehicle control at $p < 0.05$ (One-way ANOVA, followed by post-hoc Dunnet's test)

Dynamic allodynia was significantly reduced with MG17 treatment at a dose of 30mg.kg^{-1}, and the time of peak effect was observed at 60 minutes (55.8%) and effect was continued till 120 minutes with percentage reversal of pain 55.2. It did not show any significant effect at 10 and 3mg.kg^{-1}. Compounds MG16, MG18, MG19, MG20 and MG21 did not show any significant effect at 30, 10 and 3mg.kg^{-1}.

Table 6.5 Effect of triazole derivatives on cold allodynia in CCI rats

Cold allodynia_MG16 to MG21			
Compound	**30 min**	**60 min**	**120 min**
Vehicle	11.06 ± 4.96	11.93 ± 2.85	10.26 ± 4.62
Gabapentin	33.59 ± 3.66	50.48 ± 3.42*	58.07 ± 7.17*
Dose 30mg.kg^{-1} *i.p.* route			
MG16	21.3 ± 2.06	21.0 ± 1.22	15.8 ± 0.63
MG17	48.7 ± 9.8	73.4 ± 3.6*	62.6 ± 5.7*
MG18	27.3 ± 3.68	16.8 ± 1.03	15.5 ± 0.64
MG19	34.5 ± 1.32	24.3 ± 1.18	16 ± 0.71
MG20	40.8 ± 1.11	26.3 ± 1.11	17.3 ± 0.48
MG21	41.3 ± 1.65	21.3 ± 1.38	17.3 ± 0.85
Dose 10mg.kg^{-1} *i.p.* route			
MG17	27.6 ± 3.2	54.9 ± 3.6	31.6 ± 8.2
Dose 3mg.kg^{-1} *i.p.* route			
MG17	23.3 ± 9.6	47.9 ± 8.0	36.7 ± 15

Each value represents the % reversal of cold allodynia (Mean ± SEM) of five rats (N=5); Yellow highlighted values denotes significant at dose tested in comparison to their respective vehicle control at $p<0.05$ (One-way ANOVA, followed by post-hoc Dunnet's test)

Cold allodynia was significantly reduced with MG17 treatment at a dose of 30mg.kg^{-1}, and the time of peak effect was observed at 60 minutes (73.4%) and effect was continued till 120 minutes with percentage reversal of pain 62.6. It did not show any significant effect at 10 and 3mg.kg^{-1}. Compounds MG16, MG18, MG19, MG20 and MG21 did not show any significant effect at 30, 10 and 3mg.kg^{-1}.

Table 6.6 Effect of triazole derivatives on mechanical hyperalgesia in CCI rats

Mechanical hyperalgesia_MG16 to MG21			
Compound	**30 min**	**60 min**	**120 min**
Vehicle	5.71 ± 4.12	9.05 ± 3.81	5.36 ± 3.57
Gabapentin	26.19 ± 4.52	39.5 ± 8.50	56.9 ± 5.05*
Dose 30mg.kg^{-1} *i.p.* route			
MG16	17.5 ± 1.0	24.02±3.3	17.4 ± 1.2
MG17	18.3 ± 11.0	58.3±9.2*	53.3 ± 4.2*
MG18	14.0 ± 1.9	30.2±2.3	15.3 ± 8.9
MG19	11.34 ± 2.4	32.0±4.3	17.4 ± 5.9
MG20	18.5 ± 0.34	24.0±8.4	14.6 ± 4.3
MG21	17.3 ± 0.45	22.0±1.2	15.9 ± 4.4
Dose 10mg.kg^{-1} *i.p.* route			
MG17	35.0 ± 11.5	46.7 ± 6.8	44.2 ± 7.5
Dose 3mg.kg^{-1} *i.p.* route			
MG17	38.3 ± 11.5	44.2 ± 7.5	40.8 ± 10.5

Each value represents the % reversal of mechanical hyperalgesia (Mean ± SEM) of five rats (N=5); '*' highlighted values denotes significant at dose tested in comparison to their respective vehicle control at $p < 0.05$ (One-way ANOVA, followed by post-hoc Dunnet's test)

Spontaneous pain was significantly reduced with MG17 treatment at a dose of 30mg.kg^{-1}, and the time of peak effect was observed at 60 minutes (58.3%) and effect was continued till 120 minutes with percentage reversal of pain 53.3. It did not show any significant effect at 10 and 3mg.kg^{-1}. Compounds MG16, MG18, MG19, MG20 and MG21 did not show any significant effect at 30, 10 and 3mg.kg^{-1}.

Table 6.7 ED_{50} values of triazole derivatives in CCI animal model

ED_{50} (mg.kg^{-1}) values of Series-II compounds in CCI animal model				
Treatment	**TPE (min)[a]**			
	SP	**TA**	**CA**	**MH**
MG17	5.42 (60)	15.03 (60)	4.32 (60)	10.15 (60)

Table 6.7 represents median effective dose ED_{50} in mg.kg^{-1} body weight of CCI rats at time of peak effect i.e., 60 mins post treatment. SP is spontaneous pain; TA is tactile allodynia; CA is cold allodynia; MH is mechanical hyperalgesia. ED_{50} values are calculated from dose response curve with three different doses viz., 30, 10 and 3mg.kg^{-1}.

Note: 'a' is time of peak effect observed in minutes post MG17 treatment.

Based on behavioral screening scores, one compound MG17 was selected for median effective dose calculation (ED_{50}). Out of six compounds screened only compound MG17 was found pharmacologically active and hence ED_{50} was calculated employing three different doses (30, 10 and 3mg.kg^{-1}) of the active molecules. ED_{50} of MG17 in CCI model for spontaneous pain and cold allodynia was found to be <10mg.kg^{-1}, for tactile allodynia it was 15.03mg.kg^{-1} and for mechanical hyperalgesia it was 10.15mg.kg^{-1}.

6.2.4 Screening studies on Partial sciatic nerve ligation (PSNL) model

Three behavioral modules of neuropathic pain assessment were studied using this model. Spontaneous pain responses were not clear and hence were not reported as quantification was difficult. Antiallodynic and anti-hyperalgesic effect of Series-II compounds were investigated in this model as represented in tables **6.8**, **6.9** and **6.10**.

Table 6.8 Effect of triazole derivatives on dynamic allodynia in PSNL rats

Dynamic allodyna_MG16 to MG21			
Compound	**30 min**	**60 min**	**120 min**
Vehicle	11.82 ± 7.22	18.64 ± 10.95	8.64 ± 5.90
Gabapentin	26.36 ± 5.07	45.15 ± 9.82	55.15 ± 5.64*
Dose 30mg.kg^{-1} *i.p.* route			
MG16	16.0 ± 4.3	23.4 ± 4.4	17.3 ± 4.5
MG17	17.0 ± 7.0	63.5 ± 7.1*	58.5 ± 7.1*
MG18	11.99 ± 5.1	26.4 ± 5.9	19.3 ± 5.4
MG19	13.05 ± 5.0	20.3 ± 9.5	20.4 ± 6.5
MG20	14.5 ± 3.98	26.1 ± 9.4	21.4 ± 7.6
MG21	10.4 ± 4.9	15.3 ± 5.9	13.9 ± 5.5
Dose 10mg.kg^{-1} *i.p.* route			
MG17	31.7 ± 2.1	45.0 ± 11	38.3 ± 9.2
Dose 3mg.kg^{-1} *i.p.* route			
MG17	37.7 ± 9.2	42.4 ± 9.2	37.7 ± 9.2

Each value represents the % reversal of dynamic allodynia (Mean ± SEM) of five rats (N=5); '*' highlighted values denotes significant at dose tested in comparison to their respective vehicle control at $p<0.05$ (One-way ANOVA, followed by post-hoc Dunnet's test)

Dynamic allodynia was significantly reduced with MG17 treatment at a dose of 30mg.kg^{-1} and the time of peak effect was observed at 60 minutes (63.5%) and effect was continued till 120 minutes with percentage reversal of pain 58.5. It did not show any significant effect at 10 and 3mg.kg^{-1}. Compounds MG16, MG18, MG19, MG20 and MG21 did not show any significant effect at 30, 10 and 3mg.kg^{-1}.

Table 6.9 Effect of triazole derivatives on cold allodynia in PSNL rats

Cold allodyna_MG16 to MG21			
Compound	**30 min**	**60 min**	**120 min**
Vehicle	14.93 ± 7.21	15.75 ± 10.01	22.24 ± 6.54
Gabapentin	46.53 ± 5.62	67.68 ± 1.40	63.81 ± 4.45*
Dose 30mg.kg^{-1} *i.p.* route			
MG16	20.4 ± 0.4	20.4 ± 4.5	15.4 ± 4.9
MG17	31.7 ± 7.3	78.7 ± 4.1*	61.5 ± 6.3*
MG18	20.5 ± 4.4	23.4 ± 8.3	28.4 ± 6.3
MG19	15.2 ± 2	19.4 ± 3.4	25.3 ± 5.9
MG20	16.9 ± 5.9	18. ± 9.3	20.1 ± 3.3
MG21	29.4 ± 4.1	27.4 ± 11	19.4 ± 8.2
Dose 10mg.kg^{-1} *i.p.* route			
MG17	27.1±4.7	52.9 ± 4.8	29.3 ± 7.1
Dose 3mg.kg^{-1} *i.p.* route			
MG17	32.3± 6.7	45.7± 2.5	43.7±7.1

Each value represents the % reversal of cold allodynia (Mean ± SEM) of five rats (N=5); '*' highlighted values denotes significant at dose tested in comparison to their respective vehicle control at $p < 0.05$ (One-way ANOVA, followed by post-hoc Dunnet's test)

Cold allodynia was significantly reduced with MG17 treatment at a dose of 30mg.kg^{-1}, and the time of peak effect was observed at 60 minutes (78.7%) and effect was continued till 120 minutes with percentage reversal of pain 61.5. It did not show any significant effect at 10 and 3mg.kg^{-1}. Compounds MG16, MG18, MG19, MG20 and MG21 did not show any significant effect at 30, 10 and 3mg.kg^{-1}.

Table 6.10 Effect of triazole derivatives on mechanical hyperalgesia in PSNL rats

Mechanical hyperalgesia_MG16 to MG21			
Compound	**30 min**	**60 min**	**120 min**
Vehicle	7.50 ± 9.24	5.36 ± 3.88	7.86 ± 7.83
Gabapentin	24.64 ± 3.81	43.57 ± 1.55	52.14 ± 5.67
Dose 30mg.kg^{-1} *i.p.* route			
MG16	7.0 ± 2.3	23.4 ± 3.4	17.9 ± 7.3
MG17	8.3 ± 8.3	58.3 ± 4.8*	39.6 ± 9.2
MG18	8.3 ± 3.2	21.2 ± 3.2	16.3 ± 2.9
MG19	8.2 ± 3.5	24.3 ± 1.3	19.4 ± 1.5
MG20	9.0 ± 2.1	28.4 ± 1.9	15.1 ± 4.9
MG21	7.9 ± 5.2	29.0 ± 1.4	18.9 ± 4.2
Dose 10mg.kg^{-1} *i.p.* route			
MG17	31.3 ± 6.1	48.7 ± 2.5	39.7 ± 3.0
Dose 3mg.kg^{-1} *i.p.* route			
MG17	33.3 ± 5.9	49.2 ± 7.9	35 ± 6.9

Each value represents the % reversal of mechanical hyperalgesia (Mean ± SEM) of five rats (N=5); '*' highlighted values denotes significant at dose tested in comparison to their respective vehicle control at $p < 0.05$ (One-way ANOVA, followed by post-hoc Dunnet's test)

Mechanical hyperalgesia was significantly reduced with MG17 treatment at a dose of 30mg.kg^{-1}, and the time of peak effect was observed at 60 minutes (58.3%). It did not show any significant effect at 10 and 3mg.kg^{-1}. Compounds MG16, MG18, MG19, MG20 and MG21 did not show any significant effect at 30, 10 and 3mg.kg^{-1}.

Table 6.11. ED_{50} values of triazole derivatives in PSNL animal model

ED_{50} (mg.kg^{-1}) values of Series-I compounds in PSNL animal model			
Treatment	**TPE (min)[a]**		
	TA	**CA**	**MH**
MG17	9.44 (60)	5.04 (60)	6.05 (60)

Table 6.11 represents median effective dose ED_{50} in mg.kg^{-1} body weight of PSNL rats at time of peak effect ie., 60 mins post treatment. TA is tactile allodynia; CA is cold allodynia; MH is mechanical hyperalgesia. ED_{50} values are calculated from dose response curve with three different doses viz., 30, 10 and 3mg.kg^{-1}.

ED_{50} of MG17 in PSNL model for dynamic (tactile) allodynia, cold allodynia, and for mechanical hyperalgesia was found to be <10mg.kg^{-1}.

6.2.5 Screening studies in Diabetic Peripheral Neuropathy (DPN) model

Streptozotocin at a dose of 30mg.kg^{-1} was given through *i.p* route at pH 4.5 in ice cold citrate buffer as detailed protocol presented in chapter-4 (Materials and Methods). Hyperglycaemia for prolonged period will bring changes in microenvironment surrounding neurons. The damage to microvascular system affects neuronal health and led to peripheral neuropathy. Table 6.12 represents effect of MG17 on diabetic peripheral neuropathy.

Table 6.12 Effect of MG17 on rat model of diabetic peripheral neuropathy (DPN)

Behavioral screening scores of MG17 in DPN rat model				
	Behavioral module	**Group**	**0hr**	**1hr**
a	Dynamic allodynia (50% paw withdrawal threshold)	Naïve	7.24±0.63	7.43±0.52
		Control	1.85±0.22	2.05±0.13
		Gabapentin	1.63±0.12	3.63±0.30
		MG17	1.90±0.25	**4.09±0.43***
b	Nerve Conduction Velocity (Meters/second)	Naïve	66.40±3.72	65.3±2.42
		Control	49.00±1.34	50.8±1.30
		Gabapentin	51.70±1.56	51.8±0.79
		MG17	53.76±1.44	**62.71±1.08***
c	Heat Hyperalgesia (52±0.5^{0}C) (Reflex time)	Naïve	13.4±0.81	13.6±0.87
		Control	6.80±0.86	7.20±0.86
		Gabapentin	5.60±0.42	5.80±0.62
		MG17	7.40±0.74	**9.2±0.48***
d	Tail flick (0±0.5^{0}C) (Reflex time)	Naïve	4.00±0.45	3.80±0.20
		Control	7.00±0.55	7.20±0.37
		Gabapentin	8.60±0.32	8.30±0.44
		MG17	5.00±0.92	8.00±0.37

Table 6.12. Effect of MG17 treatment on DPN rats on various modules of pain assessment and neurodegeneration.

* represents values significant ($p<0.05$) at 60 min post treatment compared to control (vehicle-20% PEG 400) group (n=5). Values compared with t test between treatment group and control group.

a: Represents tactile allodynia, MG17 significantly improved 50% paw withdrawal threshold; **b**: Represents nerve conduction velocity, MG17 treatment improved NCV of diseased rats significantly represented in meter.sec^{-1}; **c**: Represents heat hyperalgesia, response to hot plate. Significant improvement was observed in MG17 treated group in hot plate test represented as response time (seconds); **d**: Represents cold water paw dip, MG17 was not effective in CWPD test represented as response time (seconds)..

MG17 was selected based on behavioral scores obtained in CCI and PSNL models. MG17 was effective in reducing tactile allodynia and improving nerve conduction velocity at an ED_{50} of <10mg.kg^{-1} and hence was considered for DPN study. Table **6.12** represents the behavioral scores of DPN rats at 30mg.kg^{-1} dose of MG17. From the behavioral screening studies it was found that compound MG17 is having potent anti-allodynic and anti-hyperalgesic properties as MG17 significantly improved paw withdrawal threshold value from 1.9 to 4.09 (generally >4 value is considered to be significant according to modified Dixon method), nerve conduction in tail from 53 to 62 m/s, delayed thermal hyperalgesia but was not effective in cold water tail flick test.

6.2.6 Screening studies in Methyl mercury (MeHg) induced neurodegeneration model

The active compound in CCI and PSNL model was selected for further screening in MeHg induced neurodegeneration rat model. Detailed protocol is presented in chapter-4 (Materials and Methods). Out of six compounds screened, MG17 compound was identified to have consistent anti-allodynic and anti-hyperalgesic effect in all the three peripheral neuropathic pain rat models (CCI, PSNL and DPN), we decided to further screen this compound on methyl mercury induced neurodegeneration to evaluate its neuroprotective activity against environmental pollutant MeHg. Below table represents the behavioral scores of MeHg treated rats at 30mg.kg^{-1} dose of MG17. Gabapentin at a dose of 10mg.kg^{-1}was used as standard drug to compare MG17 (30mg.kg^{-1}) activity.

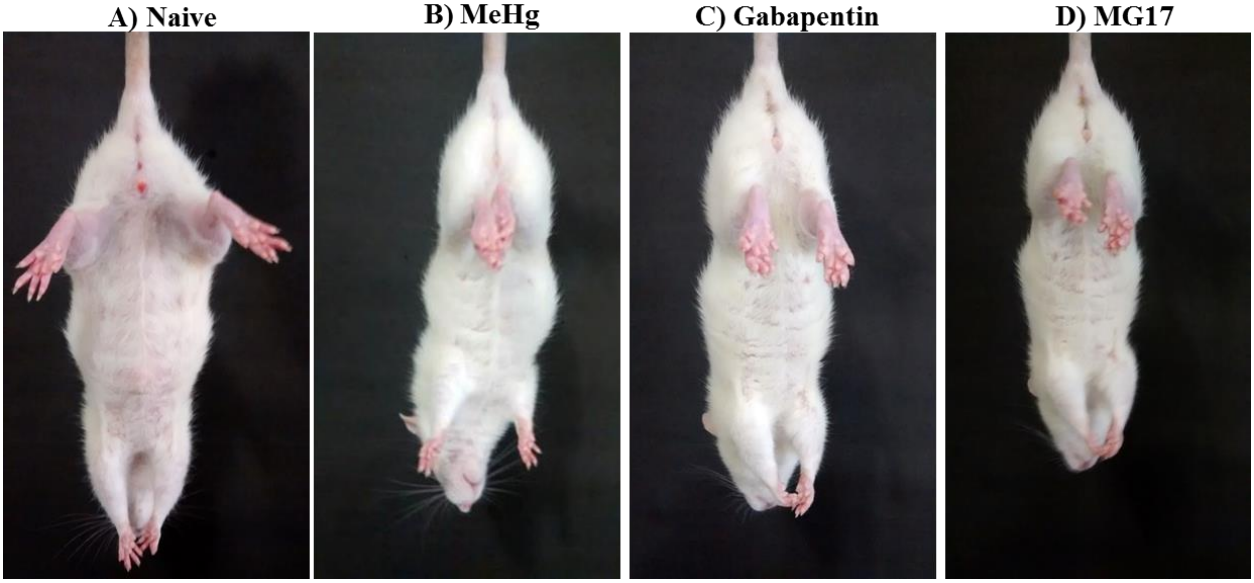


Figure 6.3: Effect of MG17 on clasping of MeHg induced neurodegenerative rats

Behavioral analysis of MeHg treated animals. a) Bodyweight analysis of animals from Day1 to Day31. b) Actophotometer graph represents the % neurotoxicity in animals from Day1to Day31. c) Clasping score of animals on each day was represented. d) Representative animals from each group clasping were shown (A=0, B=3, C=1, D=2).

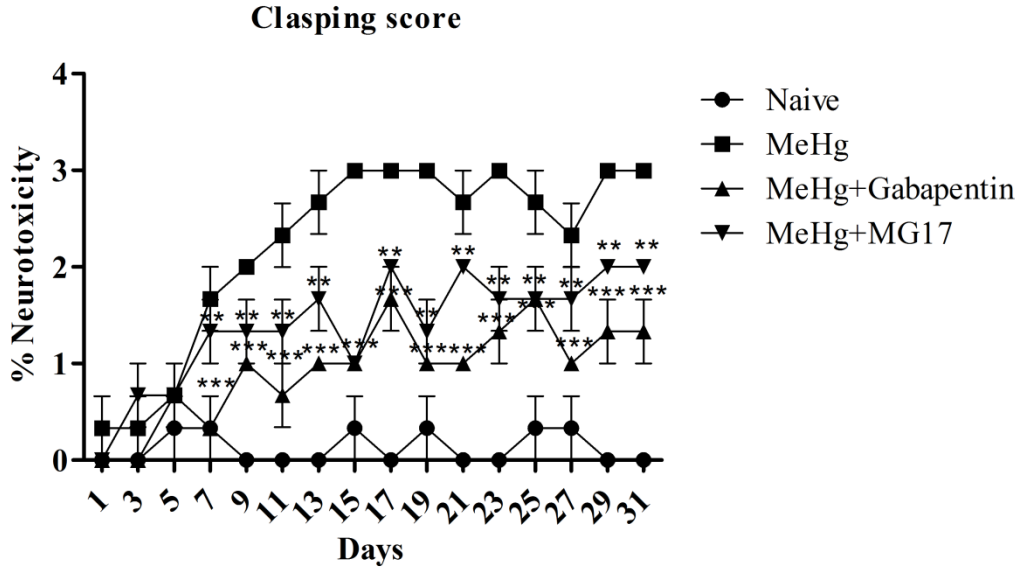


Figure 6.4: Effect of MG17 on clasping score of MeHg induced neurodegenerative rat

MG17 treatment significantly reduced clasping score and was found have more efficacy than gabapentin. Results were represented as mean ± SEM at *p<0.05, **p<0.01 and ***p<0.001. One way ANOVA followed by Dunnet's post hoc test.

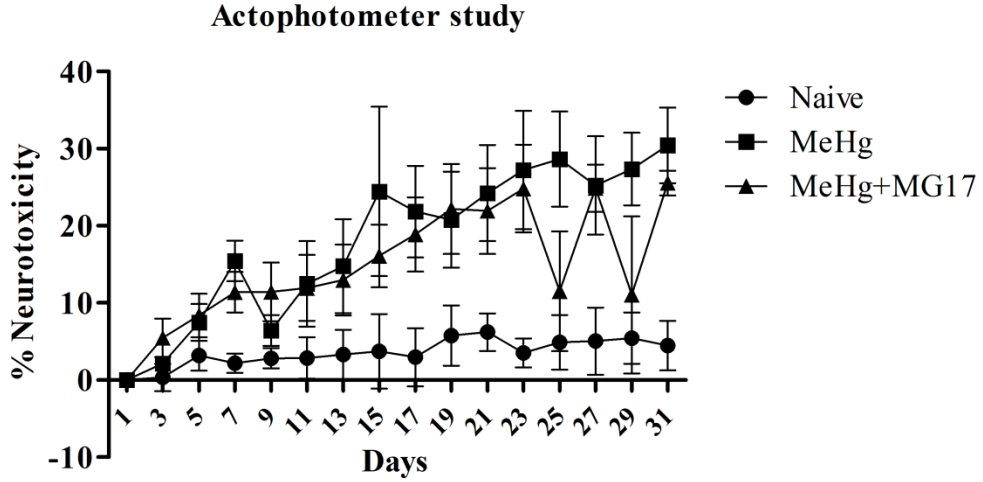


Figure 6.5: Effect of MG17 on activity score of MeHg induced neurodegenerative rats

MG17 treatment significantly improved activity score. Results were represented as mean ± SEM at *p<0.05, **p<0.01 and ***p<0.001. One way ANOVA followed by Dunnet's post hoc test.

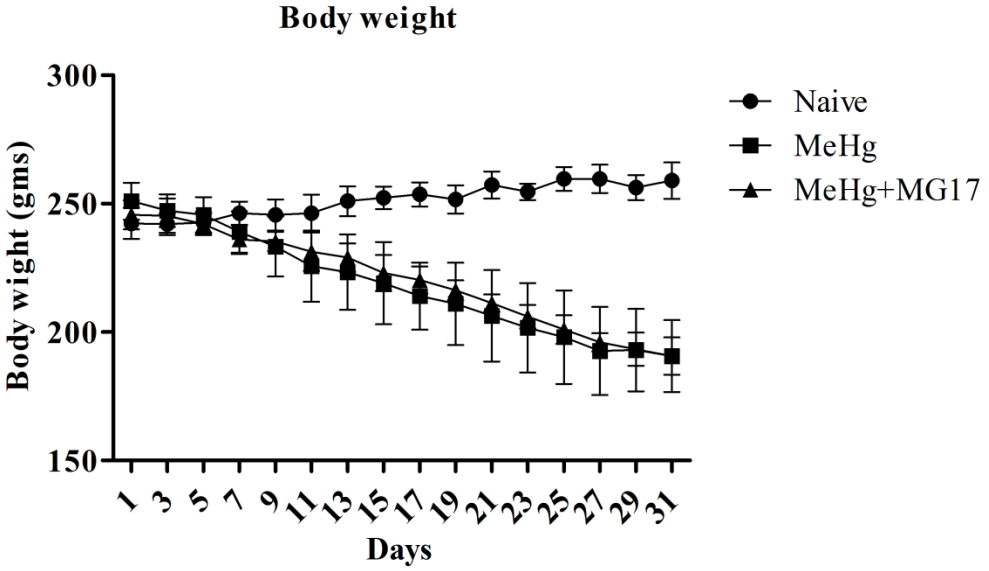


Figure 6.6: Effect of MG17 on body weights of MeHg induced neurodegenerative rats
MG17 treatment significantly improved or maintained body weights of MeHg treated rats. Results were represented as mean ± SEM at *p<0.05, **p<0.01 and ***p<0.001. One way ANOVA followed by Dunnet's post hoc test.

MG17 was taken up for *in vivo* studies to assess the neuroprotective activity in MeHg induced animal model using a cascade of behavioral assays including clasping behavior and locomotor test as it was having anti-allodynic and anti-hyperalgesic activities in mononeuropathy models and improved NCV, reduced thermal hyperalgesia in DPN model. MG17 was found to reduce MeHg induced neurodegeneration in rats more efficiently than gabapentin as observed in behavioral screening studies. MeHg treated control rats drastically reduced weight within 31days of treatment due to toxic effects of the chemical, MG17 treated group did not show such drastic drop in body weight which can be considered as its protective effect against environmental pollutant-MeHg. Both MG17 and gabapentin treatments were significantly effective in decreasing clasping score throughout the treatment period of 31days. Activity score was also improved with MG17 treatment as it was found that MeHg toxic effects reduced exploratory behaviour of rats making them more sedentary. Further the activity was also re-ascertained using histopathological studies and expression levels of inflammatory mediators.

6.3 Gene expression studies in CCI rats

Real time polymerase chain reaction cycle experiments were performed to determine the effect of MG17 treatment on dysregulated pro-inflammatory mediators such as IL-1β, IL-6 and TNF-α in the nervous tissue of CCI rats. The mRNA expression levels were determined in spinal nervous tissue collected from rats post 12 days of CCI induced neuropathic pain and after confirming neuropathy symptoms by behavioral analysis.

Figure 6.7: Effect of MG17 on IL-1β expression

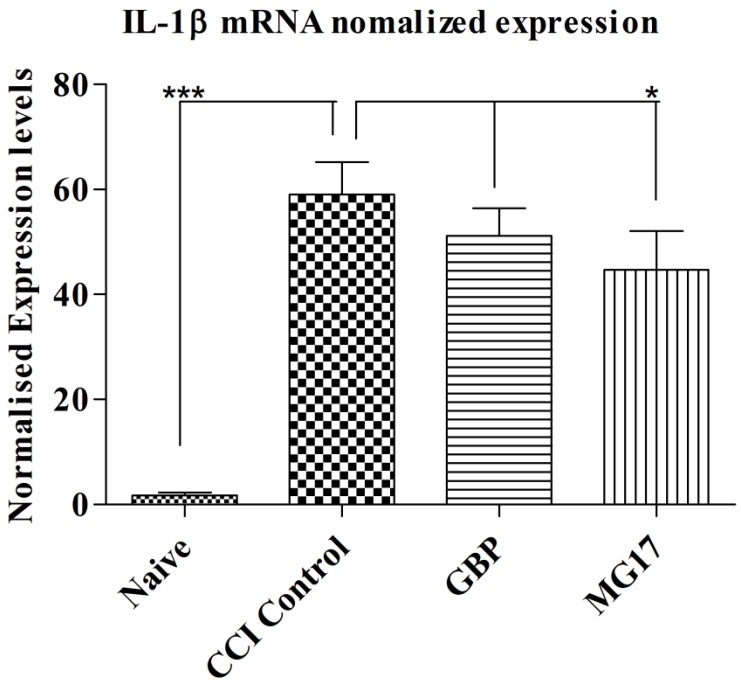


Figure 6.7: Graph depicts relative normalized expression levels of pro-inflammatory mediator IL-1β in CCI induced neuropathic pain rat spinal tissues compared to normal rats (naïve) and MG17 treated rats. GAPDH was used as housekeeping gene. Significance (*p>0.05, **p<0.01 and ***p<0.001). One way ANOVA followed by Dunnet's post-test.

Figure 6.8: Effect of MG17 on IL-6 expression

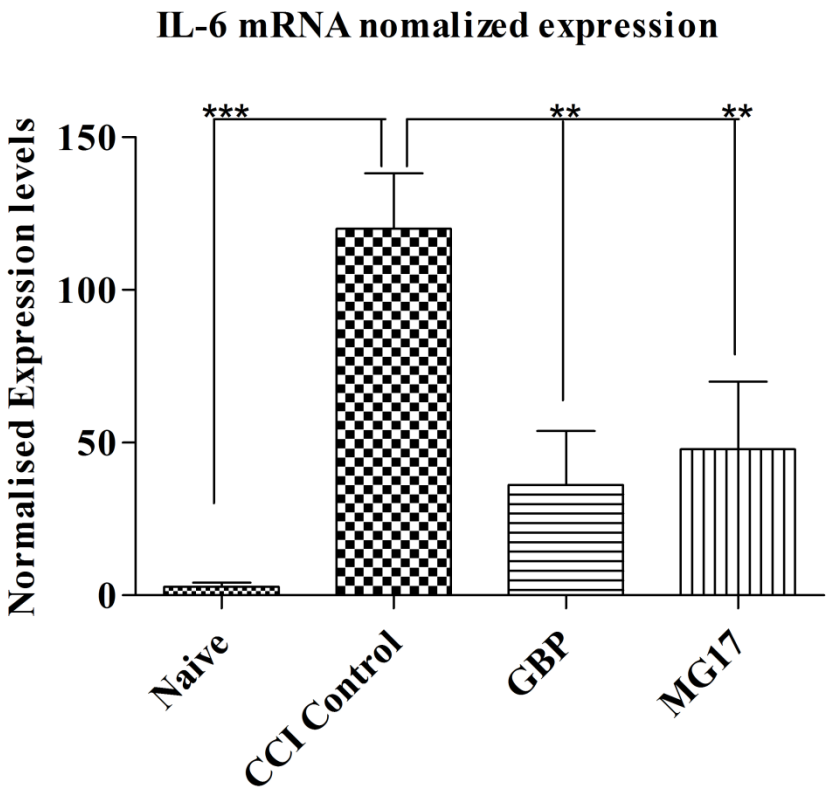


Figure 6.8: Graph depicts relative normalized expression levels of pro-inflammatory mediator IL-6 in CCI induced neuropathic pain rat spinal tissues compared to normal rats (naïve) and MG17 treated rats. GAPDH was used as housekeeping gene. Significance (*$p>0.05$, **$p<0.01$ and ***$p<0.001$). One way ANOVA followed by Dunnet's post-test.

Figure 6.9: Effect of MG17 on TNF-α expression

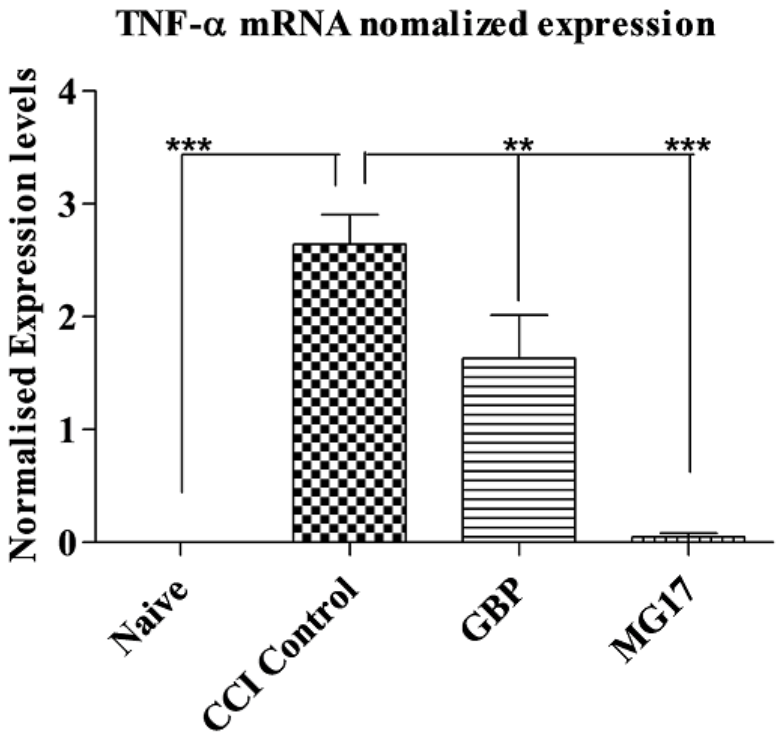


Figure 6.9: Graph depicts relative normalized expression levels of pro-inflammatory mediator TNF-α in CCI induced neuropathic pain rat spinal tissues compared to normal rats (naïve) and MG17 treated rats. GAPDH was used as housekeeping gene. Significance (*p>0.05, **p<0.01 and ***p<0.001). One way ANOVA followed by Dunnet's post-test.

The active compound MG17 was found to control or normalize the dysregulated gene expressions of TNF-α, IL-6 and IL-1β significantly compared to CCI control group. The compound MG17 was selected for *in vivo* gene expression studies as it showed consistent and very good pharmacological activity (anti allodynic, anti-hyperalgesic and neuroprotective effects) in all the four rodent models -CCI, PSNL, DPN and MeHg induced neurodegeneration. MG17 was also found to be potent ROCK-I inhibitor (Table. **6.14**) and it showed least cytotoxic effect on HEK-293 cell lines (Table. **6.13**), hence we were interested to explore the mechanism

by which MG17 is showing all these activities. We have done thorough literature search to make a list of inflammatory genes that are upregulated during neuropathic pain states. IL-1β, IL-6 and TNF-α [Austin and Moalem Taylor, 2010; Yogeeswari P *et al*., 2012] are the most commonly released proinflammatory mediators upon injury and well known mediators of inflammation and pain which can act directly on nociceptors to induce pain hypersensitivity.

6.3.2 Gene expression studies in MeHg treated rats

Real time polymerase chain reaction cycle experiments were performed to determine the effect of MG17 treatment on dysregulated pro-inflammatory mediators such as IL-1β, IL-6 and TNF-α in the nervous tissue of MeHg treated rats. The mRNA expression levels were determined in spinal nervous tissue collected from rats post four week treatment with 20ppm MeHg and after confirming neurodegenerative symptoms by clasping analysis.

Figure 6.10: Effect of MG17 on IL-1β expression

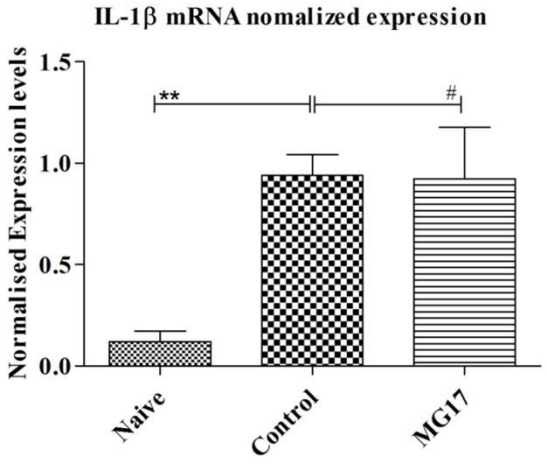


Figure 6.10: Graph depicts relative normalized expression levels of pro-inflammatory mediator Il-1β in MeHg induced neurodegeneration rat spinal tissues compared to normal rats (naïve) and MG17 treated rats. GAPDH was used as housekeeping gene. Significance (*p>0.05, **p<0.01 and ***p<0.001). One way ANOVA followed by Dunnet's post-test.

Figure 6.11: Effect of MG17 on IL-6 expression

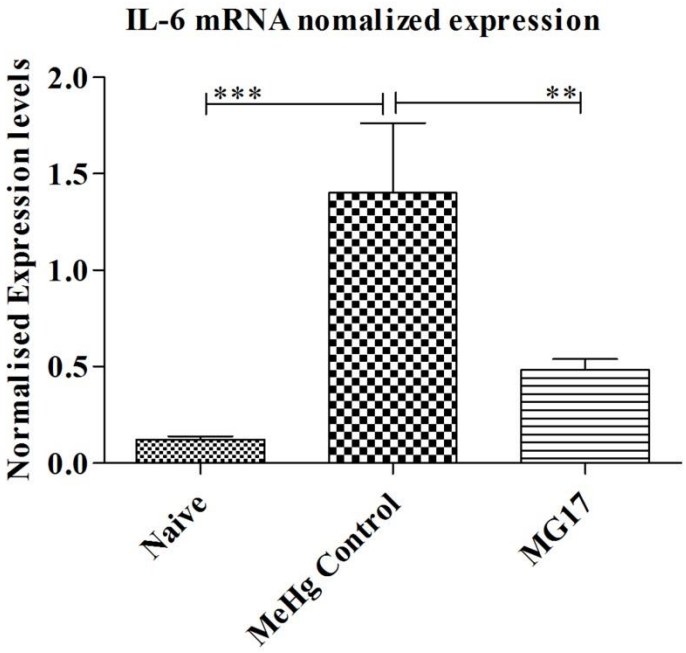


Figure 6.11: Graph depicts relative normalized expression levels of pro-inflammatory mediator IL-6 in MeHg induced neurodegeneration rat spinal tissues compared to normal rats (naïve) and MG17 treated rats. GAPDH was used as housekeeping gene. Significance (*$p>0.05$, **$p<0.01$ and ***$p<0.001$). One way ANOVA followed by Dunnett's post-test.

Figure 6.12: Effect of MG17 on TNF-α expression

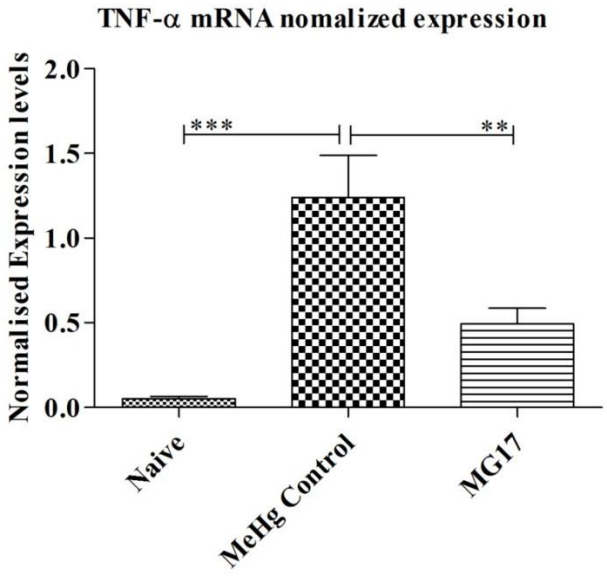


Figure 6.12: Graph depicts relative normalized expression levels of pro-inflammatory mediator TNF-α in MeHg induced neurodegeneration rat spinal tissues compared to normal rats (naïve) and MG17 treated rats. GAPDH was used as housekeeping gene. Significance (*p>0.05, **p<0.01 and ***p<0.001). One way ANOVA followed by Dunnett's post-test.

The active compound MG17 was found to control or normalize the dysregulated gene expression of TNF-α and IL-6 significantly whereas the upregulated IL-1β gene expression was not normalized and remained elevated. The compound MG17 was selected for *in vivo* gene expression studies as it showed consistent and very good pharmacological activity (anti allodynic, anti-hyperalgesic and neuroprotective effects) in all the four rodent models -CCI, PSNL, DPN and MeHg induced neurodegeneration.

6.4 Brain histopathology studies (H &E staining)

The brain tissue from rats treated with methyl mercury (20 ppm/day *P.0*) for over four weeks were collected by sacrificing them, tissues stored in formaldehyde and histopathological analysis was done on 2-4μm thin sections using hematoxylin and eosin stain. During the analysis, the extent of neurodegeneration in MeHg group was compared with Naïve group and MeHg+MG17 (30mg.kg^{-1} *i.p*) group. The histopathology images were obtained at a magnification of 20X with electron microscope under bright field.

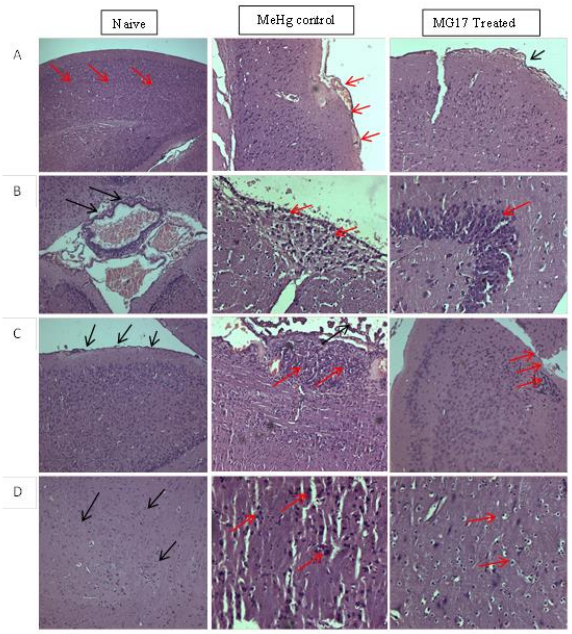


Figure 6.10: Brain sections of study rats upon hematoxylin and eosin (H&E) stain revealed the extent of degeneration that took place upon treatment with MeHg for four weeks; arrows indicate different zones of healthy tissue observed in naïve group. Arrows in MeHg control group shows degradation, meningeal haemorrhage, microglial and ependymal cell infiltration. Arrows in MG17 treated group shows no signs of degradation except small number of inflammatory cells and slight thickening of meningeal layer. In the figure A denotes cerebra hemispheres, B denotes ventricular region, C denotes meninges and D denotes cerebral cortex.

Histopathological analysis revealed that, treatment with MG17 has prevented the extent of degenerative changes caused by MeHg in positive control group (MeHg group) in comparison with healthy brain tissue of naïve rats.

6.5 Acute paw edema model

The effect of compound MG17 on acute inflammation was studied to evaluate its efficacy on inflamed paw in carrageenan induced rat paw edema model. The compound MG17 (30mg.kg^{-1}) was compared with standard anti-inflammatory drug Indomethacin (10mg.kg^{-1}) and disease control group.

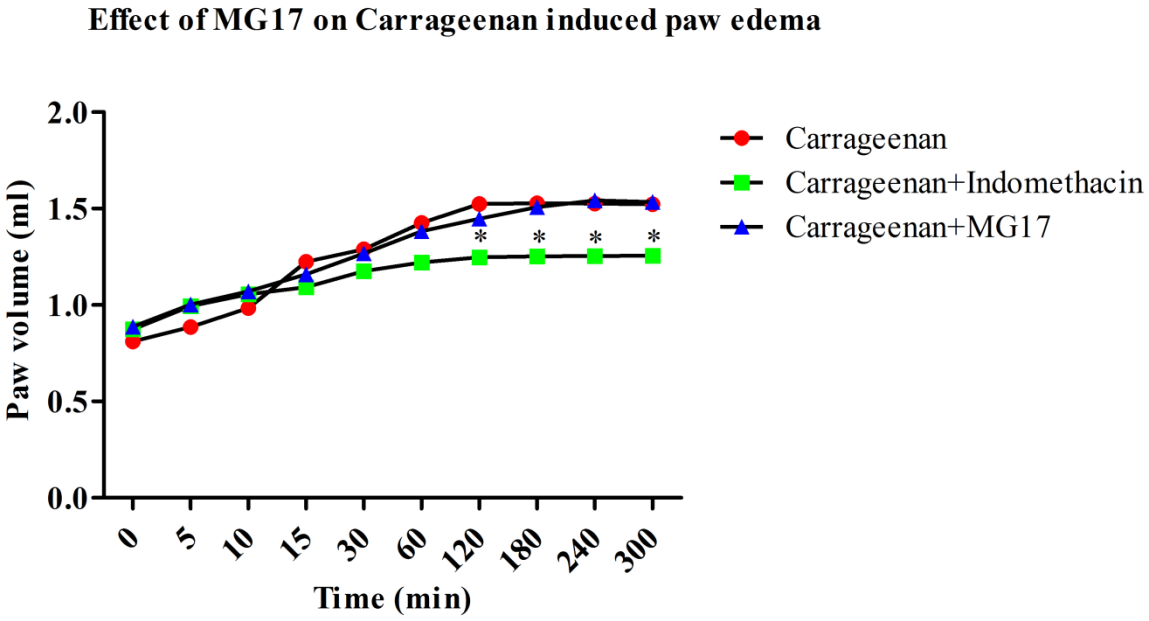


Figure 6.11: Effect of MG17 on Carrageenan induced paw edema

Effects of test compound (MG17) and Indomethacin on Carrageenan-Induced Rat Paw Edema. Each value was represented as mean ± S.E.M. *p>0.05, **p<0.01 and ***p<0.001 when compared to the carrageenan group (One-way ANOVA followed by Dunnet's post-test). There was no significant reduction in paw volume observed in MG17 treatment group.

The test compounds (Series-II Triazole derivatives) were further evaluated for its anti-inflammatory activity using carrageenan induced mice paw edema (Acute inflammation) rat model. These results suggested that **MG17** was found to be non-significant with no promising

anti-nociceptive and anti-inflammatory properties till 300 min of observation time compared to control group and standard drug (Indomethacin) treatment group. Finally, we could conclude that **MG17** is not much effective on acute inflammation; it may be further developed into a therapeutic agent for the treatment or prevention of central pain as it showed good activity score in behavioral screening studies.

6.6 MTT assay on HEK-293 cells

MTT assay was performed to test compounds cytotoxic concentration with six different concentrations ranging from 1nM to 100μM. From the IC_{50} values, it is clear that compound MG17 is having IC_{50}>100 indicating that it is more safe to use high dose.

Table 6.13 Effect of triazole derivatives on cell cytotoxicity

Compound	IC_{50}(Mean±SD)
MG16	84.22±0.24
MG17	>100
MG18	36.22±0.16
MG19	53.45±0.32
MG20	48.46±0.18
MG21	09.16±0.12

Table 6.13 Represents IC_{50} value of MG17 found to be >100 μM on HEK-293 cells, indicating that the compound has minimum or no cytotoxicity at the maximum concentration used for *in vitro* experiments.

Triazole derivatives were screened to evaluate their cytotoxic effect on human embryonic kidney cell lines (HEK-293) and most of the compounds are found to be non-toxic below 10μM concentration except MG21. Among the six compounds compound MG17 was found to be least toxic (**CC_{50}>100**). We performed MTT assay to select compounds for further *in vitro* cell based enzyme inhibition assays.

6.7 ROCK-I inhibition assay

Compounds were screened to evaluate their effect on ROCK-I inhibition using different concentrations and inhibitory concentration (IC_{50}) was determined. Rho kinase plays an important role in neurodegeneration and development of neuropathic pain. Recent literature revealed that ROCK inhibition promotes neuroprotection (Van de Velde S *et al*., 2015; Yogeeswari P *et al*., 2012). Table **6.14** shows that except 3 compound MG16, MG18 and MG19 all the compounds showed ROCK-I IC_{50}<10μM. Compounds MG17 and MG20 were found to be most potent inhibitors with an IC_{50}<1 μM.

Table 6.14 Effect of triazole derivatives on ROCK-I inhibition (ADP-glow assay)

Compound	ROCK IC_{50}(μM)
MG16	72.53±4.62
MG17	0.009±0.001
MG18	36.17±2.42
MG19	87.61±5.21
MG20	0.006±0.001
MG21	2.52±0.20

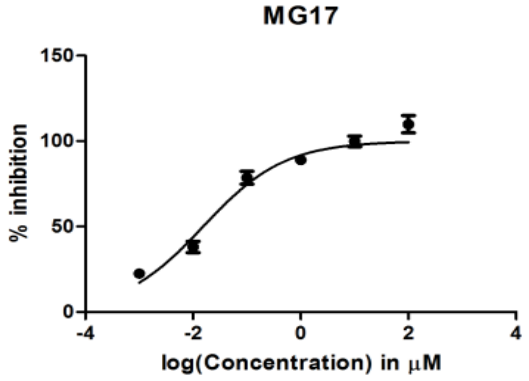


Fig 6.8: Effect of Triazole derivative (MG17) on ROCK inhibition (ADP-glow assay)

IC_{50} value of MG17 was found to be **9.22 ± 0.94 nM**, values represented as mean ± standard deviation of three independent experimental results.

6.8 ROS estimation (Flow cytometer)

The compound MG17 was evaluated for its activity on inhibiting generation of reactive oxygen species (ROS) in and around neuronal tissues as excess ROS is reported to aid in neurodegeneration and inflammation. The study was performed on RAW 264.7 cells with LPS stimulation to produce ROS and then MG17 treatment was given. MG17 treatment showed significant reduction in ROS positive cells.

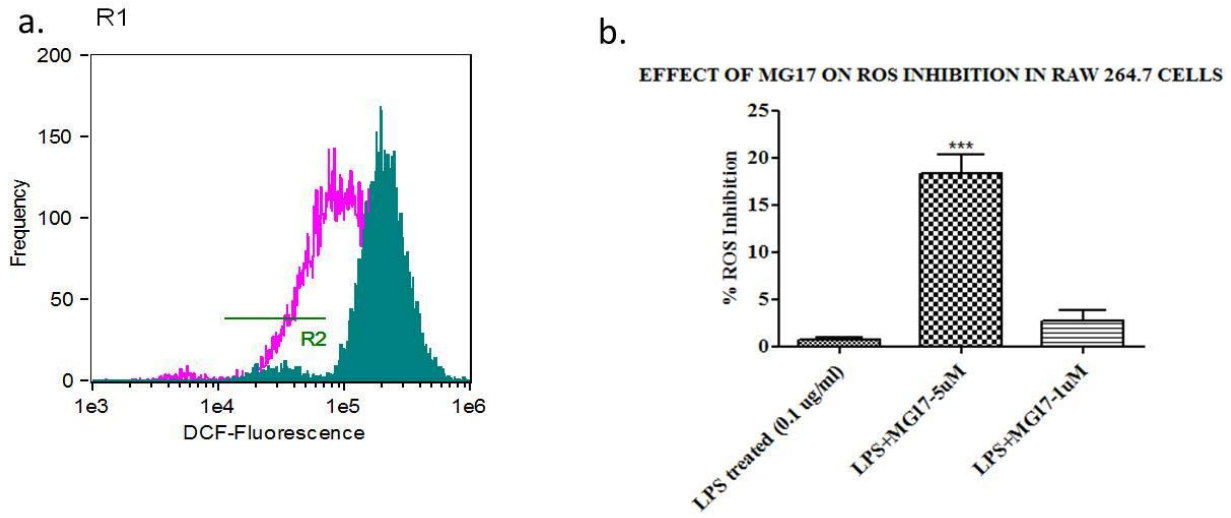


Figure 6.9: Effect of MG17 on ROS in RAW 264.7 cells. a Cells were treated with LPS (0.1 μg/ml) and with different concentrations of MG17 (0.1, 1 and 5μM) for 3h and incubated with DCFDA for 30 min. The intracellular ROS was measured by flow cytometry. b Histogram depicting the percentage of ROS positive cells of indicated concentrations of MG17 after 3h. Data were expressed as mean ± SEM of three independent experiments. ***p<0.001 versus LPS treated group.

This study was performed to evaluate MG17 compound for its potency to abrogate intracellular ROS production. We considered this study to be important as ROS production is one of the major cause of neuronal apoptosis. ROS is also produced by activation of Rho kinase and in hyperglycaemic states as in case of diabetes. We were trying to identify possible mechanisms of action of MG17 and the DCFDA (Dichloro-flourescein diacetate) assay results showed that MG17 is having potent ROS inhibitory effect at a concentration of 5μM and the effect is also concentration dependant.

6.9 Summary and conclusion

In the present study, we attempted to identify the structural requirements for a compound to act as antinociceptive agent with CNS effects. The substitution on the core triazole moiety will decide the fate of the compound. In my thesis, the six compounds selected were based on parameters obtained from *in-silico* screening studies. As the major objective of our study was to develop the compounds for neurological disorders, it was crucial to consider blood brain barrier permeability of these hit compounds. In the neurotoxicity screening studies, compound MG16 was found to have neurotoxicity at the highest dose used ($300mg.kg^{-1}$) remaining all compounds of the series was found to be safe at the tested dose levels. In the preliminary behavioral screening studies MG17 emerged as the most potent compound of the triazole series, hence was taken forward for subsequent screening studies.

Test compound MG17 was found to possess promising anti-nociceptive and anti-inflammatory properties. Though a little understanding was obtained from *in vitro* cell based assays and gene expression studies. The exact mechanism of action, whether it is central or peripheral was not known. As the compound MG17 showed normalizing effect on upregulated pro-inflammatory gene expressions in spinal nervous tissue, it can be hypothesized that MG17 may be having central actions. The ROS inhibition ability of MG17 can be attributed to its neuroprotective function as increased ROS causes cellular apoptosis in respective tissue. The compound MG17 was also found to possess rho kinase inhibition which is crucial for reducing neuroinflammation. MG17 was not effective locally as observed in carrageenan induced acute rat paw edema and was effective centrally, showed neuroprotective activity as observed in brain histopathology analyses against MeHg induced neurodegeneration.

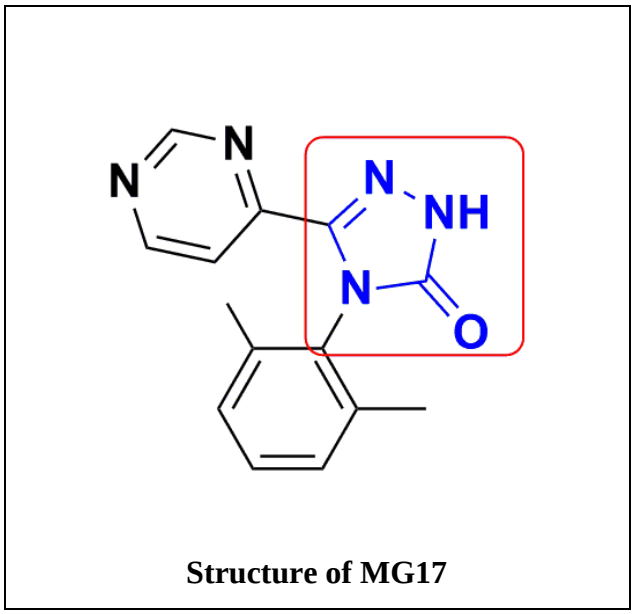


Figure 6.10 Structure of most active triazole derivative MG17

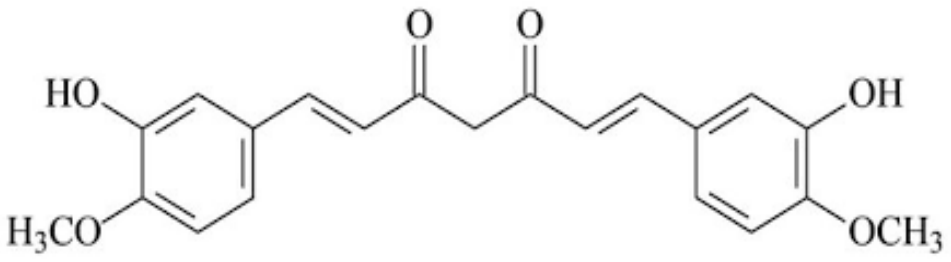
CHAPTER-7

Results and discussion: Neuropharmacological and molecular interventions on Curcumin derivatives

7.1 Series-III molecules

The compounds selected for screening studies are categorized into three groups based on the common central moiety present in them. The series-III molecules containing the central core moiety 'Curcumin' was selected to derive its analogues for screening studies. Four derivatives containing different substituents on curcumin moiety presented in this group. The following table contains the structures and molecular formula of these compounds.

7.1.1 Structure of Curcumin moiety

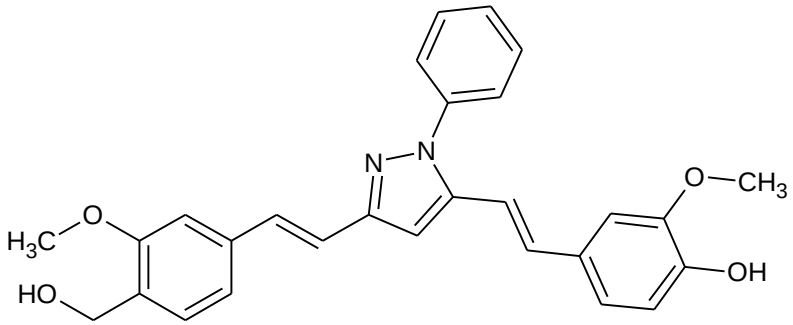
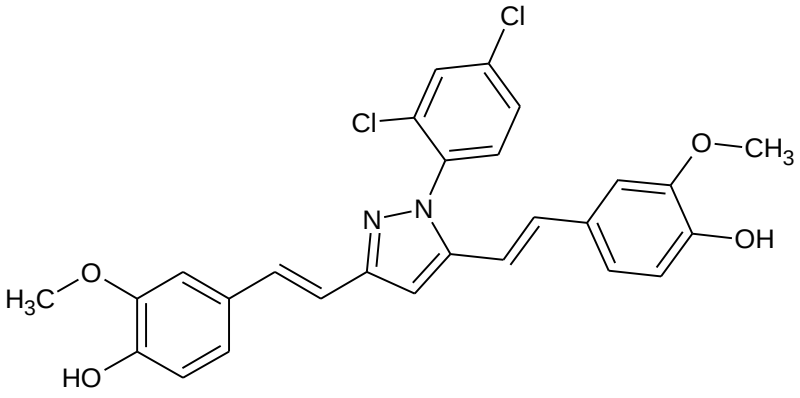


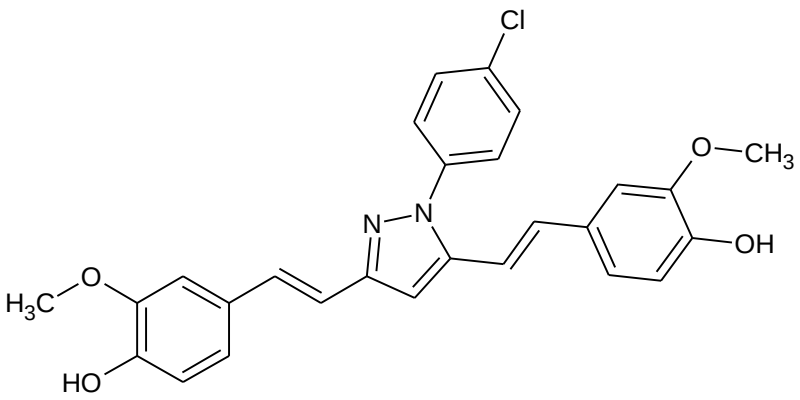
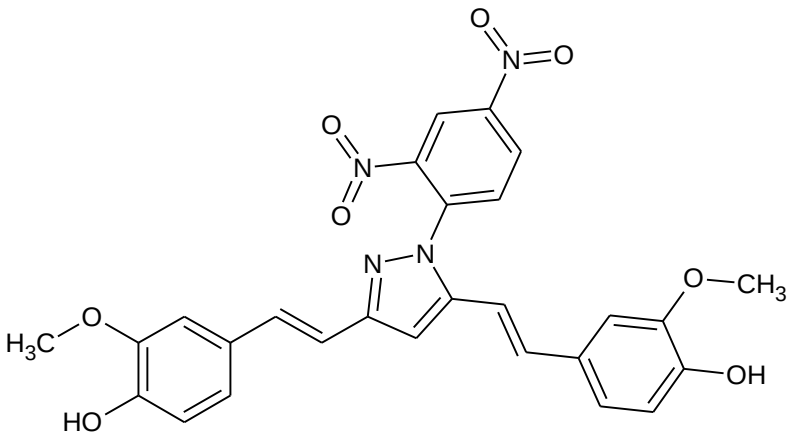
Curcumin

Figure 7.1: Structure of Curcumin moiety

7.1.2 Structures of Series-III compounds

Table 7.1 Structures of Series-III compounds

Compound Code	Structure	Molecular weight
MG22		454.51
MG23	 Satyendra Mishra *et al*., 2008	509.38

MG24	 Dinesh Kumar *et al*., 2012	474.93
MG25	 Okunieff Paul *et al.*, 2009	530.48

7.2 Pharmacological evaluation

Curcumins are versatile pharmacophores which have multiple biological effects such as anticancer, antibacterial, anti-inflammatory and anti-oxidant properties apart from its ability to modulate multiple cell signaling pathways [Subhash. *et al*., 2013], Inhibitory properties [Yogeeswari et al., 2005], hence can be further screened to evaluate their potency in alleviating neuropathic pain symptoms and neurodegeneration. Neuropharmacological screening studies were considered important and hence the thesis work was focused on exploring the biological activities of the compounds employing animal models and cell based assays.

7.2.1 Neurotoxicity screening

Neurotoxicity screening was carried out to study effect of NCEs on motor coordination using the natural fear of falling motivation (rota rod test).In the present study, neurotoxicity was assessed using two animal models *viz.* rotarod and actophotometer based screening. Compounds were administered at three dose levels (300,100 and 30 $mg.kg^{-1}$). Minimal motor impairment was measured by rotarod test and neurotoxicity was indicated by the inability of the animal to maintain balance on the rotating rod for at least 2 min. In these acute neurotoxicity assays, only one compound out of six exhibited motor deficit at the highest tested dose ($300mg.kg^{-1}$) up to 2 h post administration (**Table 7.2**). But at a dose of 30 and 100 $mg.kg^{-1}$ none of compounds (MG16 to MG21) were found to be toxic in rotarod test and actophotometer assay. Thus, we decided to proceed with a lower dose ($30mg.kg^{-1}$) for further *in vivo* experiments. The results of neurotoxicity assays clearly indicated that one compound MG16 showed neurotoxicity at high doses ($300mg.kg^{-1}$). Thus, we further carried out *in vivo* experiments with a sub neurotoxic dose ($30mg.kg^{-1}$).

Table 7.2. Neurotoxic Activity of test Compounds.

Test Group	Neurotoxicity [a]			
	Rotarod		Actophotometer	
	1h	2h	1h	2h
MG22	300	300	300	300
MG23	300	300	300	300
MG24	300	300	100	100
MG25	100	300	100	300

[a] Neurotoxicity screening of Series-III compounds. Dose of 30, 100 and $300mg.kg^{-1}$ were administered. The figures in the table indicate the dose ($mg.kg^{-1}$) whereby bioactivity (neurotoxicity) was demonstrated in half or more of the mice.

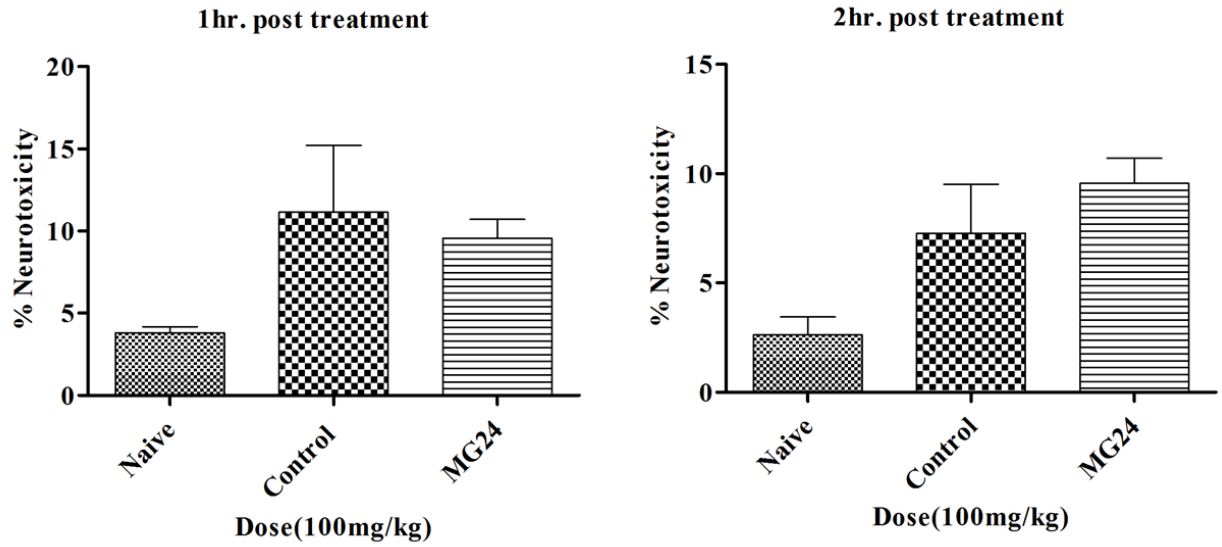


Figure 7.2: MG24 % neurotoxicity assessment

Neurotoxic effect of MG24 given intraperitoneally (*i.p*. route) on Actophotometer. Each bar represents the mean ± SEM of 5 rats; *p<0.5 statistically significant compared to their respective control. (* p < 0.05; ** p< 0.01; *** p< 0.001; One-way ANOVA followed by a Dunnet's post hoc test was used).

Compound is considered as neurotoxic if % neurotoxicity is greater than 50. In our study % neurotoxicity observed was less than 50 at a higher dose of 100mg.kg^{-1}. All the studies were performed at a much lower dose ie., 30mg.kg^{-1} with which significant pharmacological activity was observed. Since 100mg.kg^{-1} did not show neurotoxic effects, 30mg.kg^{-1} is considered as safe dose to proceed for further studies.

7.2.2 Screening studies on animal models of neuropathic pain

Compounds selected were screened for their preliminary pharmacological activities employing various behavioral screening modules. Two rodent models of neuropathic pain, chronic constriction injury and partial sciatic nerve ligation were considered for initial screening of compounds. Compounds which are pharmacologically active at 30 mg.kg^{-1} dose were selected for median effective dose (ED_{50}) determination and further screened on animal models at 10 and 3mg.kg^{-1}doses.

7.2.3 Chronic constriction injury (CCI): Rat model of mononeuropathy

Four modules of behavioral analysis were employed to identify the preliminary biological effect of Series-III compounds on peripheral mononeuropathy viz. Spontaneous pain, Dynamic allodynia, Cold allodynia and Mechanical hyperalgesia.

Table 7.3. Effect of curcumin derivatives on spontaneous pain

Spontaneous Pain_MG22 to MG25			
Compound	**30 min**	**60 min**	**120 min**
Vehicle	6.97 ± 2.88	8.90± 2.22	11.83±2.50
Gabapentin	48.34 ± 2.20	**68.50± 3.04***	**68.31±5.00***
Dose 30 mg.kg^{-1} i.p. route			
MG22	19.5 ± 0.4	25.4± 2.9	23.3±3.3
MG23	20.3 ± 3.3	21.1± 3.2	15.6±2.1
MG24	**39.1 ± 3.8**	**67.7± 3.3***	**51.6±3.8***
MG25	23.5 ± 4.3	21.4± 2.2	15.4±1.6
Dose 10 mg.kg^{-1} i.p. route			
MG24	36.1 ± 4.3	49.3± 5.3	45.8±9.7
Dose 3 mg.kg^{-1} i.p. route			
MG24	8.9 ± 7.7	41.1± 5.7	35.9±9.5

Each value represents the % reversal of spontaneous pain (Mean ±SEM) of five rats (N=5); '*' highlighted values denotes significant at dose tested in comparison to their respective vehicle control at $p < 0.05$ (One-way ANOVA, followed by post-hoc Dunnet's test)

Spontaneous pain was significantly reduced with MG24 treatment at a dose of 30mg.kg^{-1}, and the time of peak effect was observed at 60 minutes (67.7%) and effect was continued till 120 minutes with percentage reversal of pain 51.6. It did not show any significant effect at 10 and 3mg.kg^{-1}. Compounds MG22, MG23, and MG25 did not show any significant effect at 30, 10 and 3mg.kg^{-1}.

Table 7.4 Effect of curcumin derivatives on dynamic allodynia

Dynamic allodynia_MG22to MG25			
Compound	**30 min**	**60 min**	**120 min**
Vehicle	1.40 ± 1.74	2.79 ± 2.01	4.61 ± 1.98
Gabapentin	15.30 ± 6.04	48.48 ± 7.98	**60.15 ± 7.95***
Dose 30mg.kg^{-1} i.p. route			
MG22	13.3 ± 4.7	24.4 ± 2.1	16.4 ± 7.5
MG23	15.4 ± 3.6	21.9 ± 2.9	14.1 ± 5.4
MG24	26.4 ± 9.5	**56.8 ± 9.3***	**51.8 ± 9.3***
MG25	13.9 ± 8.5	29.4 ± 5.4	18.4 ± 4.7
Dose 10mg.kg^{-1} i.p. route			
MG24	36.7 ± 9.2	48.3 ± 11	41.74 ± 8
Dose 3mg.kg^{-1} i.p. route			
MG24	47.7 ± 16.8	45.7 ± 9.2	40.7 ± 14.2

Each value represents the % reversal of dynamic allodynia (Mean ± SEM) of five rats (N=5); '*' highlighted values denotes significant at dose tested in comparison to their respective vehicle control at $p<0.05$ (One-way ANOVA, followed by post-hoc Dunnet's test)

Dynamic allodynia was significantly reduced with MG24 treatment at a dose of 30mg.kg^{-1}, and the time of peak effect was observed at 60 minutes (56.8%) and effect was continued till 120 minutes with percentage reversal of pain 51.8. It did not show any significant effect at 10 and 3mg.kg^{-1}. Compounds MG22, MG23, and MG25 did not show any significant effect at 30, 10 and 3mg.kg^{-1}.

Table 7.5 Effect of curcumin derivatives on cold allodynia

Cold allodynia_MG16 to MG21			
Compound	**30 min**	**60 min**	**120 min**
Vehicle	11.06 ± 4.96	11.93 ± 2.85	10.26 ± 4.62
Gabapentin	33.59 ± 3.66	**50.48 ± 3.42***	**58.07 ± 7.17***
Dose 30mg.kg^{-1} i.p. route			
MG22	14.5 ± 5.6	31.3 ± 4.5	13.4 ± 3.2
MG23	18.8 ± 9.2	24.5 ± 5.8	26.4 ± 3.1
MG24	36.4 ± 14.3	**62.3 ± 6.1***	**50.7 ± 8.0***
MG25	17.4 ± 3.3	31 ± 2.4	25.4 ± 5.3
Dose 10mg.kg^{-1} i.p. route			
MG24	45.4 ± 11.3	40.8 ± 8.6	33.3 ± 11
Dose 3mg.kg^{-1} i.p. route			
MG24	36.1 ± 6.6	38.8 ± 5.1	25.9 ± 12.3

Each value represents the % reversal of cold allodynia (Mean ± SEM) of five rats (N=5); '*' highlighted values denotes significant at dose tested in comparison to their respective vehicle control at $p < 0.05$ (One-way ANOVA, followed by post-hoc Dunnet's test)

Cold allodynia was significantly reduced with MG24 treatment at a dose of 30mg.kg^{-1}, and the time of peak effect was observed at 60 minutes (62.3%) and effect was continued till 120 minutes with percentage reversal of pain 50.7. It did not show any significant effect at 10 and 3mg.kg^{-1}. Compounds MG22, MG23, and MG25 did not show any significant effect at 30, 10 and 3mg.kg^{-1}.

Table 7.6. Effect of curcumin derivatives on mechanical hyperalgesia

Mechanical hyperalgesia_MG22 to MG25			
Compound	**30 min**	**60 min**	**120 min**
Vehicle	5.71 ± 4.12	9.05 ± 3.81	5.36 ± 3.57
Gabapentin	26.19 ± 4.52	39.52 ± 8.50	**56.90 ± 5.05***
Dose 30mg.kg^{-1} i.p. route			
MG22	14.6 ± 3.5	26.4 ± 6.5	24.3 ± 4.8
MG23	7.5 ± 0.5	17.5 ± 5.4	17.6 ± 5.7
MG24	16.7 ± 12.5	**65 ± 7.1***	**51.7 ± 6.3***
MG25	8.6 ± 1.4	19.3 ± 2.4	25.4 ± 4.7
Dose 10mg.kg^{-1} i.p. route			
MG24	33 ± 7	49.5 ± 8.2	42.2 ± 10.3
Dose 3mg.kg^{-1} i.p. route			
MG24	24.9 ± 10.2	34.4 ± 7.9	33.2 ± 4

Each value represents the % reversal of mechanical hyperalgesia (Mean ± SEM) of five rats (N=5); Yellow highlighted values denotes significant at dose tested in comparison to their respective vehicle control at $p < 0.05$ (One-way ANOVA, followed by post-hoc Dunnet's test)

Cold allodynia was significantly reduced with MG24 treatment at a dose of 30mg.kg^{-1}, and the time of peak effect was observed at 60 minutes (65%) and effect was continued till 120 minutes with percentage reversal of pain 51.7. It did not show any significant effect at 10 and 3mg.kg^{-1}. Compounds MG22, MG23, and MG25 did not show any significant effect at 30, 10 and 3mg.kg^{-1}.

Table 7.7 ED_{50} values of MG24 in CCI animal model

ED_{50} (mg.kg^{-1}) values of Series-II compounds in CCI animal model				
Treatment	**TPE (min)[a]**			
	SP	**TA**	**CA**	**MH**
MG24	7.64 (60)	9.10 (60)	12.86 (60)	9.95 (60)

Table 7.7 Each value represents median effective dose ED_{50} in mg.kg^{-1} body weight of CCI rats at time of peak effect i.e., 60 mins post treatment. SP is spontaneous pain; TA is tactile allodynia; CA is cold allodynia; MH is mechanical hyperalgesia. ED_{50} values are calculated from dose response curve with three different doses viz., 30, 10 and 3mg.kg^{-1}.
Note: 'a' is time of peak effect observed in minutes post MG2 treatment

Based on behavioral screening scores, one compound MG24 was selected for median effective dose calculation (ED_{50}). Out of four compounds screened only compound MG24 was found pharmacologically active and hence ED_{50} was calculated employing three different doses (30, 10 and 3mg.kg^{-1}) of the active molecule. ED_{50} of MG24 in CCI model for spontaneous pain, tactile allodynia and mechanical hyperalgesia was found to be <10mg.kg^{-1}, for cold allodynia it was 12.86mg.kg^{-1}.

7.2.4 Screening studies on Partial sciatic nerve ligation (PSNL) model

Three behavioral modules of neuropathic pain assessment were studied using this model. Spontaneous pain responses were not clear and hence were not reported as quantification was difficult. Antiallodynic and anti-hyperalgesic effect of Series-II compounds were investigated in this model as represented in tables **7.8**, **7.9** and **7.10**.

Table 7.8 Effect of curcumin derivatives on dynamic allodynia

Dynamic allodynia_MG22 to MG25			
Compound	**30 min**	**60 min**	**120 min**
Vehicle	11.82 ± 7.22	18.64 ± 10.95	8.64 ± 5.90
Gabapentin	26.36 ± 5.07	45.15 ± 9.82	**55.15 ± 5.64***
Dose 30 mg.kg^{-1} i.p. route			
MG22	8.4 ± 4.5	24.3 ± 5.1	16.7 ± 7.9
MG23	13.6 ± 6.4	15.4 ± 7.8	26.3 ± 8.6
MG24	23 ± 6.7	**65.2 ± 12.4***	**56.8 ± 9.3***
MG25	14.1 ± 5.6	14.9 ± 7.8	7.1 ± 8.4
Dose 10 mg.kg^{-1} i.p. route			
MG24	36.7 ± 9.2	53.3 ± 8	43.3 ± 11
Dose 3 mg.kg^{-1} i.p. route			
MG24	41 ± 9.2	39 ± 5.2	34.4 ± 5.2

Each value represents the % reversal of dynamic allodynia (Mean ± SEM) of five rats (N=5); '*' highlighted values denotes significant at dose tested in comparison to their respective vehicle control at $p < 0.05$ (One-way ANOVA, followed by post-hoc Dunnet's test)

Dynamic allodynia was significantly reduced with MG24 treatment at a dose of 30mg.kg^{-1}, and the time of peak effect was observed at 60 minutes (65.2%) and effect was continued till 120 minutes with percentage reversal of pain 56.8. It did not show any significant effect at 10 and 3mg.kg^{-1}. Compounds MG22, MG23, and MG25 did not show any significant effect at 30, 10 and 3mg.kg^{-1}.

Table.7.9. Effect of curcumin derivatives on cold allodynia

Cold allodynia_MG22 to MG25			
Compound	**30 min**	**60 min**	**120 min**
Vehicle	14.93 ± 7.21	15.75 ± 10.01	22.24 ± 6.54
Gabapentin	46.53 ± 5.62	**67.68 ± 1.40***	**63.81 ± 4.45***
Dose 30 mg.kg^{-1} i.p. route			
MG22	17.3 ± 3.8	17.4 ± 4.3	16.5 ± 5.4
MG23	15.6 ± 3.2	19.1 ± 6.5	17.4 ± 5.9
MG24	26.3 ± 1.5	**71.3 ± 4.6***	**56.7 ± 10.2***
MG25	18.3 ± 4.2	20.3.2 ± 8.1	19.9 ± 7.4
Dose 10 mg.kg^{-1} i.p. Route			
MG24	45.4 ± 11.3	47.9 ± 7.8	40.1 ± 8.5
Dose 3 mg.kg^{-1} i.p. route			
MG24	32.8 ± 9.6	47.7 ± 4.8	42.9 ± 11.1

Each value represents the % reversal of cold allodynia (Mean ± SEM) of five rats (N=5); '*'highlighted values denotes significant at dose tested in comparison to their respective vehicle control at $p < 0.05$ (One-way ANOVA, followed by post-hoc Dunnet's test)

Cold allodynia was significantly reduced with MG24 treatment at a dose of 30mg.kg^{-1}, and the time of peak effect was observed at 60 minutes (71.3%) and effect was continued till 120 minutes with percentage reversal of pain 56.7. It did not show any significant effect at 10 and 3mg.kg^{-1}. Compounds MG22, MG23, and MG25 did not show any significant effect at 30, 10 and 3mg.kg^{-1}.

Table.7.10. Effect of curcumin derivatives on mechanical hyperalgesia

Mechanical hyperalgesia_MG22 to MG25			
Compound	**30 min**	**60 min**	**120 min**
Vehicle	7.50 ± 9.24	5.36 ± 3.88	7.86 ± 7.83
Gabapentin	24.64 ± 3.81	43.57 ± 1.55	**52.14 ± 5.67***
Dose 30 mg.kg^{-1} i.p. route			
MG22	8.9 ± 0.5	23.4 ± 7.1	14.1 ± 4
MG23	8.9 ± 5.2	13.6 ± 6.2	23.4 ± 3.5
MG24	10.4 ± 6.3	**56.3 ± 9.1***	**50 ± 2.0***
MG25	9.5 ± 5.1	18.6 ± 5.5	17.1 ± 3.4
Dose 10 mg.kg^{-1} i.p. route			
MG24	32 ± 6.9	47.5 ± 5.1	46.5 ± 11.9
Dose 3 mg.kg^{-1} i.p. route			
MG24	19.7 ± 7.5	43.8 ± 6.3	39.8 ± 9.3

Each value represents the % reversal of mechanical hyperalgesia (Mean ± SEM) of five rats (N=5); '*' highlighted values denotes significant at dose tested in comparison to their respective vehicle control at $p < 0.05$ (One-way ANOVA, followed by post-hoc Dunnet's test)

Mechanical hyperalgesia was significantly reduced with MG24 treatment at a dose of 30mg.kg^{-1}, and the time of peak effect was observed at 60 minutes (56.3%) and effect was continued till 120 minutes with percentage reversal of pain 50. It did not show any significant effect at 10 and 3mg.kg^{-1}. Compounds MG22, MG23, and MG25 did not show any significant effect at 30, 10 and 3mg.kg^{-1}.

Table 7.11. ED_{50} values of MG24 on PSNL animal model

ED_{50} (mg.kg^{-1}) values of MG24 in PSNL animal model			
Treatment	**TPE (min)[a]**		
	TA	**CA**	**MH**
MG24	7.7 (60)	5.53 (60)	8.73 (60)

Table 7.11 represents median effective dose ED_{50} in mg.kg^{-1} body weight of PSNL rats at time of peak effect i.e. 60 mins post treatment. TA is tactile allodynia; CA is cold allodynia; MH is mechanical hyperalgesia. ED_{50} values are calculated from dose response curve with three different doses viz., 30, 10 and 3mg.kg^{-1}.

ED_{50} of MG24 in PSNL model for dynamic (tactile) allodynia, cold allodynia, and for mechanical hyperalgesia was found to be <10mg.kg^{-1}.

7.2.5 Screening studies on Diabetic peripheral neuropathy (DPN) model

The compounds which showed good efficacy in CCI and PSNL models were further taken forward for diabetic peripheral neuropathy screening. In this model the compounds efficacy was assessed by four different tests. In the first test effect of compound on tactile allodynia was assessed followed by nerve conduction velocity determination using needle electrodes. Hot plate test (heat hyperalgesia) and cold water tail flick test were performed to evaluate compounds effect on hyperalgesia and allodynia.

Table 7.12 Effect of MG24 in DPN animal model

Streptozotocin at a dose of 30mg.kg^{-1} was given through *i.p* route at pH 4.5 in ice cold citrate buffer as detailed protocol presented in chapter-4 (Materials and Methods). Hyperglycaemia for prolonged period will bring changes in microenvironment surrounding neurons. The damage to microvascular system affects neuronal health and led to peripheral neuropathy. Table **7.12** represents effect of MG17 on diabetic peripheral neuropathy.

Behavioral screening scores of MG24 in DPN rat model

	Behavioral module	Group	0hr	1hr
a	Dynamic allodynia (50% paw withdrawal threshold)	Naïve	7.24±0.63	7.43±0.52
		Control	1.85±0.22	2.05±0.13
		Gabapentin	1.63±0.12	3.63±0.30
		MG24	1.28±0.11	**4.24±0.0***
b	Nerve Conduction Velocity (Meters/second)	Naïve	66.40±3.72	65.3±2.42
		Control	49.00±1.34	50.8±1.30
		Gabapentin	51.70±1.56	51.8±0.79
		MG24	53.32±0.70	**58.67±2.22***
c	Heat Hyperalgesia (52±0.5^{0}C) (Reflex time)	Naïve	13.40±0.81	13.60±0.87
		Control	6.80±0.86	7.20±0.86
		Gabapentin	5.60±0.42	5.80±0.62
		MG24	5.20±0.58	**6.60±0.50***
d	Tail flick (0±0.5^{0}C) (Reflex time)	Naïve	4.00±0.45	3.80±0.20
		Control	7.00±0.55	7.20±0.37
		Gabapentin	8.60±0.32	8.30±0.44
		MG24	9.60±0.5	8.80±0.7

Table 7.12 Effect of MG24 treatment on DPN rats on various modules of pain assessment and neurodegeneration. '*' represents values significant at 60 min post treatment compared to control (vehicle-20% PEG 400) group (n=5). Statistical significance *p<0.05, **p<0.01 and ***p<0.001 was calculated using One Way ANOVA followed by Dunnet's post-test analyses.

Dynamic (Tactile) allodynia measured as 50% paw withdrawal threshold; Motor nerve conduction velocity represented in meter.sec^{-1}; Hot plate test (heat hyperalgesia) represented as response time (seconds); Cold water tail flick (cold allodynia) represented as response time (seconds).

MG24 was selected based on behavioral scores obtained in CCI and PSNL models. MG24 was effective in reducing allodynia and hyperalgesia at an ED_{50} of <10mg.kg^{-1} and hence was considered for DPN study. Table **7.12** represents the behavioral scores of DPN rats at 30mg.kg^{-1} dose of MG24. From the behavioral screening studies it was found that compound MG24 is having potent anti-allodynic and anti-hyperalgesic properties as MG24 significantly improved paw withdrawal threshold value from 1.28 to 4.24 (generally >4 value is considered to be significant according to modified Dixon method), nerve conduction in tail from 53 to 58 m/s, delayed thermal hyperalgesia but was not effective in cold water tail flick test.

7.2.6 Screening studies on MeHg induced neurodegeneration model

The active compound in CCI and PSNL model was selected for further screening in MeHg induced neurodegeneration rat model. Detailed protocol is presented in chapter-4 (Materials and Methods). Out of four compounds screened, compound MG24 was identified to have consistent anti-allodynic and anti-hyperalgesic effect in all the three peripheral neuropathic pain rat models (CCI, PSNL and DPN), we decided to further screen this compound on methyl mercury induced neurodegeneration to evaluate its neuroprotective activity against environmental pollutant MeHg. Below table represents the behavioral scores of MeHg treated rats at 30mg.kg^{-1} dose of MG24. Gabapentin at a dose of 10mg.kg^{-1}was used as standard drug to compare MG24 (30mg.kg^{-1})

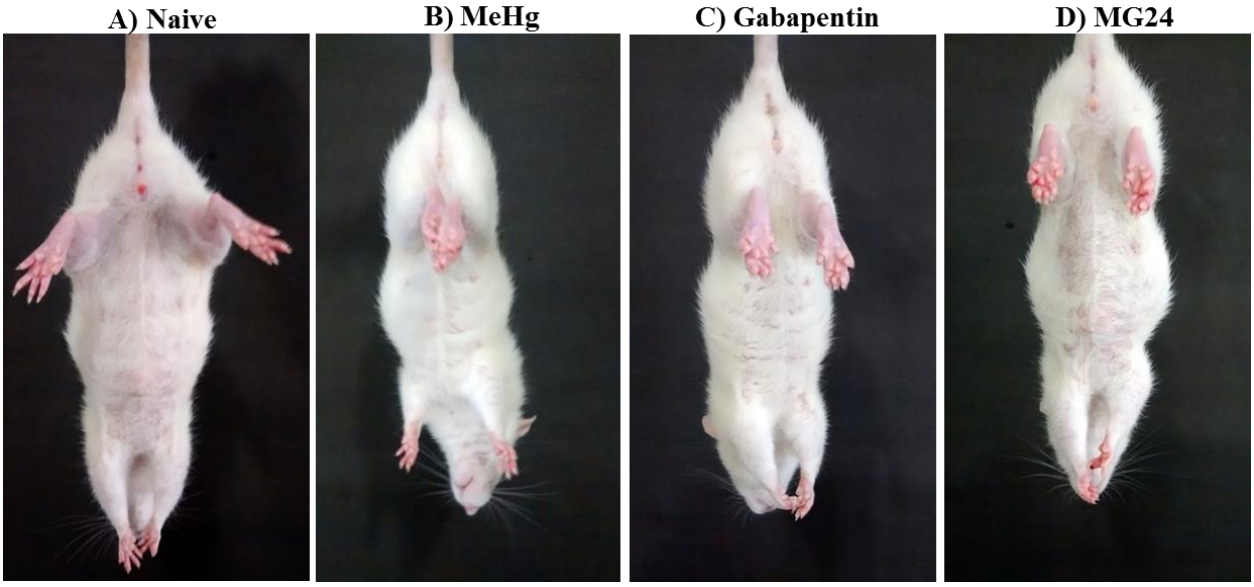


Figure 7.3: Effect of MG24 on behavioral responses of MeHg induced neurodegenerative rats Representative animals from each group clasping were shown.

Behavioral analysis of MeHg treated animals. a) Bodyweight analysis of animals from Day1 to Day31. b) Actophotometer graph represents the % neurotoxicity in animals from Day1to Day31. c) Clasping score of animals on each day was represented. d) Representative animals from each group clasping were shown (A=0, B=3, C=2, D=1).

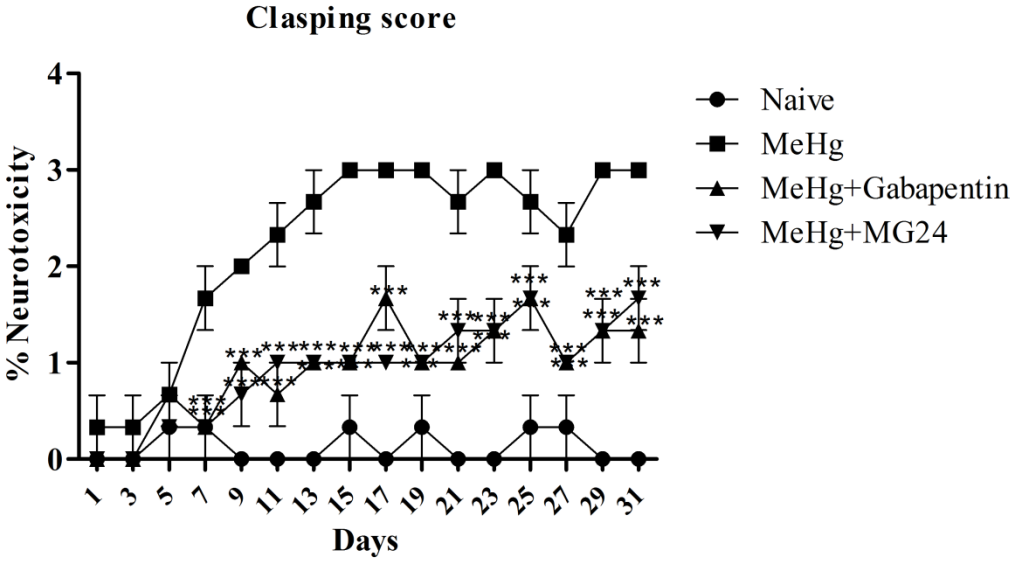


Figure 7.4: Effect of MG24 on clasping score of MeHg induced neurodegenerative rats
MeHg induced neurodegeneration: MG24 treatment significantly reduced clasping score and was found to have more efficacy than gabapentin. Results were represented as mean ± SEM at *p<0.05, **p<0.01 and ***p<0.001. One way ANOVA followed by Dunnet's post hoc test.

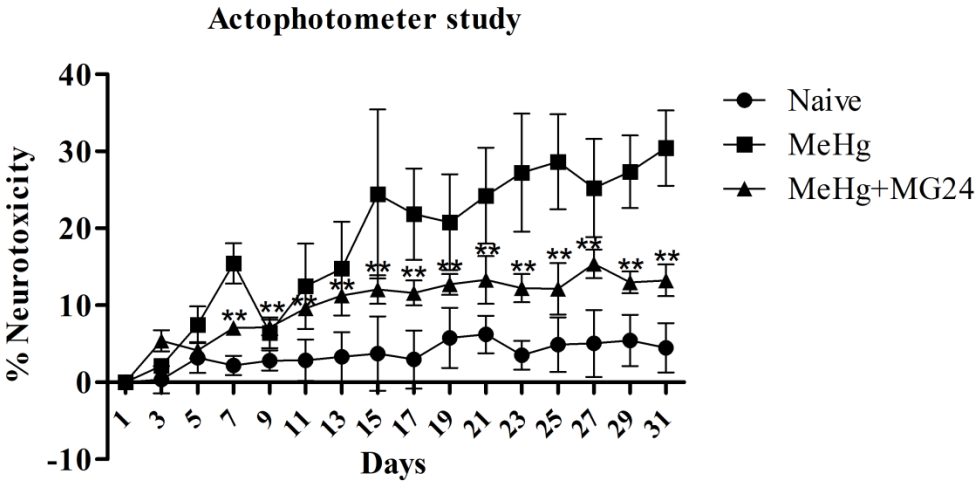


Figure 7.5: Effect of MG24 on activity score of MeHg induced neurodegenerative rats
Actophotometer graph represents the % neurotoxicity in animals from Day1to Day31.
MG24 treatment significantly improved activity score. Results were represented as mean ± SEM at *p<0.05, **p<0.01 and ***p<0.001. One way ANOVA followed by Dunnet's post hoc test.

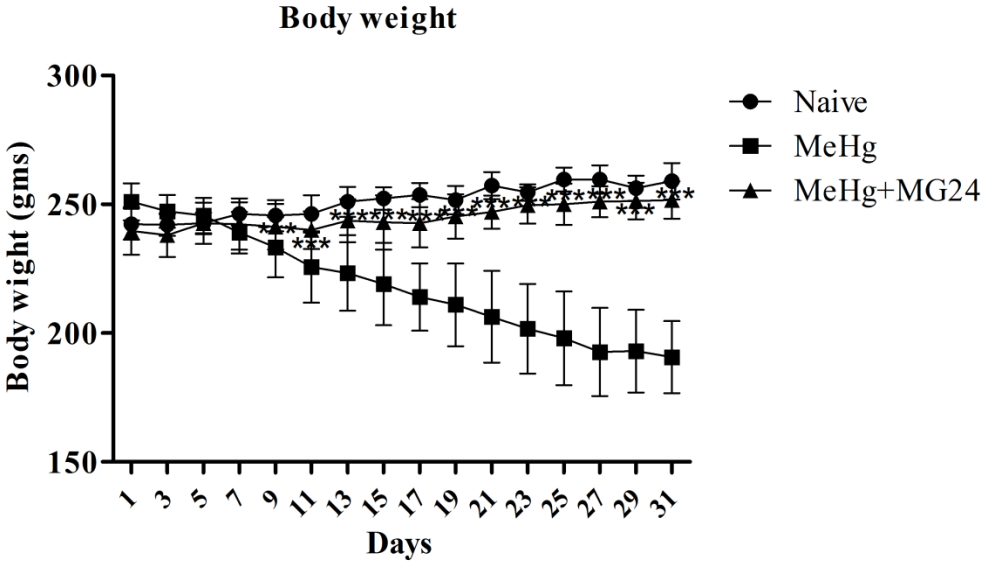


Figure 7.6: Effect of MG24 on body weight of MeHg induced neurodegenerative rats
Body weight analysis graph represents the drop in body weight with MeHg from Day1 to Day31 MG24 treatment significantly improved or maintained body weights of MeHg treated rats. Results were represented as mean ± SEM at *p<0.05, **p<0.01 and ***p<0.001. One way ANOVA followed by Dunnet's post hoc test..

The compound MG24 showed significant protective effects against neurodegeneration which is evident from results. MG24 was taken up for *in vivo* studies to assess the neuroprotective activity in MeHg induced animal model using a cascade of behavioral assays including clasping behavior and locomotor test as it was having anti-allodynic and anti-hyperalgesic activities in mononeuropathy models and improved NCV, reduced thermal hyperalgesia in DPN model. MG24 was found to reduce MeHg induced neurodegeneration in rats more efficiently than gabapentin as observed in behavioral screening studies. MeHg treated control rats drastically reduced weight within 31days of treatment due to toxic effects of the chemical, MG24 treated group did not show such drastic drop in body weight which can be considered as its protective effect against environmental pollutant-MeHg. Both MG24 and gabapentin treatments were significantly effective in decreasing clasping score throughout the treatment period of 31days. Activity score was also improved with MG24 treatment as it was found that MeHg toxic effects reduced exploratory behaviour of rats making them more sedentary. Further the activity was also reascertained using histopathological studies and expression levels of inflammatory mediators.

7. 3 Gene expression studies-RT-PCR

7.3.1 Gene expression studies in CCI rats

Real time polymerase chain reaction cycle experiments were performed to determine the effect of MG24 treatment on dysregulated pro-inflammatory mediators such as IL-1β, IL-6 and TNF-α in the nervous tissue of CCI rats. The mRNA expression levels were determined in spinal nervous tissue collected from rats post 12 days CCI induction and after confirming neuropathy symptoms by behavioral analysis.

Figure 7.7: Effect of MG17 on IL-1β expression

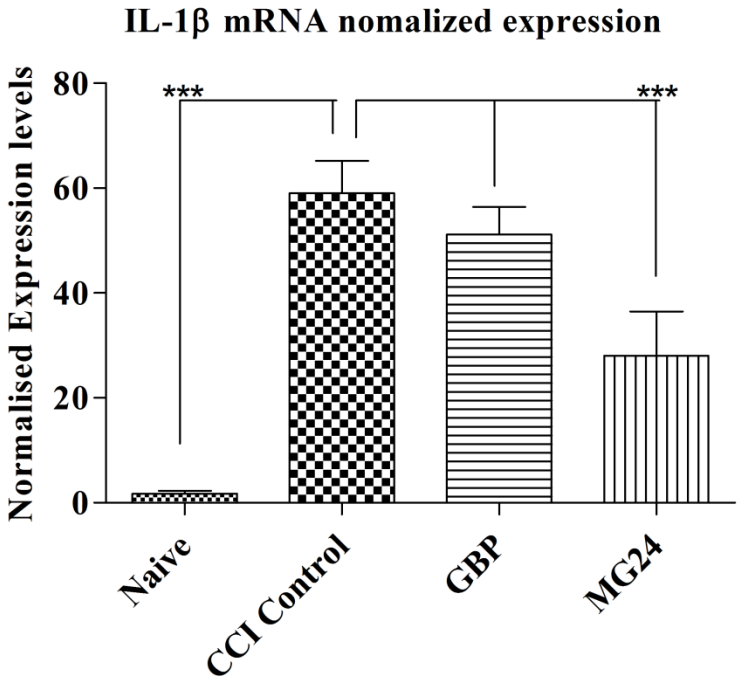


Figure 7.7: Graph depicts relative normalized expression levels of pro-inflammatory mediator IL-1β in CCI induced neuropathic pain rat spinal tissues compared to normal rats (naïve) and MG24 treated rats. GAPDH was used as housekeeping gene. Significance (*p>0.05, **p<0.01 and ***p<0.001). One way ANOVA followed by Dunnet's post-test.

Figure 7.8: Effect of MG17 on IL-16 expression

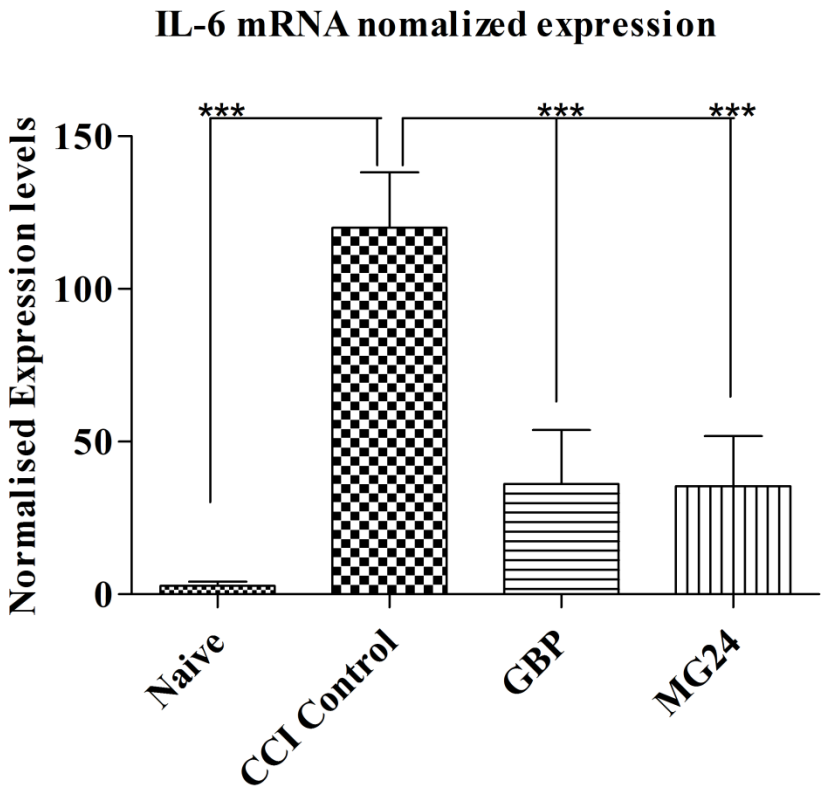


Figure 7.8: Graph depicts relative normalized expression levels of pro-inflammatory mediator IL-6 in CCI induced neuropathic pain rat spinal tissues compared to normal rats (naïve) and MG24 treated rats. GAPDH was used as housekeeping gene. Significance (*p>0.05, **p<0.01 and ***p<0.001). One way ANOVA followed by Dunnet's post-test.

Figure 7.9: Effect of MG17 on TNF-α expression

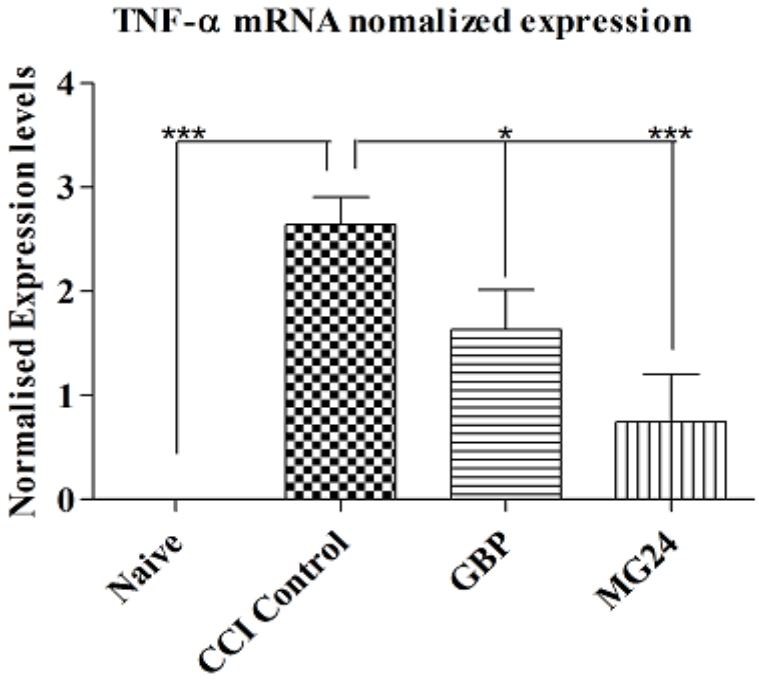


Figure 7.9: Graph depicts relative normalized expression levels of pro-inflammatory mediator TNF-α in CCI induced neuropathic pain rat spinal tissues compared to normal rats (naïve) and MG24 treated rats. GAPDH was used as housekeeping gene. Significance (*p>0.05, **p<0.01 and ***p<0.001). One way ANOVA followed by Dunnet's post-test.

The active compound MG24 was found to control or normalize the dysregulated gene expression of IL-1β, TNF-α and IL-6 significantly compared to CCI control group. The compound MG24 was selected for *in vivo* gene expression studies as it showed consistent and very good pharmacological activity (anti allodynic, anti-hyperalgesic and neuroprotective effects) in all the four rodent models -CCI, PSNL, DPN and MeHg induced neurodegeneration.

7. 3.2 Gene expression studies in MeHg treated rats

Real time polymerase chain reaction cycle experiments were performed to determine the effect of MG24 treatment on dysregulated pro-inflammatory mediators such as IL-1β, IL-6, NFkB and TNF-α in the nervous tissue of MeHg rats. The mRNA expression levels were determined in spinal nervous tissue collected from rats post four week treatment with 20ppm MeHg and after confirming neurodegenerative symptoms by clasping analysis.

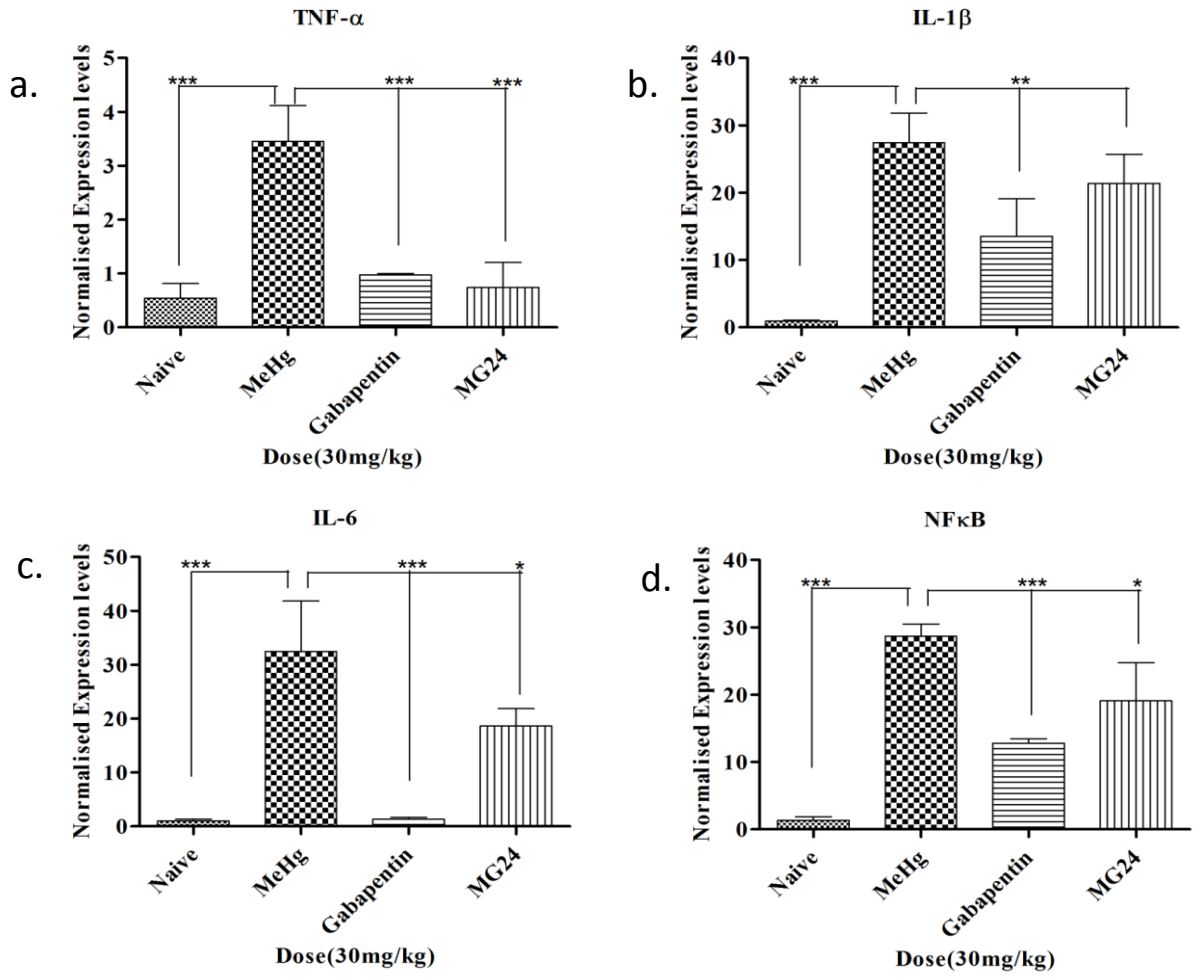


Figure 7.10**:** Effect of MG24 on mRNA expression levels of pro-inflammatory mediators

Graph depicts relative normalized expression levels of pro-inflammatory mediator TNF-α, IL-1β, IL-6 and NFkB in MeHg induced neurodegeneration rat spinal tissues compared to normal rats (naïve) and MG24 treated rats. GAPDH was used as housekeeping gene. (*$p>0.05$, **$p<0.01$ and ***$p<0.001$). One way ANOVA followed by Dunnet's post-test.

The active compound MG24was found to control or normalize significantly the dysregulated gene expression of TNF-α, IL-6 and NFkB whereas the upregulated IL-1β gene expression was not normalized and remained elevated. The compound MG24 was selected for *in vivo* gene expression studies as it showed consistent and very good pharmacological activity (anti allodynic, anti-hyperalgesic and neuroprotective effects) in all the four rodent models -CCI, PSNL, DPN and MeHg induced neurodegeneration. MG24 was also found to be potent ROCK-I inhibitor (Table. **7.14**) and it showed least cytotoxic effect on HEK-293 cell lines (Table. **7.13**), hence we were interested to explore the mechanism by which MG24 is showing all these activities. We have done thorough literature search to make a list of inflammatory genes that are upregulated during neuropathic pain states. IL-1β, IL-6, NFkB and TNF-α [Austin and Moalem Taylor, 2010; Yogeeswari P *et al*., 2012; Marissa de Mos *et al*., 2009] are the most commonly released proinflammatory mediators upon injury and well known mediators of inflammation and pain which can act directly on nociceptors to induce pain hypersensitivity.

7.4 Brain histopathology studies (H &E staining)

The brain tissue from rats treated with methyl mercury (20 ppm/day *P.0*) for over four weeks were collected by sacrificing them, tissues stored in formaldehyde and histopathological analysis was done on 2-4 µm thin sections using hematoxylin and eosin stain. During the analysis, the extent of neurodegeneration in MeHg group was compared with Naïve group and MeHg+MG24 ($30mg.kg^{-1}$ *i.p*) group. The histopathology images were obtained at a magnification of 20X with electron microscope under bright field.

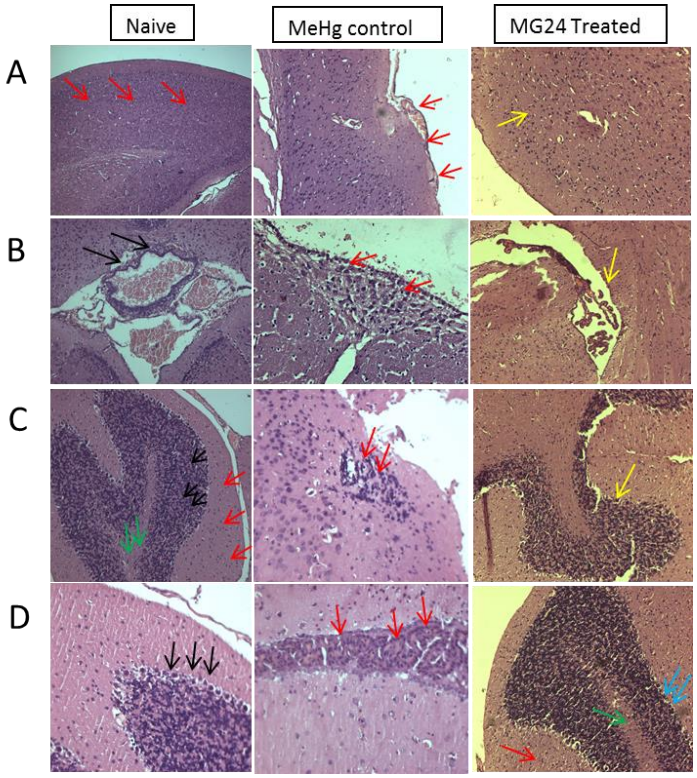


Figure 7.8: Effect of MG24 on MeHg treated rat Brain tissue

Brain sections of study rats upon hematoxylin and eosin stain revealed the extent of degeneration that took place upon treatment with MeHg for three weeks arrows indicate different zones of healthy tissue observed in naïve group. Arrows in MeHg control group shows degradation, meningeal haemorrhage, microglial and ependymal cell infiltration. Arrows in MG24 treated group shows no signs of degradation except small number of inflammatory cells and slight thickening of meningeal layer.

7.5 Acute paw oedema model

The effect of compound MG24 on acute inflammation was studied to evaluate its efficacy on inflamed paw in carrageenan induced rat paw edema model. The compound MG17 (30mg.kg^{-1}) was compared with standard anti-inflammatory drug Indomethacin (10mg.kg^{-1}) and disease control group.

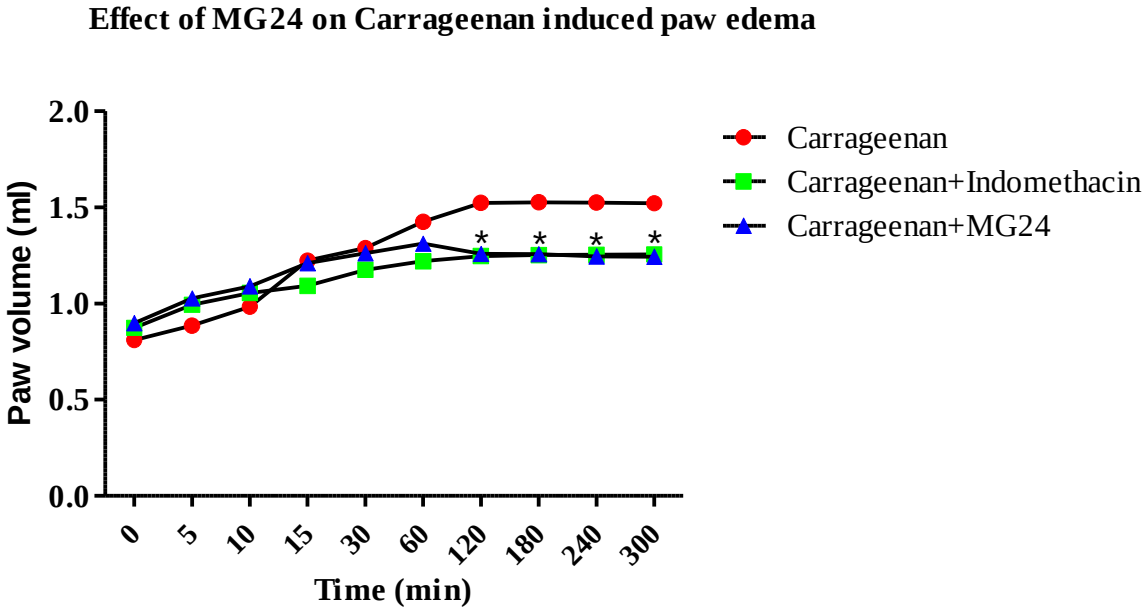


Figure 7.9: Effect of MG24 on carrageenan induced acute paw edema in rats

Carrageenan Vs Carrageenan + Indomethacin = * significance

Carrageenan Vs Carrageenan + MG24 = *** significance

Effect of treatment of compound Indomethacin, MG24 on Carrageenan induced paw edema in rat. Data is represented as mean ± SEM of n=5 rats and analyzed by one way ANOVA followed by Dunnett's test. Significance values are represented as ***$p<0.001$ and *$p<0.05$.

The test compounds (Series-III Curcumin derivatives) were further evaluated for its anti-inflammatory activity using carrageenan induced mice paw edema (Acute inflammation) rat model. These results suggested that **MG24** was significantly active in reducing acute inflammation over a period of 5h with promising anti-nociceptive and anti-inflammatory

properties compared to control group and standard drug (Indomethacin) treatment group. Finally, we could conclude that **MG24** is most effective on acute inflammation; it may be further developed into a therapeutic agent for the treatment and prevention of acute inflammation besides having efficacy on central pain as it showed good activity score in behavioral screening studies.

7.6 MTT assay on HEK-293 cells

MTT assay was performed to test compounds cytotoxic concentration with six different concentrations ranging from 1nM to 100μM. From the IC_{50} values, it is clear that compound MG24 is having IC_{50}>100 indicating that it is more safe to use high dose.

Table 7.13.Effect of Curcumin derivatives on cytotoxicity

Compound	IC_{50}(Mean±SD) (μM)
MG22	32.24±0.22
MG23	46.62±0.32
MG24	10.14±0.16
MG25	30.34±0.28

Table 7.13 Represents IC_{50} value of MG24 found to be 10.14 μM on HEK-293 cells, indicating that the compound has minimum cytotoxicity at the maximum concentration used for *in vitro* experiments

Curcumin derivatives were screened to evaluate their cytotoxic effect on human embryonic kidney cell lines (HEK-293) and all the four compounds are found to be non-toxic below 10μM concentration. Among the four compounds screened, compound MG24 was found to be least toxic (**CC_{50}>10μM**). We performed MTT assay to select compounds for further *in vitro* cell based enzyme inhibition assays.

7.7 Effect of curcumin derivatives on ROCK-I inhibition assay (ADP-glow assay)

ROCK-I plays an important role in neuroinflammation, we examined whether our compounds inhibit ROCK-I enzyme. To test our hypothesis, the compounds were screened for ROCK-I enzymatic activity at different concentrations from 100 μM -1 nM utilizing standard enzyme assay protocol and the relative inhibition of ROCK-I activity was evaluated for IC_{50} calculations. Rho kinase plays an important role in neurodegeneration and development of neuropathic pain. Recent literature revealed that ROCK inhibition promotes neuroprotection (Van de Velde S et al., 2015; Yogeeswari P et al., 2012). Table **7.14** shows that except one compound MG23 all the three compounds showed ROCK-I IC_{50}<1μM. Compound MG24 was found to be most potent inhibitors with an IC_{50}<0.1 μM.

Table 7.14 Effect of Curcumin derivatives on ROCK-I inhibition assay

Compound	ROCK IC_{50}(Mean±SD)(μM)
MG22	0.622±0.022
MG23	485.4±12.021
MG24	≤0.001
MG25	0.834±0.242

All data in triplicates was used for deriving and represented in Mean ± SEM (n=3)

7.8 ROS estimation (Flow cytometer)

The compound MG24 was evaluated for its activity on inhibiting generation of reactive oxygen species (ROS) in and around neuronal tissues as excess ROS is reported to aid in neurodegeneration and inflammation. The study was performed on RAW 264.7 cells with LPS stimulation to produce ROS and then MG24 treatment was given. MG17 treatment showed significant reduction in ROS positive cells.

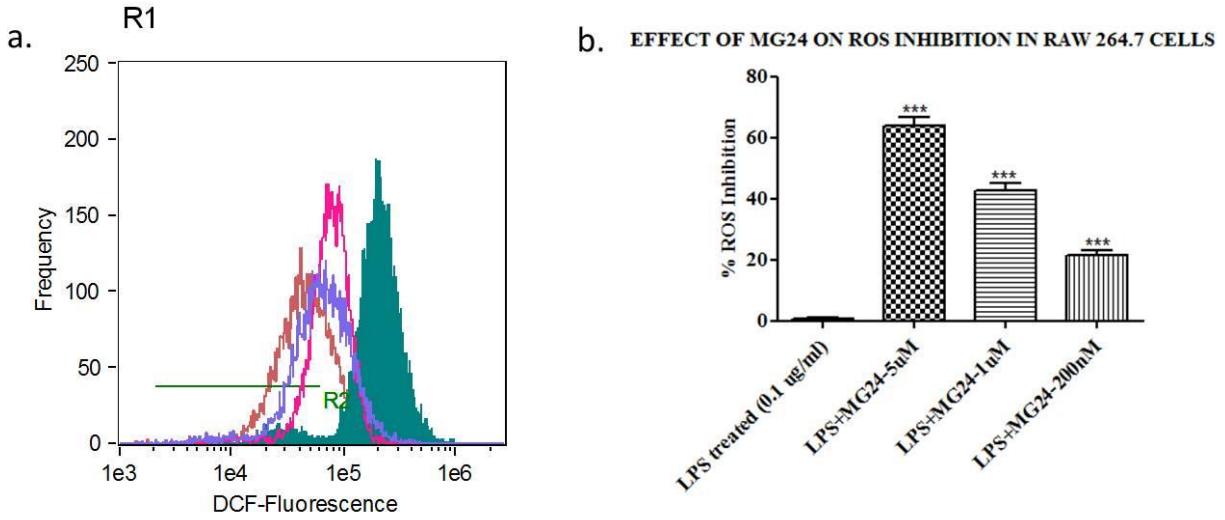


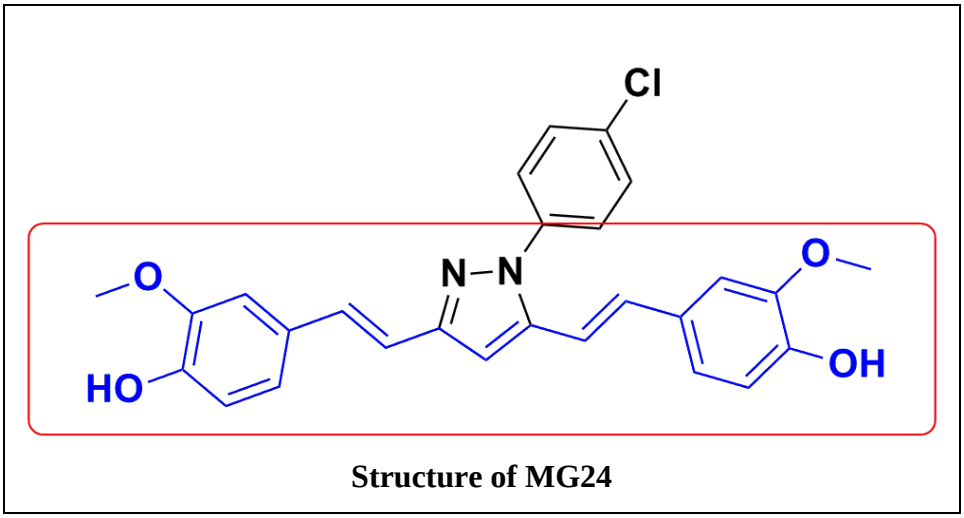
Figure 7.10: Effect of MG24 on ROS in RAW 264.7 cells. a Cells were treated with LPS (0.1 μg/ml) and with different concentrations of MG24 (0.1, 1 and 5μM) for 3h and incubated with DCFDA for 30 min. The intracellular ROS was measured by flow cytometry. b Histogram depicting the percentage of ROS positive cells of indicated concentrations of MG24 after 3h. Data were expressed as mean ± SEM of three independent experiments. ***p<0.001 versus LPS treated group.

This study was performed to evaluate MG24 compound for its potency to abrogate intracellular ROS production. We considered this study to be important as ROS production is one of the major cause of neuronal apoptosis. ROS is also produced by activation of Rho kinase and in hyperglycaemic states as in case of diabetes. We were trying to identify possible mechanisms of action of MG24 and the DCFDA (Dichloro-flourescein diacetate) assay results showed that MG24 is having potent ROS inhibitory effect at a concentration of 5μM and the effect is also concentration dependant.

7.9 Summary and conclusion

In the present study, we attempted to identify structural requirements or substitutions on curcumin moiety as it has been shown to exhibit beneficial activities in a plethora of human diseases with minimal toxicities [Subhash. *et al*., 2013]. Since this polyphenol has shown tremendous CNS effects due to its high lipophilic nature, we selected curcumin based analogues from BITS in-house database for behavioral pharmacological screening studies.

Out of the four curcumin analogues (MG22 to MG25), MG24 was identified to be most effective for neuroinflammation and neurodegeneration related diseases. In this study MG24 has shown > 50% pain reversal in four rodent models of neuropathic pain. The mean effective dose (ED_{50}) calculated was below 15 $mg.kg^{-1}$ in CCI and PSNL model. All compounds were found to be nontoxic at low concentrations. The compound MG24 was found to inhibit ROS production and was able to block the enzyme activity of Rho kinase which is a key trigger of neuroinflammation. Further MG24 was able to normalize most of the upregulated pro-inflammatory mediator mRNA expression levels such as TNF-α, IL-1β, IL-6 and NFkB. The compound MG24 showed both peripheral and central effects. Local acute inflammation in carrageenan induced rat paw edema was reduced by MG24 within 2hours after i.p administration. In brain histopathological studies MG24 has shown neuroprotection against MeHg induced neuroinflammation and neurodegenerative changes.



Structure of MG24

Figure 7.11: Structure of most active curcumin derivative

CHAPTER-8

Discussion

8.1 Semicarbazone derivatives

Neuropathic pain is an inflammatory condition involving a complex interaction of multiple pathways, which leads to fatal outcomes. The array of outcomes due to neuropathic pain may lead to paralysis and ultimately death. Pharmacological agents currently being employed in the intervention of neuropathy and related conditions in both clinical and experimental settings are GABA analogues, glutamate receptor agonists, transient receptor potential vanilloid modulators etc.

Semicarbazone moiety and its various derivatives studied frequently in the past and found potent having various pharmacological activities. This article is mainly focused on bromo isatin derivative (MG2) of semicarbazone and its biological activity. MG2 is having anticonvulsant activity with GABA agonistic actions.

The reason behind choosing MG2 for these studies is its effectiveness on convulsions/seizures [Yogeeswari p *et al.,* 2005]. This compound was selected for neuropathic pain and neurodegenerative diseases as it has shown good GABA inhibition in our previous studies. Since GABA activation has an instrumental role in suppressing pain generation and propagation. We thought this might be having beneficial effects in neurodegenerative disorders. As expected MG2 has shown good preliminary activity in rodent models of neuropathic pain and has regulated the expression of key mediators which are overexpressed in neuropathic pain states.

Before exploring further we wanted to check its biological safety levels. As we know MG2 is a highly nonpolar hydrophobic molecule having CNS activity, we decided to perform neurotoxicity studies first. In neurotoxicity studies MG2 did not show neurotoxic effects even at a high concentration of 300mg/kg whereas its potency ED_{50} was observed at far low concentration ie., 30, 10 and 3 mg/kg. Further cytotoxic effects of MG2 were also evaluated on three different cell types viz..HEK, IMR32 and U87 cell lines. Where MG2 has shown IC_{50} > 100 μM. in HEK-293 cell line studies.

MG2 was able to pass rota rod test at 100mg/kg as all the five mice sustained to maintain their balance on rotating rod for the initial one min and three out of five sustained till end time (5min). This indicates 30 mg/kg which is much lower than 100 mg/kg will be safer to work with other behavioral screening protocols.

Sciatic nerve of rats was ligated with sutures and kept for observation for 12 days. On the 12th day, pain assessment was done to estimate the effect of compound MG2 when treated at a dose of 30mg.kg^{-1}. MG2 showed a significant reduction in pain threshold levels as indicated by the reduction in cold allodynia, mechanical hyperalgesia, tactile allodynia and spontaneous pain responses. Since 30mg.kg^{-1} dose was active we decided to see effectiveness at lower doses 10 and 3 respectively in both CCI and PSNL models. To our surprise, MG2 was able to reverse pain responses even at 3mg.kg^{-1} dose. We then calculated the median effective dose of MG2 in all the pain assessment modalities and found that the ED_{50} values obtained were less than the highest dose employed. Since MG2 was active in CCI and PSNL pain we thought of checking its efficacy in other models i.e. POP, DPN and MeHg induced neurodegenerative rat model. MG2 was able to improve nerve conduction velocity, paw withdrawal threshold and stimulus evoked response latency (hot plate and tail flick). This significant improvement in all these values indicates that MG2 is having molecular effects or on pain sensing receptors with chronic dosing. Hence we decided to go for expression studies using rat tissue samples. Rats were sacrificed after alternate dosing for 10 days, brain, spinal cord and sciatic nerve were isolated, snap frozen and stored for PCR analysis. There was a significant reduction ($p<0.01$) in BDNF, TNFα, IL-6 in brain, sciatic nerve and spinal cord, when rats were treated with MG2 compared to control. However, expression levels of ERK1 were reduced in spinal cord and sciatic nerve but not in brain. Expression levels of IL-1β were reduced in brain and sciatic nerve but not in spinal cord samples of CCI rats. Expression studies in MeHg treated rats revealed that MG2 was able to regulate IL-6, IL-1β, TNF-α and NFκB upregulated gene expression levels (**Figure 5.14**). This indicates that MG2 is having anti-inflammatory effect which is useful to alleviate local pain responses (IL-6, IL-1β and TNF-α) whereas the regulating effect of MG2 on gene expression levels of BDNF, NFκB and ERK1 in brain and spinal cord samples indicates that MG2 is also having its role in central sensitization. Hence MG2 is able to block peripheral (nociceptors) and spinal (spinal cord) pain responses.

From the RT-PCR results it is clear that MG2 is having down regulatory effect on neuro-inflammatory mediators like IL-6, TNF-α, NFκB, BDN, ERK1 and IL-1β, which have their significant role in generation and propagation of neuropathic pain.

To understand the mechanism in protein level, we estimated the ability of MG2 to inhibit in various enzymes (**Figure 5.17**). As shown in figure, MG2 was found to inhibit PKMζ Akt, ROCK kinase at IC_{50} concentrations of **48.6** nM, **652** nM and **2935** nM respectively**.**

Based on results it is clear that MG2 is having potent neuropathic pain inhibition activity. Though its mechanism of action is not clear, in preliminary screening studies it has shown Akt inhibition at an IC_{50} concentration of **0.6**μM. Hence we can assume that neuropathic pain inhibition activity of MG2 is may be through the inhibition of all these kinase enzymes which has to be studied further to explore the intermediates involved in this process. Further we can also test its anticancer activity as it is a potent Akt inhibitor. Literature says semicarbazone moiety possess anti-convulsant properties through action at the sodium channels and might also act through glycine receptors. These properties also aid for its anti-nociceptive nature which has to be further validated.

The main problem with most of the experimental agents is their poor PK properties, which may present a significant hurdle in the development of these agents into a potential clinical candidate. Other reasons are the poor correlation in outcomes between preclinical studies and clinical trials hindering the development of experimental

We were also enthusiastic to know the active compound's binding properties with some important enzymes viz., Akt, NFκB and BDNF, we have performed docking studies for MG2 on these three enzymes where it has shown decent binding score.

8.2 Triazole derivatives

The present work mainly focus on evaluating test compound's (**MG17**) potency against neurodegeneration and other neurological symptoms. The purpose of the study was also to investigate compound's effect on expression levels of key mediators involved in neurodegeneration and pain propagation.

As MG17 has a strong antinociceptive potential and also shown beneficial effect against surgically induced mononeurpathy pain models in our previous studies it was taken for further investigation to get better insights on molecular mechanism of action.

Before moving on to actual investigations we carried out neurotoxicity and cytotoxicity studies to check the test compound's safety profile. Since most of the protocols obtained from the literature states that % neurotoxicity of a drug to be accepted should not cross 50. In both the neurotoxicity screening studies viz., Actophotometer and Rotarod, MG17 did not show neurotoxicity (Actophotometer % reduction in activity score was < 20; Rotarod falling time was beyond the time of observation), hence MG17 has no undesirable central effects. In cytotoxicity studies on IMR32 cell lines the IC_{50} was found to be more than 100 μM, hence allowing maximum range to select concentrations for further in vitro studies.

We have listed key mediators involved in neurodegeneration and pain propagation and based on the facilities available and feasibility of performing experiments, we decided to look for compound's activity on ROCK1 enzyme, effect on reactive oxygen species and pro inflammatory cytokines such as IL-1β , Il-6 and TNF-α (Gangadhar et al., 2014). Since it was found from earlier literature that ROCK play an important role in neurodegeneration especially in diabetes conditions, we conducted ROCK1 enzyme inhibition studies and to our surprise inhibitory concentration (IC_{50}) of MG17 was found to be **9.22 nM** (Bernhard *et al*., 2005). Leucocyte migration to CNS is observed in neurodegenerative diseases and it was found that preventing ROCK activation ameliorated the disease progression. Leukocyte migration to CNS require active RhoA and ROCK for their journey beyond brain endothelium, reports say that their trans-endothelial migration was prevented by the ROCK inhibitor Y-2763294 (Honing *et al.*, 2004).

Effect of MG17 on reactive oxygen species release in RAW 264.7 cells was studied since ROS estimation is an important parameter as it was identified to cause degeneration, necrosis and cell death (Eveliina *et al*., 2014). In all inflammatory conditions one or more of these reactive oxygen species are found especially Oxidative RNA damage has been recently found to be involved in the pathogenesis of several human diseases such as chronic degeneration [Carmela Fimognari *et al*., 2015]. MG17 at a concentration of 5μM has significantly reduced the percentage of ROS production from RAW 264.7 cells measured using flow cytometer.

Gene expression (RT-qPCR studies)

The systematic study of simultaneous changes in gene expression for several pro inflammatory cytokines chronic constriction induced mononeuropathic rat brain tissues was performed using RT gene expression studies. Comparing the CCI induced mononeuropathic rat brain samples and untreated samples, IL-1β, Il-6 and TNF-α expression showed a statistically significant upregulation. MG17 treatment at a dose of 30mg.kg^{-1} given through intraperitoneal route every alternate day for 10 days showed significant reduction in IL-6 and TNF-α mRNA expression levels but was not effective in regulating IL-1β levels.

Research in molecular cellular biology reveals that neural plasticity may be the result of changes in the number of one or several functional proteins that occur in turn through long-lasting transcription-dependent changes in the nucleus. In the cytoplasm, some properties of functional proteins can be rapidly changed via posttranslational changes. Cytokines, in addition to other inflammatory agents, influence these intracellular modulating processes. IL-6 in particular modulates the presence of several extracellular and intracellular mediators that are also known to be active during pain. A correlation between induced allodynic response and number of IL-6 positive cells was studied by Cui et al. It is already proven that IL-6 exerts an initial potential beneficial effect after nerve injury, including protection against neuronal cell death, promotion of growth, and protection against axotomy caused by the nerve injury [Arruda *et al*., 1998]. It is found that neurons interactions with adjacent cells through IL-6 and its receptors is crucial then subsequent detrimental and aberrant sensory effects accompany the IL-6 increase, but further research on the exact role played by IL-6 in the modulation of nociception is warranted.

Cytokines in particular, tumor necrosis factor alpha (TNF-α) [Wagner et al., 1996], interleukin-1 (IL-1) [Sommer *et al.*, 2004; Zelenka *et al*., 2005; Sweitzer *et al*., 1999] and interleukin-6 (IL-6) [Arruda *et al.*, 1998; DeLeo *et al*., 1996] have been associated with the development of neuropathic pain in various animal models [Wells *et al*., 1992].

TNF-α upregulation is observed in inflammatory states, the increase in this cytokine level will potentiate the conductance of TTX-R Na+ channels via p38-MAPK pathway and also found to increase conductance of non-voltage gated K+ channels. TNF-α aid in apoptosis by activating the caspase signalling. MG17 treatment has controlled the upregulation of TNF-α but it has to be investigated through which mechanism this cytokine has elucidated its effect.

In vivo studies

The effect of MG17 was observed in vivo in Streptozotocin induced diabetic peripheral neuropathy model of rats (Yasunori Kanazawa *et al*., 2001). Prolonged hyperglycemic states will cause nerve damage and also reported that will activate ROCKI and other cytokines. Hence we selected DPN model to screen our compound MG17. We employed various modules to assess pain and extent of neurodegeneration such as tactile allodynia using von Frey filaments to measure 50 percent paw withdrawal threshold (PWT). It is reported that the paw withdrawal threshold value less than 4 is allodynic state. MG17 treated group showed PWT value above 4 indicating that the compound is active against tactile allodynia. MG17 was able to prolong the response time in hot plate test indicating its efficacy on heat hyperalgesia, the reaction time in MG17 treated groups increased significantly ($p < 0.05$-0.001) in comparison to the control group. MG17 could not attenuate the delayed response in cold water paw dip test but was found to be significantly effective in improving nerve conduction velocity (NCV), the reaction time in test compound (MG17) treated group was not significantly different ($p < 0.05$-0.01) in comparison to the control group The normal NCV in rats was reported as 60-64 meters/second and the alternate day treatment with MG17 for 10 days reversed the decreased NCV values indicating that it has some role in neuronal regeneration which has to be explored further.

We wanted to explore MG17 activity on acute inflammation using carrageenan induced rat paw edema. During the five hour observation time the inflammation induced by 0.1 ml 1% carrageenan solution injected in rat paw was not decreased by MG17 administration. This indicates that MG17 has not much local effect. This can be attributed to its high lipophilic nature.

High lipophilic nature of molecule helps in increasing the permeability to higher centers of brain which is desirable for centrally acting drug.

Histopathology studies

Dosing a rodent with a new drug up to one month identifies over 90% of adverse effects that will ever be detected in conventional animal studies. However, these animal studies do not predict all drug adverse effects that can occur in clinical practice and there remains significant over and under prediction of human toxicity. Overall the true positive concordance rate (sensitivity) is of the order of 70% with 30% of human toxicities not predicted by safety pharmacology or conventional toxicity studies. Moreover this concordance varies with different organ and tissue. Therefore, each drug induced pathological finding needs to be assessed on a case by case basis for its likely clinical relevance. For some systems histopathology remains crucial and for some it may not. For example, animal studies are poor predictors of subjective neurological symptoms but histopathological examination of the nervous system in laboratory animals treated with centrally acting drugs detects potential serious neurotoxic effects in humans. Hence, to understand the protective effect of MG17 on rat brain tissue, MeHg (20ppm in water) was given for three weeks to develop neurodegeneration. Neurodegeneration was identified with clasping score and when clasping score was good, brain was isolated and histopathological examination was done. MG17 was given through i.p route during last ten days of induction time (21 days) to test the group. Histopathology reports indicated mild meningeal haemorrhage in the meninges surrounding the cerebral hemispheres; mild to moderate foci of necrosis and degeneration of tissues surrounding the ventricular region; multiple foci of inflammation with infiltration of inflammatory cells was observed in the cerebral cortex; choroid plexus epithelial cells (ependymal cells) in the third ventricle of brain showed hyperplasia and disintegration which indicates changes in the blood brain barrier function observed in MeHg treated group whereas moderate foci of inflammation noticed surrounding the ventricles of brain with evenly distribution of inflammatory cells spreading over the cerebral hemispheres having normal ependymal cells in the ventricle appeared in MG17 group. This indicates that MG17 is having neuro-protective effect against insults causing neurodegeneration.

8.3 Curcumin derivatives

In the present study behavioral pharmacological screening studies of curcumin derivative MG24 on various rat models of neuropathic pain and inflammation has revealed MG24 test compound's preliminary activity on neuroinflammation and pain. To further understand the mode of action in vitro ROCK-I enzyme inhibition assay, mRNA expression studies of pro-inflammatory genes and brain histopathological analysis were performed. MG24 has potent ROCK-I inhibition activity, it has also reduced the upregulated expression levels of TNF-α, IL-1β, IL-6 and NFkB mRNA. It has been reported that the gene expression levels of all these mediators are dysregulated during neuroinflammatory conditions. Curcumin is well known for its strong anti-inflammatory activity and its ability to inhibit the elevated spinal mature IL-1β protein levels in neuropathic pain conditions . In our study interleukin-1 beta levels were not affected by MG24 treatment.

ROCK-I plays prominent role in neuroinflammation and recent studies have reported that the inhibition of Rho kinase (ROCK-I) is essential to ameliorate progressive neurodegeneration as phosphorylated ROCK was found to activate NOX2 assembly via Ras related C3 botulinum toxin substrate (Rac) in neuroinflammatory disease conditions .

It is always advantageous to synthesize one molecule which is multipotent and can target broad range of factors or mediators which influence the disease progression.

Hyperglycemia induces peripheral neuropathy via activation of Rho kinase. Increased glucose concentration in extracellular fluid triggers activation of Rho kinase enzyme which is responsible for the production of reactive oxygen species (ROS). ROS is essential for phagocytosis during infections but production of ROS for prolonged duration can cause apoptosis. Hence limiting ROS production will be beneficial to control neuronal tissue apoptosis.

MG24 has shown neuroprotective activity in brain tissue of MeHg induced neurodegeneration rats. MG24 may be potent anti-inflammatory as it has shown anti-inflammatory properties in both in vitro and in vivo screening studies. Neuroprotection and improvement in nerve conduction velocity may be attributed to MG24's beneficial effects to nervous tissue (repair and regeneration).

Finally our random screening studies revealed many beneficial pharmacological effects of MG24, based on these preliminary studies it is expected that MG24 could become a better therapeutic approach provided molecular mechanistic studies to be performed to understand its mechanism of action in a better way.

CHAPTER-9

Future prospects

FUTURE PERSPECTIVES

The present work elucidates the potential of some multifunctional leads to possess antinociceptive activities in various animal models of neuropathic pain.

These active molecules have also shown anti-inflammatory effects in carrageenan induced acute rat paw oedema. In View of the preliminary pharmacological activity the studies could be extended further to various other nerve injury models involving the spared nerve injury (SNI) and spinal nerve ligation (SNL) models as well as disease induced neuropathy (cancer induced neuropathy, trigeminal neuralgia). All the potent compounds (MG2, MG17 and MG24 each one from the three series) demonstrated regulatory effect on various upregulated pro-inflammatory mediators in neurological disease conditions. Detailed *in vitro* molecular studies are required to understand the mechanism of action. Effect of compounds on electrophysiological parameters using whole-cell patch clamp experiments, radio-ligand binding assays for GABA receptors and study of active compounds on rat brain monoamines will be helpful to understand cellular and receptor activities.

Other CNS effects such as sedation and mental disorientation need to be tested. Curcumin derivatives were found to have excellent wound healing property which has to be explored further in relation to neuronal damage. Overall survival rate need to be find out with dose ranges (100 to 300 mg/kg body weight).

Oral efficacies of the compounds along with pharmacokinetic studies to determine bioavailability, half-life, C_{max} etc. should be undertaken. Metabolism and elimination pattern of the compounds are yet to be explored.

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APPENDIX

LIST OF PUBLICATIONS

FROM THESIS WORK

1. **Gangadhar M**, Mishra R K, Sriram D, Yogeeswari P. Future Directions in the Treatment of Neuropathic Pain: A Review on Various Therapeutic Targets. CNS & Neurological Disorders-Drug Targets, 2014, 13, 1871-5273, 2014 Bentham Science Publishers. (http://www.ncbi.nlm.nih.gov/pubmed/24152326).

2. **Gangadhar M**, Battu M B, Joseph S, Brahmam M, Reshma A, Ravi P R, Sriram D and Yogeeswari P. Evaluation of a novel multipotent semicarbazone derivative having a therapeutic potential for neuroinflammation therapy. Neuropathology and Applied Neurobiology. (MG2) (Communicated)

3. **Gangadhar M**, Ganesh S, Yogeeswari P. MG17, a novel triazole derivative abrogated neuroinflammation and related neurodegenerative symptoms in rodents. Current Molecular Pharmacology, Bentham Science Publishers. (Accepted).

4. **Gangadhar M**, Ravi S, Ashwini S, Mishra R K and Yogeeswari P. Neuroprotective potential of curcumin analogue; Therapeutic approach for neurodegeneration and neuroinflammation related diseases. Bangladesh Journal of Pharmacology. (MG24) (Revised).

OTHER PUBLICATIONS

1.Sharma M, Suman Dash S, Matharasala G, Deekshith V, Sriram D, Yogeeswari P. Novel piperazinyl derivatives with anti-hyperalgesic, anti-allodynic and anti-inflammatory activities useful for the treatment of neuropathic pain. Anti-Inflammatory & Anti-Allergy Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Inflammatory and Anti-Allergy Agents). 2012 Sep 1;11(2):182-90.

2.Yogeeswari P, Patel SK, Reddy IV, Semwal A, Sharma M, Gangadhar M, Sai MS, Sriram D. GABA derivatives for the treatment of epilepsy and neuropathic pain: A synthetic integration of GABA in 1, 2, 4–Triazolo–2H–one nucleus. Biomedicine & Aging Pathology. 2012 Jun 30;2(2):31-40.3.

3.Veesam H, Avula PR, Challa SR, Matharasala G, Perumal Y, Dharmarajan S. Relationship between plasma levels and the anti-neuropathic pain effect of lamotrigine in rat model. Journal of Pharmacy Research. 2013 Jul 31;6(7):780-4.7.

4.Racharla S, Reshma A, Gangadhar M, Sushruth J, Pushkar K, Raghavender M, Swapna Y, D Sriram D, Yogeeswari P. Design, Synthesis, and Pharmacological Evaluation of Novel 1,3,5-triazine-2,4,6-triamine derivatives as Potent and Selective A2A Adenosine Receptor Antagonists in the treatment of Epilepsy. Journal of Medicinal Chemistry.

5.Racharla S, Priyanka S, Gangadhar M, Reshma A, Radhika N, Manoj C, Sriram D, Yogeeswari P. Identification of novel A2BR antagonists by using Pharmacophore-Based 3D-QSAR and its Anti-nociceptive and Anti-inflammatory effects. European Journal of Pharmaceutical Sciences.

6.Reshma A, Srikanth R, Madhu Babu B, Gangadhar M, Sriram D, Yogeeswari P. Design of Dual inhibitors of ROCK-I and NOX-2 for the treatment of neuroinflammatory disorders. Journal of Neuroscience Research.

7.Ram K Mishra, Srikanth R, Reshma A, Sarthak M S, Aquib G, Geethasai S, Gangadhar M, Sriram D, Yogeeswari P. 2,6-Diaminopyrimidines as Rho-Kinase Inhibitors Useful for the Treatment of Neurological Disorders Including Diabetic Neuropathy.

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1. Gangadhar. M, Isaeva. O, Perumal. Y. Anti-inflammatory compound with sIPSC blocking potential, a promising therapeutic approach for neurological pain disorders. Journal of Neurochemistry © 2017 International Society for Neurochemistry, J. Neurochem. (**2017**) 142 (Suppl. 1), 78-164. **Paris, France**.

2. Ashwini. S, Gangadhar. M, Yogeeswari. P, Sriram. D. Pharmacological intervention to study the effect of antinociceptive curcumin analogue in a rat model of migraine. Journal of Neurochemistry © 2017 International Society for Neurochemistry, J. Neurochem. (**2017**) 142 (Suppl. 1), 78-164. **Paris, France**.

3. Screening studies of new chemical entities to evaluate their efficacy on rat model of Haemorrhoids. Pharmaceutical Education-Academia Relation to Industry-Current Scenario, 17th-18th March **2017**, JNTU-Hyderabad, Telangana, India.

4. Screening studies of new chemical entities to evaluate their efficacy on rat model of migraine. 68th Indian Pharmaceutical congress (IPC), 16th-18th Dec **2016**, Vizag, Andhra Pradesh, India

5. Yeast-derived β-D-glucans as a supplement with combination of anti-cancer therapies and for immunotherapy. IKMC conference, 2nd & 3rd Nov **2015**, Novotel, Hyderabad, India.

6. MG2, a novel semicarbazone derivative, reduces neuropathic pain by inhibiting the expression levels of neuro-inflammatory mediators. Pathways of Neurodevelopmental Disorders, March 16-20, **2015**, Granlibakken Resort, Tahoe City, California, USA.

7. Novel Semicarbazone derivatives effective against Diabetic Peripheral Neuropathy. Indian Pharmacological Society NIMHANS convention centre, 16-18 December **2012**, Bangalore.

8. Evaluation of Anti-Nociceptive Properties of Novel Rho Kinase Inhibitors Using Animal Models of Neuropathic Pain Indian Pharmacological Society NIMHANS convention centre, 16-18 December **2012**, Bangalore.

9. Preclinical evaluation of some novel heterocycles against postoperative pain, XXXXIVth Annual Conference of Indian Pharmacological Society, 19-21 December **2011**, Manipal, India.

10. Pharmacological profiling of triazolone derivatives as new leads for neuropathic pain treatment, XXXXIVth Annual Conference of Indian Pharmacological Society, 19-21 December **2011**, Manipal, India.

11. Pharmacological screening of some novel heterocycles for the treatment of Neuropathic Pain. Current Trends in Pharmaceutical Sciences, 12 November **2011**, Birla Institute of Science and Technology, Pilani-Hyderabad Campus, Hyderabad, India.

12. Discovery of Novel in vivo TNF-alpha inhibitors for the treatment of Neuropathic Pain: Possible NO modulatory and antioxidant mechanisms. Current Trends in Pharmaceutical Sciences-2011, 12 November **2011**, Birla Institute of Science and Technology, Pilani-Hyderabad Campus, Hyderabad, India.

13. Discovery of Novel Tetrahydropyridopyrazoles as new leads for the treatment of Neuropathic Pain. RSC MedChem-2011, 25-26th February **2011**, Indian Institute of Chemical Technology, Hyderabad, India.

14. Discovery of new leads more effective than Lamotrigine for the treatment of febrile seizures. RSC MedChem-2011, 25-26th February **2011**, Indian Institute of Chemical Technology, Hyderabad, India.

BIOGRAPHY OF MATHARASALA GANGADHAR

Mr. Gangadhar Matharasala completed his Bachelor of Pharmacy from Nirmala College of Pharmacy affiliated to Jawaharlal Nehru Technological University, Ananthapur (JNTUA) in the year 2009 and Master of Pharmacy (General pharmacy) from Birla Institute of Science and Technology (BITS-Pilani), Hyderabad Campus in 2012. He has been appointed as a Senior Research Fellow at Birla Institute of Technology and Science, Pilani, Hyderabad campus from 2012-2015 under the supervision of Prof. P. Yogeeswari. He has published five scientific publications in well-renowned international journals. He had presented papers at various national and international conferences. He was awarded "Young researcher award" for his research work and publications on behavioral pharmacological screening studies by world's renowned custom pharmacology research instrumentation provider Ugo Basile, Italy on 26th June 2014. He was awarded Department of Science and Technology (DST)-International travel scholarship, New Delhi to present his part of research work on title " MG2, a novel semicarbazone derivative, reduces neuropathic pain by inhibiting the expression levels of neuro-inflammatory mediators" at Keystone Symposia on Pathways of Neurodevelopmental disorders, Tahoe city, California, USA on 18thth March, 2015. He was recently awarded ICMR travel grant [3/2/TG-83/HRD-2017(14)] to participate and present his research work at ISN-ESN Meeting, Paris, France.

BIOGRAPHY OF PROF. P. YOGEESWARI

Prof. P. Yogeeswari is presently working in the capacity of Professor and Associate Dean (Sponsored Research and Consultancy Division), Department of Pharmacy, Birla Institute of Technology and Science, Pilani, Hyderabad Campus. She received her Ph.D. degree in the year 2001 from Banaras Hindu University; Varanasi. She has been involved in research for the last 16 years and in teaching for 17 years. APTI honoured her with YOUNG PHARMACY TEACHER AWARD for the year 2007. In 2010, ICMR honored her by awarding "Shakuntala Amir Chand Award" for her excellent biomedical research. She has been awarded for IASP 2014 "Excellence in Pain Research and Management in Developing Countries" under the basic science research category at Argentina in October 2014. Recently she received BITSAA best faculty award from BITS in 2017. She has collaborations with various national and international organizations that include National Institute of Health, Bethesda, USA, National Institute of Mental Health and Neurosciences, Bangalore, Karolinska Institute, Stockholm, Sweden, National Institute of Immunology, New Delhi, India, Pasteur Institute, University of Lille, France, Bogomoletz Institute of Physiology National Academy of Science, Ukraine, and Faculty of Medicine of Porto, Porto, Portugal,. She has to her credit more than 300 research publications and 5 Indian Patent, Applications. She is an expert reviewer of many international journals like Journal of Medicinal Chemistry (ACS), Journal of Chemical Information & Modeling (ACS, USA), Bioorganic Medicinal Chemistry (Elsevier), Recent Patents on CNS Drug Discovery (Bentham), etc. She has also co-authored a textbook on organic medicinal chemistry titled "Medicinal Chemistry" published by Pearson Education and one book chapter in Jan 2013 published by IGI Global. She is a lifetime member of Association of Pharmacy Teachers of India and Indian Pharmacological Society. She has successfully completed many sponsored projects and currently handling projects sponsored by DBT INDO-BRAZIL, ICMR-INSERM, and DST Indoportugal. She has guided seventeen Ph.D students and currently three students are pursuing their Ph.D. work.