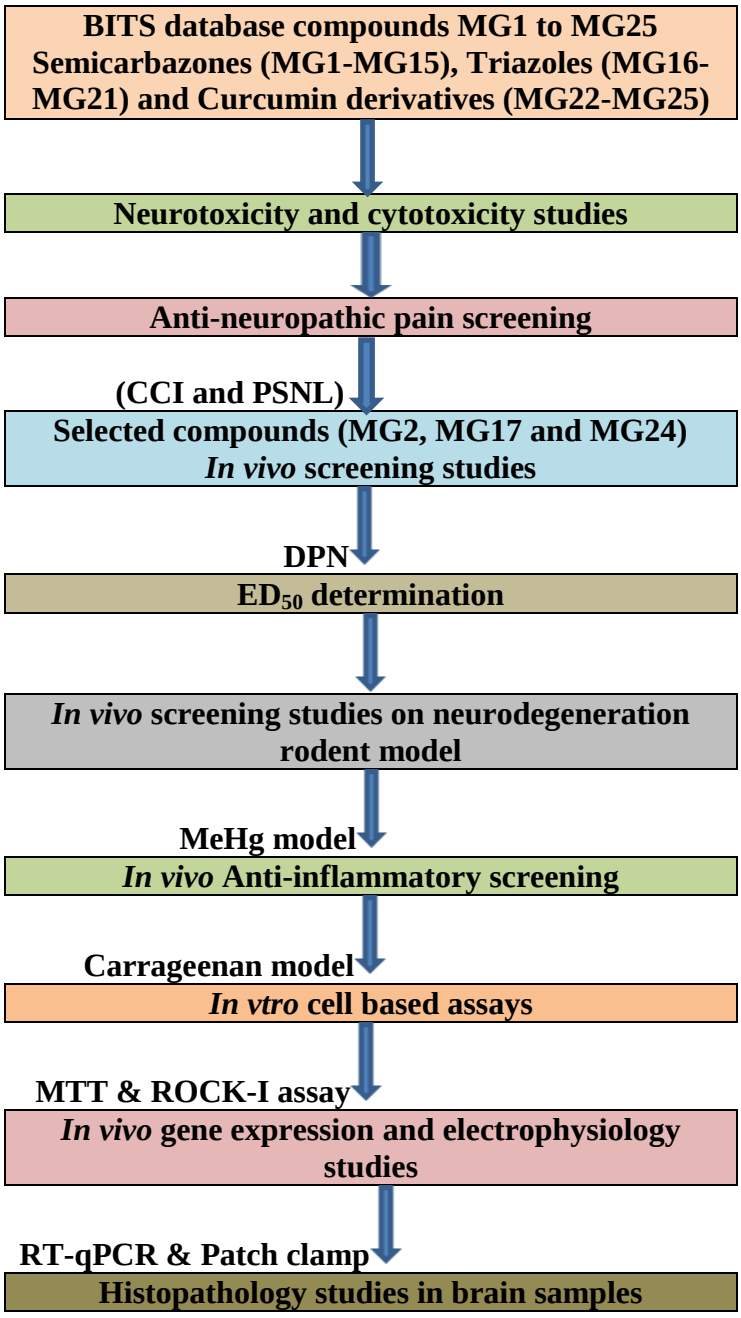
SYNOPSIS

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

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 sensitivity initially 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. The present unmet need for the treatment of neuropathic pain stimulated us to conduct screening studies to find out suitable molecules for the treatment of this disorder 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.

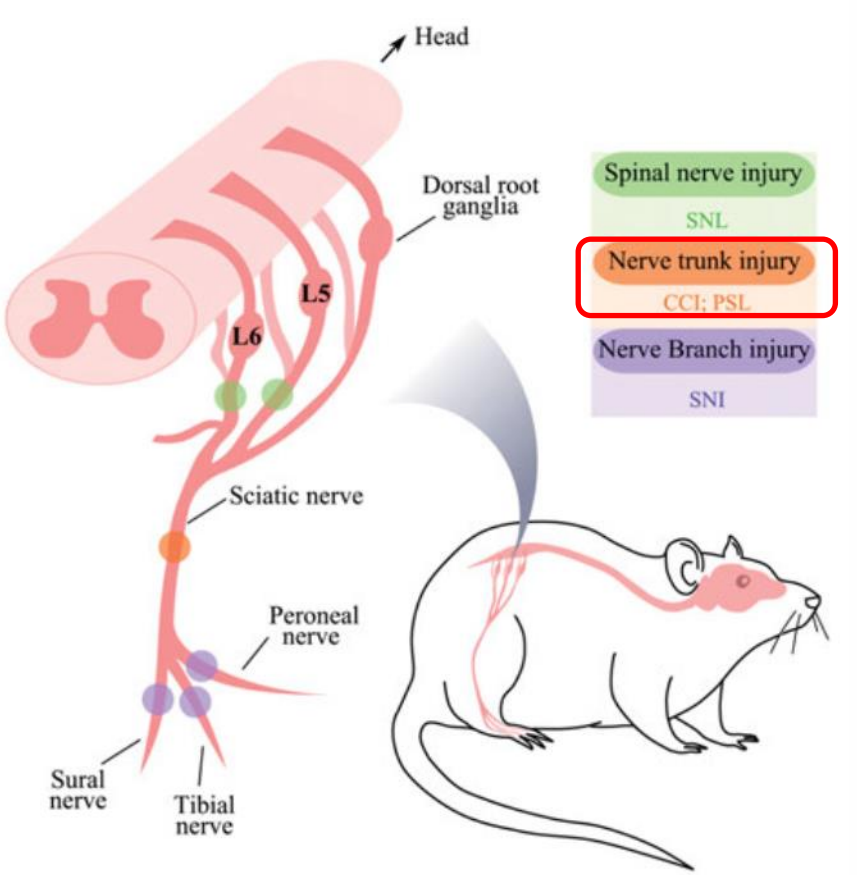
The selection of above three core moieties for carrying out screening studies is based on their reported activities. All the three moieties semicarbazone, triazole and curcumin are lipophilic in nature with potent anti-oxidant and anti-inflammatory properties. These molecules are proven antiepileptic agents which can calm down neuronal hyper excitability. All these properties are essential for compound to act as an anti-neuropathic agent. For this thesis work we have selected fifteen semicarbazone derivatives, six triazole derivatives and four curcumin derivatives from BITS database, synthesized and characterized before screening on animal models. Initial neurotoxic and cytotoxic assessment of all the 25 molecules was done to evaluate their safety profile to be able to use them for further *in vivo* and *in vitro* screening studies. *In vivo* screening studies were performed on rat models of peripheral neuropathy and neurodegeneration. *In vivo* screening studies followed by electrophysiology studies, cell based enzyme inhibition studies,

RT-qPCR studies, acute inflammation studies and histopathology studies were performed as represented in the flow chart.



Neurotoxicity assessment of the selected 25 compounds was done with rotarod and actophotometer studies using maximum dose of 300mg.kg^{-1} and minimum 30mg.kg^{-1}. Most of the compounds were neurotoxic at 300mg.kg^{-1} and 100mg.kg^{-1} dose but none of them were neurotoxic at 30mg.kg^{-1} dose. Hence behavioral screening studies were performed starting with

30mg.kg^{-1} maximum dose followed by 10 and 3mg.kg^{-1} based on the efficacy shown by the compound. Two animal models of chronic pain (neuropathic pain), the chronic constriction injury (CCI) and partial sciatic nerve ligation (PSNL) models were used to evaluate the selected compounds for antineuralgic efficacy. Following neuropathic pain models were employed for behavioral screening studies.



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

Most of the compounds were active in preliminary behavioral screening studies in chronic constriction injury and partial sciatic nerve models of mononeuropathic pain. Four behavioral modules of neuropathic pain assessment-spontaneous pain, dynamic allodynia, cold allodynia and mechanical hyperalgesia were studied in CCI model whereas spontaneous pain responses were not clear and hence were not reported as quantification was difficult in PSNL model.

Out of 25 compounds screened, ten compounds **MG2**, **MG6**, **MG9**, **MG10**, **MG11**, **MG13**, **MG14**, **MG15**, **MG17** and **MG24** were active on spontaneous pain at 60min post treatment.

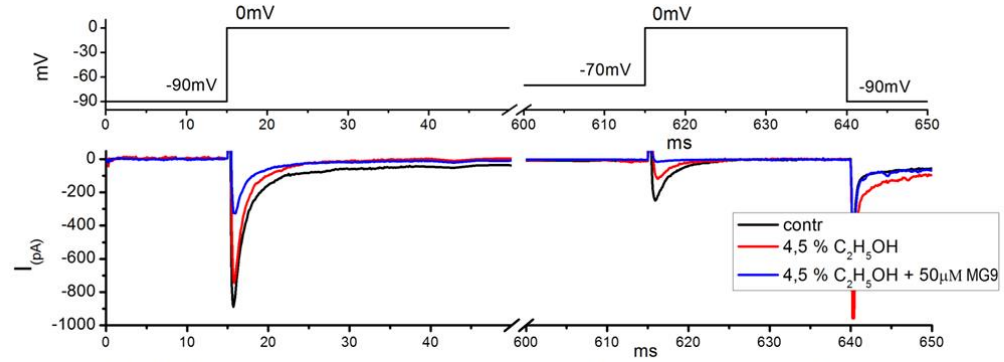
Four compounds **MG2**, **MG8**, **MG17** and **MG24** were active on dynamic allodynia at 60min post treatment. Five compounds **MG9**, **MG11**, **MG14**, **MG17** and **MG24** were active on cold allodynia and two compounds **MG17** and **MG24** were active on mechanical hyperalgesia at 60min post treatment in CCI model.

Out of 25 compounds screened, four compounds **MG2**, **MG10**, **MG17** and **MG24** were active on dynamic allodynia at 60min post treatment. Twelve compounds **MG2**, **MG4**, **MG6**, **MG9-MG15**, **MG17** and **MG24** were active on cold allodynia and three compounds **MG9**, **MG17** and **MG24** were active on mechanical hyperalgesia at 60min post treatment in PSNL model. Based on the behavioral screening scores, three compounds **MG2**, **MG17** and **MG24** (one from each series) showed consistent antiallodynic and anti-hyperalgesic properties and were selected for further screening on diabetic peripheral neuropathy (DPN) and methyl mercury induced neurodegeneration (MeHg) model.

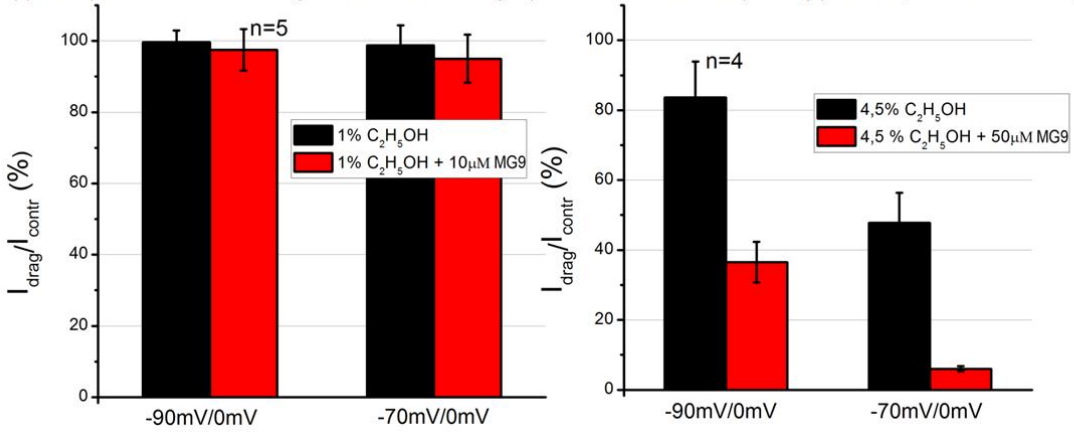
The compounds **MG2**, **MG4** and **MG9** were active in alleviating pain responses and improving nerve conduction velocity.

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 grey matter and suppression of γ- aminobutyric acid (GABA)ergic neurotransmission in the spinal cord .

Compound **MG9** showed blocking effect on voltage gated sodium channels >50% (**MG9**) as shown in the figure at two holding potentials (-90 mV and -70 mV).



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).



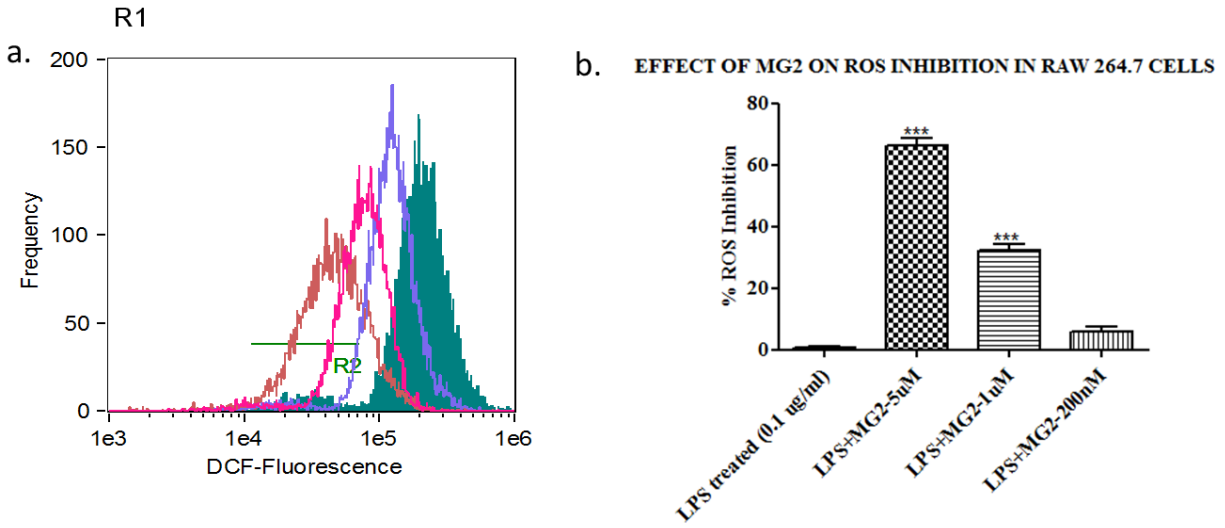
Blockade effect of 10μM and 50μM MG9 on transient voltage gated sodium currents in hippocampal CA1 PC cells

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 over excitation or hyperactivity of sodium channels. The activated sodium channels cause ectopic firing, which is responsible for neuropathic 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.

The compounds **MG2**, **MG17** and **MG24** from each series were extensively screened as they are the most potent compounds from each series. These three compounds showed very good Rho kinase inhibition at IC_{50} concentrations of <1µM (**MG2 2.935±0.022**; **MG17 0.009±0.001**; **MG24 ≤0.001 µM**) 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 disease rats. These compounds were able to reduce mRNA expression levels of THF-α, IL-6 and NF-kB but were unable to reduce IL-1β expression levels at 30mg.kg^{-1} dose. In addition to above properties all the three compounds showed neuroprotective effects on brain tissue of methyl mercury induced neurodegeneration in rats.



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, MG17 and MG24 compounds for their 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 the active compounds (MG2, MG17 and MG24) and the DCFDA (Dichloro-flourescein diacetate) assay results showed that MG2, MG17 and MG24 have potent ROS inhibitory effect at a concentration of 5μM and the effect is also concentration dependant.

All the three active compounds being potent alleviators of neuropathic pain, their 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.

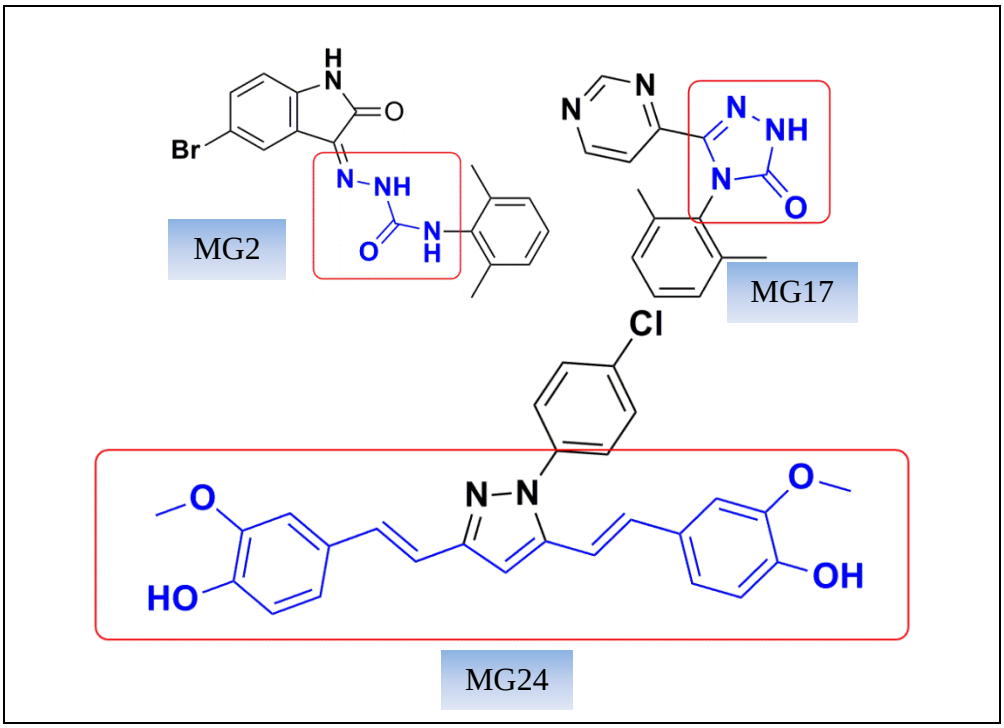
Finally, we conclude that MG2 emerged as a novel semicarbazone derivative possessing multiple effects with therapeutic benefits against neuropathic pain and neurodegenerative disorders and it may be further developed into a therapeutic agent for the treatment or prevention of acute inflammation, neuropathic 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. 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.

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. 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.

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 NF-kB. 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.

Thus the identified active molecules MG2, MG17 and MG24 constitute a prototypical molecule 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 treatment of neuroinflammation related diseases.



Structures of most active compounds