CHAPTER 1

INVESTIGATION OF ANTI-NOCICEPTIVE EFFECTS OF BIRM IN ANIMAL MODELS OF NOCICEPTIVE PAIN

INTRODUCTION

Nociception is the process by which intense thermal, mechanical or chemical stimuli are detected by a subpopulation of peripheral nerve fibers, called nociceptors (Basbaum and Jessell, 2000) and nociceptive refers to the potential of a stimulus to produce a tissue lesion and a reaction from the organism. Nociceptive pain results from activity in neural pathways caused by actual tissue injury or potential tissue damaging stimulus. Nociceptive pain acts as an alarm which helps to protect the organism from further damage. It both triggers reactions and induces learned avoidance behaviors, thus limiting the damaging consequences (Le Bars *et al.*, 2001). As per Dennis and Melzack (1983), pain/nociception has three major functions:

i. To warn the individual of the existence of real tissue damage
ii. To warn the individual of the probability that tissue damage is about to occur by realizing that a stimulus has the potential to cause such damage
iii. To warn a social group about the danger as soon as it exists for any one of its members.

Mechanism of Nociceptive pain

It mainly results from activation of nociceptors (primary afferent fibers - Aδ - and C- fibers) in peripheral tissues through intense thermal, mechanical or chemical stimuli (Basbaum *et al.*, 2009). The nociceptors detect the stimuli and transduce this information into electrical current /signals and release chemical messengers. These messengers travel through superfast nerve conduits to the spinal cord where they are passed directly to the thalamus and into the cerebral cortex and increase the perception of pain signal. The somatosensory cortex in brain identifies the site of injury. Part of the signal reaching limbic system and hypothalamus triggers the behavioral and emotional response to pain stimulus (Basbaum *et al.*, 2009).

Animal Models of Nociceptive Pain

Animal models have been used extensively in basic pain research based on the premise that these models can serve as surrogate assays that can reliably predict the potency and efficacy

of the pharmacologic action of agents that can work in human pain states (Yaksh, 1999).

Experimental models of nociception (pain sensitivity) include tests of response thresholds to high-intensity stimuli such as thermal, chemical and mechanical (e.g. hot plate test, tail flick test, etc.) and of changes in spontaneous or evoked behavioral responses in animals with peripheral injury or inflammation (persistent pain models e.g. formalin test). They are used to assess the anti-nociceptive properties of drugs and can be classified into three types based on the type of stimulus:

i. Thermal assays of nociception: The tail withdrawal test (D'Amour and Smith, 1941; Ben-Bassat *et al.*, 1959), the hot plate test (Woolfe and MacDonald, 1944; Eddy and Leimbach, 1953), Hargreaves' test of paw withdrawal (Hargreaves *et al.*, 1988).
ii. Mechanical assays of nociception: The von Frey fiber test of mechanical sensitivity (von Frey, 1922), the tail clip test (Haffner, 1929), the Randall-Selitto paw pressure test (Randall and Selitto, 1957).
iii. Chemical assays of nociception: The abdominal constriction assay (Van der Wende and Margolin, 1956), the formalin test (Dubuisson and Dennis, 1977; Hunskaar *et al.*, 1986; Abbott *et al.*, 1995).

RATIONALE FOR THE CURRENT STUDY

Neurotransmitters and neuromodulators mediating pain responses may differ in each model. As a result, different pharmacological agents may be effective in attenuating pain in different models. In order to assess the efficacy and further the mechanism of action of the botanical in question the Biological Immune Response Modulator (BIRM) in ameliorating both acute and persistent pain, we selected one animal model from each phase as screening assays namely

(1) Abdominal constriction assay (writhing test) as a model of acute pain which is selectively used to assess the efficacy of peripherally acting analgesics and
(2) Formalin test as a model for persistent pain and it is largely accepted as a surrogate model to assess the efficacy of centrally acting drugs.

The experimental protocols employed as well as the results of both these tests are described separately hereunder for better comprehension.

1. Abdominal Constriction Assay: A model of acute pain

Abdominal constriction assay commonly known as writhing assay is the chemical assay of nociception featuring spontaneous behavior. The substances most commonly used as chemical stimulants (e.g. acids, formaldehyde, etc.,) are virtually never encountered by

organisms in the wild, but it is believed that pain due to trauma and inflammation is mediated by chemical algogens released from damaged cells (Dray, 1995). A wide variety of substances, both exogenous and endogenous have been demonstrated to be painful when injected into humans, and produce nociceptive behaviors in animals when injected into the skin (e.g. capsaicin, bradykinin, prostaglandins, formalin) or peritoneal cavity (e.g. non-isotonic saline, acetic acid, phenylquinones, magnesium sulphate, acetylcholine). It is the major nociceptive test most widely developed and still being used as a screening test in mice. Writhing is described as lengthwise stretches of the torso accompanied by concave arching of the back in response to phenylquinone or acetic acid and is sensitive to abolition by a wide range of analgesics including NSAIDs (Hendershot and Forsaith, 1959; Mogil *et al.*., 2001). Writhing phenomenon, produced by a caudally directed wave of abdominal wall muscle constrictions and elongation, and often followed by a characteristic hind-limb extension is thought to be reflexive in nature.

It was first reported by Van der Wende and Margolin (1956) that intraperitoneal injections of iodinated contrast agents elicited a characteristic stretching response in rats which could be abolished by narcotic analgesics but it was observed that this test was not sensitive enough to detect weak analgesic activity. However, in late 1950s, it was established through certain modifications in test, that this test could be used for testing weak and strong analgesic drugs (Siegmund *et al.*, 1957a; Siegmund *et al.*, 1957b; Hendershot and Forsaith, 1959). The abdominal constriction test is widely used as an assay of visceral nociception, which represents a major clinical problem and it may differ from somatic nociception in its mediation (McMahon, 2006).

We selected acetic acid as a chemical algogen to induce the nociceptive pain exhibiting spontaneous behavior. The recommended concentration of acetic acid to be used for writhing test is 60 mg/kg (Koster *et al.*, 1959). Acetic acid as an algogen and the recommended concentration were selected due to its sensitivity to weak analgesics. Abdominal constrictions from acetic acid start to appear within 5 minutes of injections, peak from 5 to 10 minutes post injections and decline thereafter. We scored for 20 minutes post acetic acid injection. Since time course for producing writhing differs with algogens, it was proposed by Collier *et al.* (1968) that short acting substances act directly on nociceptors whereas delayed acting substances like acetic acid may work indirectly by releasing endogenous mediators.

MATERIAL AND METHOD

Animals and Housing Conditions

Male Swiss albino mice (20-35g) were procured from CPCSEA and AAALAC approved vivarium facility at GVK Biosciences Pvt. Ltd., Hyderabad, India. They were allowed to acclimatize for a minimum duration of one week prior to initiation of testing. They were housed in groups of four in polypropylene cages under ambient conditions. Room temperature and humidity were maintained at 20-25°C and 65-70%, respectively. 12h light/dark cycle was maintained. Standard laboratory rodent diet and potable drinking water were provided *ad libitum*. Experimental protocols were approved by IAEC (Institutional Animal Ethics Committee) according to CPCSEA (Committee for the purpose of Control and Supervision of Experiments of Animals), India. All animal procedures were performed in accordance with the guidelines of CPCSEA. All efforts were made to minimize animal suffering, and to utilize minimum number of animals in this study.

Test Compound and Treatment Regimen

BIRM was a gift from BIRM Inc. (Quito, Ecuador). It is an aqueous extract of dried roots of a plant *Solanum dulcamara* grown in Ecuador, and marketed as a greenish-brown suspension with a mild bitter-sweet smell. The inactive ingredients in BIRM comprise 16% solid particles, likely root fibers, and the remainder, a lipid-free liquid. BIRM is prepared by aqueous extraction of dried roots followed by oxidation/reduction of the extract. During this process, the amount of roots and the timing of oxidation/reduction are carefully controlled to minimize batch-to batch variation.

In the present study, BIRM samples from lot number 18.09.09-003PR were used and it was clarified by centrifugation at 10,000g prior to usage as described by Dandekar *et al.* (2003). Diclofenac (Sigma Aldrich, USA) and glacial acetic acid (Merck) were obtained commercially.

To optimize the treatment regimen of BIRM for screening its peripheral analgesic activity, several trials of writhing assay were conducted. Main assay was conducted using thirty two male Swiss albino mice divided into four groups: Group I - Vehicle control (4 ml/kg, p.o., distilled water), Group II - BIRM (4 ml/kg, p.o., seven days pre-treatment), Group III - Diclofenac (20 mg/kg, p.o., single dose at 30 minutes pre-treatment) and Group IV - BIRM + Diclofenac (BIRM: 4 ml/kg, p.o., seven days pre-treatment + Diclofenac: 20 mg/kg, p.o., single dose at 30min. pre-treatment on day 7)

Test Procedure

The test was carried out according to method described by Koster *et al.* (1959). BIRM was administered orally through gavage needle for seven days prior to acetic acid treatment. Diclofenac was administered orally at a dose level of 20 mg/kg as a single dose on the day of assessment (day 7).Thirty minutes later, acetic acid (0.6% v/v in distilled water, 10 ml/kg, i.p.) was administered to mice to induce the characteristic writhing. Animals were placed in a plexiglass box immediately post acetic acid administration and writhing response namely abdominal constriction, trunk twisting and extension of hind limbs was counted for 20 minutes and expressed as the pain response.

Statistical Analysis

Results were expressed as mean ± SEM (standard error of mean) of the pain response measured. Data was analysed using Graphpad Prism (version 4.1). One way ANOVA followed by Tukey's multiple comparison test was used to analyse data generated from acetic acid induced writhing assay. $p \leq 0.05$ was considered statistically significant. For ease of reading, the basic statistical values are shown in the text while the more extensive statistical information can be found in the figure legends.

RESULTS:

Intraperitoneal injection of 0.6% acetic acid caused an average of 57 writhes in a 20 minute interval. The treatment with BIRM alone, repeatedly for seven days could generate significant inhibition of writhes (41%) as compared to vehicle control. However, BIRM when administered as combination therapy with standard analgesic Diclofenac, could appreciably inhibit the occurrence of writhes (63%) as compared to vehicle control as well as Diclofenac alone (Table 1, Figure 1).

Table 1: Nociceptive effect of repeated BIRM administration in abdominal constriction assay

Group	Total number of writhes	Inhibition (%)
Vehicle Control	56.75 ± 3.56	-
Diclofenac	33.63 ± 2.25***	44
BIRM	32 ± 3.06 ***	41
BIRM + Diclofenac	21.13 ± 2.78*** #	63

Data represented as mean ± S.E.M *** $p \leq 0.001$ as compared to vehicle control; #$p \leq 0.05$ as compared to Diclofenac (ANOVA followed by Tukey's multiple comparison test)

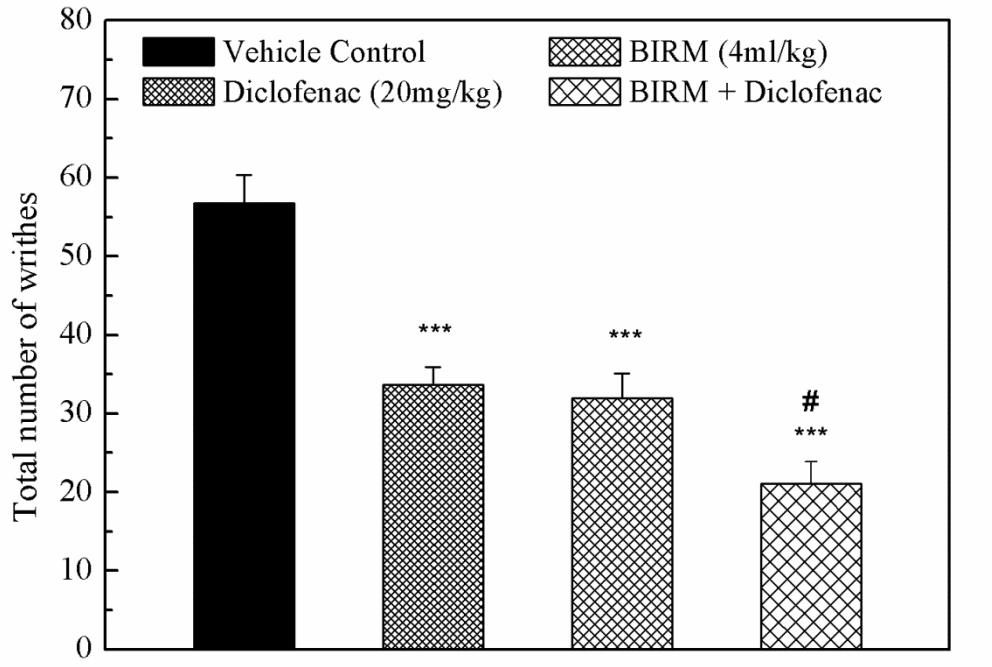

Figure 1: Effect of repeated administration of BIRM (4 ml/kg, seven days p.o.) on nociception induced by acetic acid in abdominal constriction assay as a standalone and in combination with standard drug, Diclofenac (20 mg/kg, single dose p.o.). ***p≤0.001 as compared to vehicle control group, #p≤0.05 as compared to Diclofenac group. Data was analysed using ANOVA followed by Tukey's multiple comparison test.

DISCUSSION

The acetic acid induced abdominal constriction test is carried out to confirm the peripheral analgesics. In general, acetic acid causes spontaneous pain by secretion of endogenous substances such as serotonin, histamine, prostaglandins (PGs), bradykinins and substance P. It is also postulated that abdominal constriction response may be mediated by local peritoneal receptors (Bentley *et al.*, 1983). This test is also associated with acute peritoneal inflammation and increase in the prostaglandin level (mainly PGE_2) in the peritoneal fluid of the mice (Derardt *et al.*, 1980). Prostaglandins induce abdominal constriction by activating and sensitizing the peripheral chemo-sensitive nociceptors (Dirig *et al.*, 1998) which are mostly responsible for causing inflammatory pain (Bley *et al.*, 1998). BIRM significantly reduces the occurrence of writhing in mice in response to acetic acid administration (i.p.), similar to the potent diclofenac sodium (conventional NSAIDs). This significant increase in the pain threshold by BIRM and Diclofenac suggests involvement of central pain pathways. Pain is centrally modulated via number of complex processes including opiate, dopaminergic descending noradrenergic and serotonergic systems (Wigdor and Wilcox, 1987). This anti-nocicpetive effect produced by BIRM and Diclofenac may be via central mechanisms or via peripheral mechanisms involved in the inhibition of prostaglandins, leukotrienes and other endogenous substances that are key players in inducing pain. This observation is also in agreement with observation made by Jaggi *et al.* (2004) that *Solanum dulcamara* extract inhibits PGE_2 production via inhibition of COX activity. Furthermore, it was also observed in the current study that BIRM as standalone treatment could reduce the pain at par with Diclofenac but BIRM when administered as combination therapy along with Diclofenac was found to be more effective in terms of inhibition of writhes as compared to BIRM or Diclofenac alone. This could be attributed to a synergistic effect of the combination therapy possibly acting through effective inhibition of cyclooxygenase enzymes.

2. Formalin Test: A model for persistent pain

Formalin test initially proposed by Dubuisson and Dennis (1977) as a chronic pain model (Cowan, 1990) sensitive to centrally active analgesic agent, is a tonic model of continuous pain resulting from formalin induced tissue injury and is the most commonly used chemical assay of nociception. It was modified by Hunskaar *et al.* (1985) to be adapted in mice. It is a useful model, particularly for the screening of novel compounds, since it encompasses inflammatory, neurogenic and central mechanisms of nociceptors (Tjølsen *et al.*, 1992; Lee *et*

al., 2000b). The formalin test identifies mainly centrally active drugs, whereas peripherally acting analgesics are almost ineffective. Studies by Chau (1989) and Hunskaar and Hole (1987) indicate that the formalin test may allow differentiation between inflammatory and non inflammatory pain, a rough classification of analgesics according to their site and their mechanism of pain.

In formalin test, tissue injury due to subcutaneous administration of formalin evokes a spontaneous behavior, characterized by paw licking and flinching that persists for approximately 1 hour and produces firing of Aδ- and C- dorsal horn convergent neurons (Dickenson and Sullivan, 1987; Heapy *et al.*, 1987). The nociceptive response produced by formalin is biphasic in nature. The early or acute phase (0 to 5 minutes post injection) is thought to reflect direct activation of nociceptors (particularly C fibres) and the late or tonic phase (approximately 15 to 60 minutes) has been attributed to central sensitization dependent on N-methyl-D-aspartate receptor (NMDAR) activation (Coderre *et al.*, 1990) and/or ongoing inflammation-related afferent input (peripheral inflammation) (Dallel *et al.*, 1995). The interphase period (5 to 14 minutes) is attributed to active inhibition at the supraspinal or spinal level (Franklin and Abbott, 1993; Henry *et al.*, 1999). Hence acute and tonic pain can be modeled using a single noxious stimulus in this test.

MATERIAL AND METHOD

Animals and Housing Conditions

Male Sprague Dawley rats (200-230g) were procured from CPCSEA and AAALAC approved vivarium facility at GVK Biosciences Pvt. Ltd., Hyderabad, India. They were allowed to acclimatize for a minimum duration of one week prior to initiation of testing. They were housed in groups of five in polypropylene cages under ambient conditions. Housing conditions were similar to as mentioned earlier.

Treatment Regimen

Total of fifteen male SD rats were selected for the study and were divided in three groups (n=5): Group I - vehicle control, Group II - BIRM (4 ml/kg, seven days, p.o.) and Group III - Gabapentin (50 mg/kg, single dose, i.p. on day 7) (Heughan and Sawynok, 2002). BIRM was a gift from BIRM Inc. (Quito, Ecuador). Gabapentin was procured commercially (Sigma Aldrich, USA).

Test Procedure

On day 7, 30 minutes post administration of BIRM and gabapentin, animals were administered with formalin (50µl of 2.5% concentration) as described by Ellis *et al.* (2008), subcutaneously into the plantar surface of the rat's left hind paw using a 27-gauge needle. Prior to formalin administration, animals were acclimatized in an open plexiglass chamber for 30 minutes.

Post formalin administration, animals were returned back to the observation chamber (open plexiglass chamber) with a mirror angled at 45° positioned behind to allow an unobstructed view of the paws. The frequency of formalin induced pain response like paw lifting, flinching, biting and licking was recorded continuously for 60 minutes (phase 1: 0-10 minutes, phase 2: 11-60 minutes).

Statistical Analysis

Results were expressed as mean ± SEM (standard error of mean) of the pain response measured. Data was analysed using Graphpad Prism (version 4.1). One way ANOVA followed by Tukey's multiple comparison test was used to analyse data generated from formalin test. $p \leq 0.05$ was considered statistically significant. For ease of reading, the basic statistical values are shown in the text while the more extensive statistical information can be found in the figure legends.

RESULTS

The per oral repeated administration of BIRM for seven days prior to formalin administration produced a significant ($p \leq 0.001$) reduction in overall pain response which includes frequency of flinching, biting, licking and paw lifting in both phase 1 and phase 2 as compared to vehicle control rats. Gabapentin, when administered as a single intraperitoneal dose also reduced the pain response in phase 1 and phase 2 as compared to vehicle control rats that received subcutaneous injection of formalin but to a lesser extent as compared to BIRM in phase 2 (Table 2; Figure 2). This observation with respect to gabapentin is in line with the reported data (Yoon and Yaksh, 1999; Heughan and Sawynok, 2002). It is apparent therefore from the study that BRIM is more effective in ameliorating the acute as well as tonic phases of formalin induced nociceptive response when compared to traditional analgesic compounds like gabapentin.

Table 2: Effect of BIRM on pain behavior in Formalin induced nociception assay

Group	Cumulative pain response observed post formalin administration	
	Phase 1 (0-10 minutes)	Phase 2 (11-60 minutes)
Vehicle control	28.60 ± 2.86	351.80 ± 10.41
BIRM (4 ml/kg, p.o.)	11.80 ± 1.66***	10.00 ± 1.38*** #
Gabapentin (50 mg/kg, i.p.)	17.00 ± 1.30**	46.20 ± 3.18***

Data represented as Mean ± SEM **p ≤ 0.01 and ***p ≤ 0.001 as compared to vehicle control. # p≤0.01 as compared to Gabapentin. ANOVA followed by Tukey's multiple comparison test.

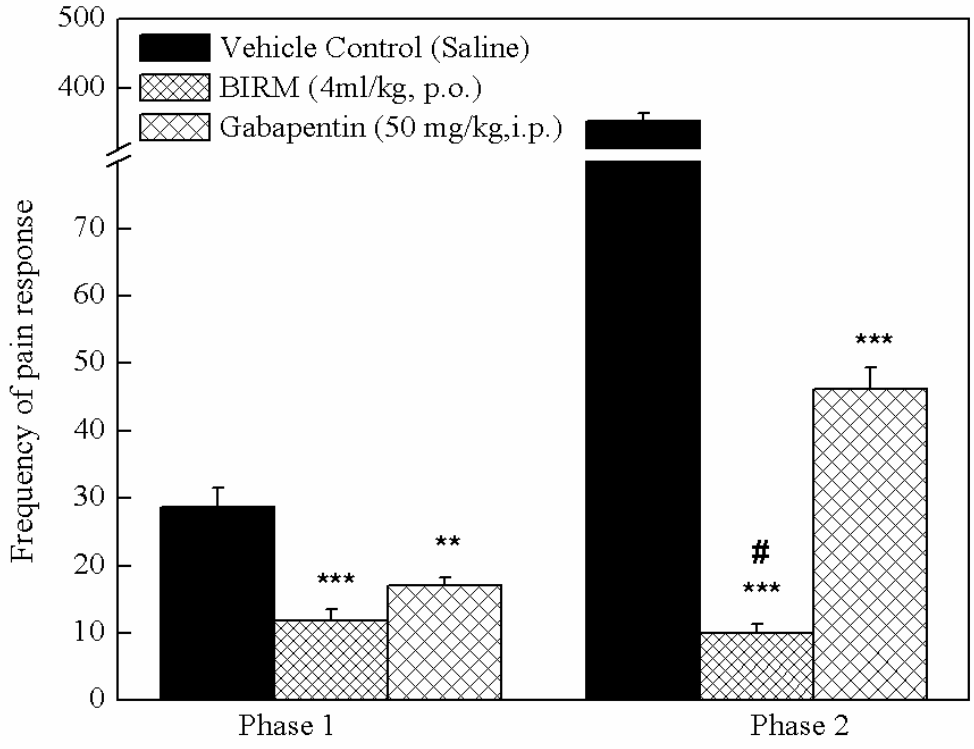

Figure 2: Effect of repeated administration of BIRM (4 ml/kg, p.o.-seven days) on pain behavior induced by formalin in formalin test. **p≤0.01 and ***p≤0.001 as compared to vehicle control. # p≤0.01 as compared to Gabapentin. ANOVA followed by Tukey's multiple comparison test

DISCUSSION

As observed in the present study, BIRM when administered orally for seven days prior to formalin administration in rats inhibits overall pain response (biting, paw licking, lifting and flinching behavior). Contrary to reported results, reference compound Gabapentin used too showed unusual inhibition of pain response in phase I as compared to vehicle control (Heughan and Sawynok, 2002; Yoon and Yaksh, 1999). The reason for this change is not immediately apparent but could be attributed to any of the following procedures adopted in the current study such as age of the animal, environmental stress, ambient temperature and formalin injection site compared to the former studies (Tjølsen *et al.*, 1992; Lariviere *et al.*, 2006). Another criteria leading to variability in the results of various laboratories for formalin test is the type of nociceptive behavior measured such as time spent in exhibiting the pain response and the type and number of behavioral end points considered for the study (Abbott *et al.*, 1999).

As we know, formalin test is capable of discerning between neurogenic pain (early phase which is considered to CNS modulated and non-inflammatory) and inflammatory pain (chronic and peripheral pain). The neurogenic pain which is caused by direct chemical stimulation of nociceptive afferent fibers (predominantly C fibers) can be suppressed by opiates like morphine (do Amaral *et al.*, 2007). Whereas inflammatory pain caused by the sensitization of spinal cord mediated activation of NMDA receptors and release of inflammatory mediators like histamine, prostaglandins, bradykinin, serotonin in the peripheral tissue could be suppressed by opiates, NSAIDs and the likes (Dalal *et al.*, 1999).

Prostaglandins which are thought to play important role in nociceptive transmission at peripheral sites and in the spinal cord (Malmberg and Yaksh, 1992; Yamamoto and Nozaki-Taguchi, 1996; Vane *et al.*, 1998) are synthesized in tissues by cyclooxygenase (COX), an enzyme responsible for the conversion of arachidonic acid into PGs. COX-2 an isoform of COX is highly inducible in response to cytokines, growth factors or other inflammatory stimuli (Vane *et al.*, 1998). There are reports indicating that COX-2 inhibitors are effective in producing an anti-nociceptive effect in rat inflammatory pain models thus proving that COX-2 has a major role in nociceptive transmission in both the spinal cord and at the peripheral sites (Yamamoto and Nozaki-Taguchi, 1996; Yamamoto and Sakashita, 1998). Now, since BIRM is found to inhibit COX-2 (Jaggi *et al.*, 2004), this could be the reason for its anti-nocieptive effect observed in phase I and phase 2. Hence, it is prudent to presume that BIRM

exhibits its anit-nociceptive effect through suppressing neurogenic pain as well as inflammatory pain.

In conclusion, BIRM is found to be effective in attenuating pain occurring due to CNS activation and peripheral inflammatory mediators thus showcasing its analgesic role as peripherally and centrally acting compound.