CHAPTER 9:

INTERACTIONS BETWEEN CYCLOOXYGENASE INHIBITORS AND TRAMADOL IN ACUTE NON-INFLAMMATORY AND INFLAMMATORY PAIN
9.1. INTERACTIONS BETWEEN CYCLOOXYGENASE INHIBITORS AND TRAMADOL IN ACETIC ACID-INDUCED WRITHING IN MICE: AN ISOOLOGOGRAPHIC ANALYSIS

9.1.1. INTRODUCTION

Over the decades, NSAIDs and opioids are the most commonly used analgesics in the management of acute and chronic pain. It is well known that PGs, both centrally and peripherally, play a key role in pain and inflammatory processes (Malmberg and Yaksh, 1992; McCormack, 1994; Willingale et al., 1997). The NSAIDs act by inhibiting the COX enzyme that catalyzes conversion of AA to generate PGs (Vane et al., 1998; Svensson and Yaksh, 2002). Owing to the differential inhibitory activity for COX-1 and COX-2, NSAIDs are now classified as nonselective COX (COX-1/2; e.g. ibuprofen, naproxen), preferential COX-2 (e.g. meloxicam, nimesulide), and selective COX-2 (e.g. celecoxib, rofecoxib) inhibitors, respectively (Kulkami et al., 2000; Simmons et al., 2005). It is well known that the use of NSAIDs is limited by ceiling effects and by adverse events, especially gastrointestinal and renal effects produced by the inhibition of COX-1 isoform (Allison et al., 1992; Murray and Brater, 1993).

Opioid analgesics produce antinociception through their action on both the peripheral and central opioid receptors in acute and chronic pain states (Levine and Taiwo, 1989; Hong and Abbott, 1995). Tramadol, a synthetic opioid, is a racemic mixture of two enantiomers that have actions on other neurotransmitters. (-)-Tramadol preferentially inhibits noradrenaline (NA) uptake (Driessen and Reimann, 1993), whereas (+)-tramadol inhibits serotonin (5-HT) uptake, enhances 5-HT release, and binds to μ-opioid receptors (Driessen and Reimann, 1992). Both these, opioid and non-opioid mechanisms independently contribute to the analgesic effect of tramadol (Raffa et al., 1993). Further, its therapeutic use is not associated with classical side effects of opiate drugs such as respiratory depression, sedation, and constipation.

Intraperitoneal administration of a solution of acetic acid produces characteristic writhing response in animals. This test is commonly employed to evaluate visceral pain since acetic acid directly activates visceral and somatic nociceptors innervating the peritoneum and induces inflammation not only in subdiaphragmatic visceral organs, but also in subcutaneous muscle walls. The acetic acid-induced visceral pain is generally diffuse (poorly localized) and often referred to other intact tissues. Further, there are evidences for the presence of polymodal Aδ- and C-fibers in the gut...
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(Cervero, 1994 and 1995). Acetic acid causes tissue damage and releases pain-producing substances including PGs that activate nociceptors on the terminals on the sensory nerve fibers. Such painful stimuli caused by acetic acid reach higher centers by a number of spinal nerve pathways (Millan, 1999). Moreover, the pain modulators are involved at the spinal level during nociception processing, thus making the spinal cord as the most suitable target to deliver drugs in alleviating pain.

The development of new pain strategies involves combining analgesics that target both central and peripheral pain pathways to deliver greater analgesia at reduced and tolerable doses of individual drugs (Kehlet and Dahl, 1993; Donald, 2002). Further, co-administration of two or more drugs may results in additive (i.e. the sum of the effects produced by the each agent alone), sub-additive (i.e. less than the sum of the effects produced by the each drug alone), or supra-additive (i.e. synergistic; greater than the sum of the effects produced by the each drug alone) interaction. Moreover, advances in the preclinical pain research have led to the recognition of the spinal cord as a key target for alleviation of both acute and chronic pain. Further, the spinal administration requires extremely small quantities of drugs than that cause central as well as peripheral effects (Malmberg and Yaksh, 1992).

The accumulating evidence for the presence of both the COX enzymes and opioid receptors in the spinal cord prompted us to carry out pharmacodynamic analgesic interaction between NSAIDs and tramadol to assess beneficial interactions, if any existed, in acetic acid-induced writhing test. Further, the nature of interactions was analyzed by isobolograms.

9.1.2. MATERIALS AND METHODS

9.1.2.1. Experimental animals

As per 1.2.1.

Albino Laka mice of either sex (20 – 25 g; bred in Central Animal House of Panjab University, Chandigarh, India) were used. Each mouse was used for a single treatment and each group consisted of 5 or 6 animals.

9.1.2.2. Acetic acid-induced writhing

As per 1.2.2.1.

9.1.2.3. Drugs and treatment schedule

The drugs used in the study were naproxen, rofecoxib, tramadol (all from Panacea Biotec Ltd., Lalru, India) and naloxone HCl (Sigma, USA).
All the drug solutions for oral administration were freshly prepared by suspending them in 0.5% carboxy methylcellulose (CMC) except tramadol HCl, which was dissolved in distilled water and administered 1 ml/100 g body weight 30 min before algesiometric test.

For intrathecal administration, all NSAIDs alone or in combination with tramadol were dissolved in a vehicle containing DMSO and 30% normal saline and tramadol HCl was dissolved in normal saline. Drugs for spinal administration were mixed such that all doses were delivered in a total volume of 5 μl intrathecally 15 min before algesiometric test with the help of a microsyringe attached with 30-gauge, 1/2 inch needle according to the Hylden and Wilcox (1980) technique. Control animals received equivalent volume of 0.5% CMC orally (p.o.) or 70% DMSO in normal saline intrathecally.

For antagonistic studies, naloxone HCl dissolved in normal saline was administered intraperitoneally 15 min before algesiometric assay for both drug-treated and control animals.

9.1.2.4. Experimental design
9.1.2.4.1. Dose-response studies for NSAIDs and tramadol
Six to eight animals were used at each of at least four doses to determine a dose-response curve. Dose-response curves were constructed to assess antinociceptive activity of NSAIDs and tramadol administered orally or intrathecally. Several doses of naproxen (5, 10, 20, 50 or 100 mg/kg, p.o., 100, 200, 500 or 1000 nmol, i.t.), rofecoxib (1, 2, 5, 10, 20 or 50 mg/kg, p.o., 10, 50, 100 or 200 nmol, i.t.), and tramadol (1, 2, 5, 10 or 20 mg/kg, p.o.; 50, 100, 200 or 400 nmol, i.t.) were administered to plot dose-response curve. ED$\text{50}$ (effective dose estimated to produce 50%MPE) with its 95% confidence intervals (CI) using standard linear regression analysis of log dose-response curve was calculated for each drug.

9.1.2.4.2. Drug-interaction studies
An isobolographic analysis of interactions was used to determine the nature of the drug interaction between NSAIDs and tramadol (Tallarida et al., 1989). The method is based on comparison of dose combinations in which the dose combinations are made of doses of each of the two agents that are determined to be equipotent. The respective ED$\text{50}$ values were determined from the dose-response curve of each of the two agents alone. In the present study, fixed ratio proportions of ED$\text{50}$ of a NSAID and tramadol
(1:1) were selected and then constructing a dose-response curve for interaction studies in which first combining the respective ED₅₀ values and then ED₅₀ fractions of (1/2, 1/4 and 1/8) of each drug were co-administered. From the dose-response curves of the combined drugs, the ED₅₀ with its 95% confidence intervals of the combination was calculated using standard linear regression analysis of log dose-response and these dose combinations were used for plotting the isobologram. The isobologram was constructed by connecting the ED₅₀ of the corresponding NSAID plotted on the abscissa with the ED₅₀ of tramadol on the ordinate to obtain the additive line. The theoretical additive dose (Z_add) for a combination was calculated from the following equation: \[ Z_{add} = Z_1^*/(p_1 + R \cdot p_2) \]. In this equation, \( Z_1 \) is the ED₅₀ of NSAID, \( Z_2 \) is the ED₅₀ of tramadol, \( p_1 \) and \( p_2 \) are the proportions of each NSAIDs and tramadol in the total mixture, respectively, and \( R \) is the relative potency i.e the ratio of ED₅₀ of NSAID alone to ED₅₀ of tramadol alone. In this study, equal proportions of two agents (1:1) were used in the combination, hence \( p_1 \) and \( p_2 \) becomes 0.5. For comparison of \( Z_{add} \) with experimental ED₅₀ of the mixture (\( Z_{mix} \)), confidence limits are necessary. Such limits are easily obtained from variance (V) of the total dose. Confidence limits for \( Z_{mix} \) are directly from the regression line of the mixture.

Alternatively, a modified method described by Porreca et al. (1990) was used to characterize the interaction between drugs when one drug lacks efficacy. In brief, mixtures of fixed ratio (1:1) of ineffective drug and effective drug were used in combination studies and dose-response curve was constructed as mentioned above. The doses for ineffective drug(s) were taken as equimolar or equal amount (weight) of ED₅₀ fractions of the effective drug for intrathecal and oral administration, respectively. In this interaction study, \( Z_{add} \) becomes equal to the ratio between ED₅₀ of the effective drug and its proportion in combination i.e. \( Z_{add} = Z_2/p_2 \) considering drug 1 is ineffective. The remaining procedure is same as above mentioned to analyze statistical significance.

**9.1.2.5. Statistical analysis**

The doses of the drugs alone or in combination employed in the antagonistic studies were those that produced 50% of maximal effect (ED₅₀) as determined in the dose-response studies. Naloxone, 2 mg/kg was administered intraperitoneally (i.p.) 15 min before algesiometric assay for both drug(s) and vehicle-treated animals after oral and spinal administration.
As per 2.1.2.5.
In interaction studies, Student’s ‘r’-test was used to compare statistical difference between the theoretical additive dose and experimentally derived ED$_{50}$ of the mixtures. An experimental ED$_{50}$ of the mixture that was significantly less than the theoretical additive ED$_{50}$ of the mixture ($P < 0.05$) was considered to indicate a supra-additive or synergistic interaction between the drugs used in combination.

9.1.3. RESULTS

9.1.3.1. Dose-response studies for NSAIDs and tramadol

Intrathecal administration of NSAIDs and tramadol did not produce any motor impairment in animals. Approximately 30% of control as well as drug-treated animals, which received intrathecal NSAIDs alone or combination with tramadol in 70% DMSO as vehicle showed brief vocalization but looked normal during the period of experimentation.

Intraperitoneal administration a solution of 1% acetic acid resulted in characteristic writhing response in control animals that received saline or DMSO orally or intrathecally with mean writhes count of $39.83 \pm 1.45$ and $40.83 \pm 1.56$, respectively during 20 min period of observation. The oral or intrathecal administration of tramadol and naproxen produced dose-dependent antinociceptive effect, as there was significant reduction in the number of writhes as compared to control animals in the algesiometric assay (Fig. 9.1.1A and 1B). However, rofecoxib did not show antinociceptive activity in this algesiometric assay after both oral and intrathecal administration (Fig. 9.1.1A and 1B). The corresponding ED$_{50}$ with its 95% confidence intervals for each NSAID and tramadol after oral and intrathecal administration are shown in Table 9.1.1.

9.1.3.2. Drug-interaction studies

Interaction studies between tramadol and NSAIDs except rofecoxib, on the writhing response showed dose dependent analgesic activity when administered orally or intrathecally (Fig. 9.1.2A and 2B). Isobolographic analysis, using fixed ratio (1:1) ED$_{50}$ fractions revealed a significant supra-additive or synergistic interaction between naproxen and tramadol after oral (Fig. 9.1.3A) and intrathecal (Fig. 9.1.3B) administration in the writhing test. The calculated theoretical ED$_{50}$ with 95% CI and the corresponding experimental ED$_{50}$ with 95% CI obtained for such combinations are given in Table 9.1.2. The points for experimental ED$_{50}$ for these combinations were
obtained below the theoretical additive line indicating possible synergistic interaction. Further, the experimental ED$_{50}$ doses were significantly less ($P < 0.05$) than the calculated additive ED$_{50}$ doses thereby demonstrating a synergistic interaction (Table 9.1.2).

A modified method as described by Porreca et al. (1990) was used to characterize the interaction between rofecoxib and tramadol, as the former drug alone did not show analgesic efficacy in acetic acid-induced writhing. The theoretical ED$_{50}$ for this combination was calculated differently. As rofecoxib alone lacked analgesic effect, it was assumed that the total analgesic effect observed in combination study could be attributed to tramadol alone. Thus, theoretical ED$_{50}$ for this combination becomes ED$_{50}$ of tramadol. Interestingly, the experimental ED$_{50}$ value was significantly higher than theoretical ED$_{50}$ value for this combination when administered orally or intrathecally. On careful examination of results revealed that the analgesic effect (%MPE) of tramadol in the mixture was same as that was observed with the tramadol alone in dose-response studies. This was further confirmed by isobolographic analysis clarifying that rofecoxib merely increased the bulk of the combined dose rather than any significant effect on writhing response (Fig. 9.1.4A and 4B). Hence, the amount of tramadol in experimental ED$_{50}$ value was compared with the theoretical ED$_{50}$ value, which was not significantly different (Table 9.1.2).

9.1.3.3. Antagonist studies

The intraperitoneal administration of naloxone (2 mg/kg) per se had no effect in control animals that received respective vehicle either orally or intrathecally. Further, pretreatment with naloxone did not reverse the antinociceptive effect produced by ED$_{50}$ of naproxen alone when administered orally or intrathecally. In case of rofecoxib that lacked efficacy in this assay, an amount equal to ED$_{50}$ of tramadol for oral (5.15 mg/kg) or intrathecal administration (158.08 nmol) was employed, which was not affected by naloxone. In contrast, naloxone pretreatment significantly increased the number of writhes in oral as well as spinal tramadol-treated animals (Fig. 9.1.5 and 9.1.6). In the interaction studies also, naloxone pretreatment partially blocked the analgesic activity of drugs in combination when administered orally (Fig. 9.1.5) and intrathecally (Fig. 9.1.6).
Fig. 9.1.1. Dose-response curves for (A) oral and (B) intrathecal administration of naproxen (Nap), rofecoxib (Rof), and tramadol (Tra) on the inhibition of writhing response. Each dose point on graph represents mean %MPE ± SEM. % MPE: Percent maximal possible effect.
Fig. 9.1.2. Dose-response curves for (A) oral and (B) intrathecal co-administration of naproxen and tramadol (Nap+Tra) and rofecoxib and tramadol (Rof+Tra) on the inhibition of writhing response. Each dose point on graph represents mean %MPE ± SEM. % MPE: Percent maximal possible effect.
Fig. 9.1.3. Isobolograms for (A) oral and (B) intrathecal co-administration of naproxen and tramadol. The filled point on the theoretical additive line corresponds to theoretical ED$_{50}$ ± SEM and the open point corresponds to experimental ED$_{50}$ ± SEM of the mixture. The experimental points were significantly different from the calculated additive points, indicating a synergistic interaction (p < 0.05; Student’s t-test).
Fig. 9.1.4. Isobolograms for (A) oral and (B) intrathecal co-administration of rofecoxib and tramadol. The straight solid line is the theoretical additive line and the filled point corresponds to theoretical ED$_{50}$ ± SEM and the open point corresponds to experimental ED$_{50}$ ± SEM of the mixture. The experimental points were not significantly different from the calculated additive points, indicating an additive interaction (Student’s $t$-test).
Table 9.1.1. ED$_{50}$ values with 95% confidence intervals (CI) for the antinociceptive effect of NSAIDs and tramadol administered orally or intrathecally.

<table>
<thead>
<tr>
<th>Drugs</th>
<th>ED$_{50}$ (mg/kg; 95% CI), oral</th>
<th>ED$_{50}$ (nmol; 95% CI), intrathecal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naproxen</td>
<td>30.3 (25.9 – 36.98)</td>
<td>486.21 (388.53 – 644.65)</td>
</tr>
<tr>
<td>Rofecoxib</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>(Up to 50 mg/kg, p.o.)</td>
<td>(Up to 200 nmol, i.t.)</td>
</tr>
<tr>
<td>Tramadol</td>
<td>5.15 (4.24 – 5.73)</td>
<td>158.08 (136.87 – 183.13)</td>
</tr>
</tbody>
</table>

NA: Not achieved

Fig. 9.1.5. Effect of naloxone (2 mg/kg, i.p.) on writhing response after oral administration of drug(s). The bars represent the mean ± SEM of the writhes. The open bars represent writhes observed after oral administration of vehicle, naproxen (Nap; 30.3 mg/kg), rofecoxib (Rof; 5.15 mg/kg), tramadol (Tra; 5.15 mg/kg) alone, and co-administration of Nap+Tra (30.3 mg/kg + 5.15 mg/kg), and Rof+Tra (5.15 mg/kg + 5.15 mg/kg). The striped bars demonstrate the effect of naloxone on the agents listed above. *Statistical significant reversal of the drug(s) effect by naloxone (p<0.05; one-way ANOVA followed by Dunnett’s test).
### Table 9.1.2. Theoretical and experimental ED\(_{50}\) values with 95% confidence intervals (CI) for the antinociceptive effect of combinations of NSAIDs and tramadol administered orally or intrathecally

<table>
<thead>
<tr>
<th>Drugs</th>
<th>ED(_{50}) (mg/kg; 95% CI), Oral</th>
<th></th>
<th>ED(_{50}) (nmol; 95% CI), Intrathecal</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Theoretical</td>
<td>Experimental</td>
<td>Theoretical</td>
<td>Experimental</td>
</tr>
<tr>
<td>Rofecoxib + Tramadol</td>
<td>5.15 (4.24 – 5.73)</td>
<td>11.34 (10.10 – 13.53)</td>
<td>158.05 (136.87 – 183.13)</td>
<td>339.73 (319.93 – 350.20)</td>
</tr>
<tr>
<td>Tramadol #</td>
<td>-----</td>
<td>5.67 (5.05 – 6.76)</td>
<td>-----</td>
<td>169.86 (159.96 – 175.10)</td>
</tr>
</tbody>
</table>

# Proportion of tramadol in experimental ED\(_{50}\) value of the mixture of rofecoxib and tramadol; *p < 0.05 between theoretical and experimental ED\(_{50}\) values (Student’s \(t\)-test).

**Fig. 9.1.6.** Effect of naloxone (2 mg/kg, i.p.) on writhing response after intrathecal administration of drug(s). The bars represent the mean ± SEM of the writhes. The open bars represent writhes observed after intrathecal administration of vehicle (70% DMSO), naproxen (Nap; 486.21 nmol), rofecoxib (Rof; 158.08 nmol), tramadol (Tra; 158.08 nmol) alone, and co-administration of Nap+Tra (486.21 nmol + 158.08 nmol), and Rof+Tra (158.08 nmol + 158.08 nmol). The striped bars demonstrate the effect of naloxone on the agents listed above. *Statistical significant reversal of the drug(s) effect by naloxone (p<0.05; one-way ANOVA followed by Dunnett’s test).
9.1.4. DISCUSSION

The present study examined the emerging aspect of spinal opioid and non-opioid analgesia and the application of drug combinations for spinal analgesia. Oral and intrathecal administration of naproxen or tramadol alone resulted in the dose-dependent antinociception without any behavioral change. However, rofecoxib, a selective COX-2 inhibitor, lacked analgesic efficacy in this test. This observation implicates that both COX-1 inhibition and μ-receptors may be involved in decreasing nociceptive inputs at the spinal level. It is expected that certain concentration (doses) of systemically administered drugs reach effective levels in the spinal cord so as to produce antinociception compared to the effects observed after spinal administration.

The oral co-administration of naproxen, a nonselective COX inhibitor with tramadol showed dose-dependent antinociceptive activity that produced a synergistic interaction up on isobolographic analysis. Moreover, the synergism was also observed after spinal administration of drugs demonstrating that the spinal cord was also a site for such interaction. In contrast, there was no additive or synergistic interaction between rofecoxib and tramadol suggesting that the PGs derived from COX-1 but not by COX-2 play role in this interaction.

Role of inhibition of COX isoforms in synergistic interaction

COX isoforms have been identified as COX-1 and COX-2 in the brain and spinal cord of humans and rodents (Beiche et al., 1996; Kulkarni et al., 2000). It is possible that PGs released from both the COX isoforms are involved in processing of acetic acid-induced visceral nociception. However, the failure of systemically administered rofecoxib in reducing writhes suggesting that acetic acid-induced visceral nociception may be preferably COX-1 dependent rather than both COX isoforms. It is possible that the failure of rofecoxib to alleviate pain may be due to the drug levels that achieved at spinal cord are not sufficient to counteract noxious stimuli. However, this speculation can be eliminated, as the spinal administration of rofecoxib up to 200 nmol did not show antinociceptive activity. Unlike other non-selective COX inhibitors, which are highly polar and cross the blood-brain barrier with difficulty, rofecoxib is less polar and readily cross the blood-brain barrier (Merck and Co. Vioxx information, issue 2000; Dembo et al., 2005) and its central effects have been observed after systemic administration (Schwartz et al., 1999). So the lack of efficacy of this drug is not due to sufficient drug concentration at the vicinity of
spinal cord, but it may be due to absence of adequate levels of COX-2 expression to release PGs or ineffective participation of COX-2.

Our results are consistent with previous reports in which selective COX–2 inhibitors have been shown to be ineffective to alter acute visceral pain (Jain et al., 2001; Matsumoto et al., 1998). Recently it has been reported that the number of writhing responses induced by acetic acid in the COX-1 knockout mice, but not in the COX-2 knockout mice, was less than that in the wild-type mice (Ballou et al., 2000). Further, COX-2 mRNA and protein in spinal cord increased 3 – 6 hr after injection of carrageenan in the hind paw (Beiche et al., 1996). In the present study, the number of writhes as a measure of nociception was observed for the first 20 minutes after the administration of a solution of acetic acid and this time would not be sufficient for activation of COX-2 mRNA and generation of COX-2. Thus, the presence of constitutive COX-2 or its induction may not play a role in the spinal processing of acetic acid-induced writhing. Further, it was expected that if tramadol when combined with rofecoxib might result in enhancement of total analgesic efficacy that would clarify any substantial involvement of constitutive spinal COX-2 in this test. A synergistic interaction was resulted when naproxen and tramadol were combined, instead no such synergism was observed between rofecoxib and tramadol. The isobolographic analysis for this combination showed no significant difference between ED$_{50}$ values derived theoretically and experimentally. This was further evidenced by the antagonistic studies in which the number of writhes observed with combination was not significantly different from that observed with ED$_{50}$ dose of tramadol alone. This confirms that the observed antinociceptive effect of the combination contributed to tramadol alone and rofecoxib, which lacked efficacy alone, merely increased the bulk of the mixture. In contrast, the synergistic interaction between naproxen and tramadol when combined in a fixed ratio (1:1) of their ED$_{50}$ was only partially reversed by naloxone, an opioid antagonist. These observations clearly demonstrated the beneficial interaction of COX-1, but not COX-2 inhibition with µ-opioid receptors in this synergistic effect.

Role of tramadol in synergistic interaction
It is well reported that peripheral opioid receptors play an important role in the spinal nociceptive processing. Opioid receptors are present at the primary afferent neurons and local administration of µ- and δ agonists suppress spontaneous activity
following peripheral inflammation (Levine and Taiwo, 1989; Hong and Abbott, 1995). μ-opioid agonists act to inhibit tetrodotoxin-resistant sodium channels, adenylyl cyclase produced by prostaglandin E₂ and 5-HT, the release of depolarization evoked C-fiber peptides and open adenosine triphosphate-sensitive potassium channels via Gi proteins resulting in hyperpolarisation thereby inhibiting the excitability of primary afferent nerves (Yaksh, 1988; Ocana et al., 1990; Gold et al., 1996). Naloxone partially blocked the antinociceptive activity of tramadol alone or its combination with naproxen suggesting possible involvement other neurotransmitters and/or neuromodulators in the synergism along with μ-opioid agonistic activity. Tramadol, which is a racemic mixture with (-)-tramadol preferentially inhibits NA uptake (Driessen and Reimann, 1993), whereas (+)-tramadol inhibits 5-HT uptake, enhanced 5-HT release, and binds to μ-opioid receptors (Driessen and Reimann, 1992). In addition, a marked antinociceptive synergy exists between these two enantiomers (Raffa et al., 1993). In the present study, the roles of serotoninergic and adrenergic modulation were not studied because NA and 5-HT reuptake inhibitors and the antagonists of α₁-adrenergic and 5-HT₂ receptors displayed analgesia and produced synergism with tramadol in ameliorating the acetic acid-induced visceral nociception (Pinardi et al., 1998). It has been reported that 5-HT, NA, and endogenous opioids in the descending inhibitory system modulates pain at the level of dorsal horn besides stimulation of serotoninergic system activates endogenous opioids (Aimone et al., 1987; Heinricher et al., 1992). Thus, both opioid and non-opioid components of tramadol contributed to the synergistic interaction with naproxen.

The mechanism of synergistic interaction between naproxen and tramadol is difficult to explain. Naproxen is known to act by inhibiting PGs formation when given peripherally or spinally. Further, synergism was observed after both oral as well as spinal administration of naproxen and tramadol. It has been suggested that spinal PGs may exert a presynaptic inhibition of the release of NA from spinal noradrenergic terminals. Thus, COX-1 inhibition may augment the spinal noradrenergic terminal activity by blocking PG formation, thereby facilitating NA release (Taiwo and Levine, 1988). Further, synergistic interactions have been reported between NSAIDs and adrenergic agents (Miranda et al., 2001; Jain et al., 2002). Vaughan et al. (1997) has hypothesized a mechanism that involves opioid modulation of arachidonate metabolites in GABA interneurons. These authors have been demonstrated that μ-
opioids receptors coupled to a voltage-dependent potassium conductance via a pathway involving PLA₂, AA, and 12-lipoxygenase. COX inhibitors potentate opioid inhibition of GABA synaptic transmission presumably because more arachidonic acid is available for enzymatic conversion to 12-lipoxygenase products. Therefore, inhibition of COX-1, rather than COX-2 potentate the inhibitory actions of opioids on GABA synaptic transmission (Vaughan, 1998). A functional interaction may result from distinct drug effects at separate anatomical sites that may act independently as well as together to inhibit spinal nociceptive processing (Roerig and Fugimoto, 1989). It is expected that a pharmacodynamic interaction is more probable as COX-1 inhibition reduced nociceptive inputs reaching the central nervous system, therefore enhancing the efficacy of the central action of the tramadol.

In conclusion, isobolographic analysis indicated a synergistic interaction between non-selective COX inhibitor and tramadol when administered peripherally or intrathecally. Further, opioid and non-opioid mechanisms of tramadol and inhibition of COX-1, but not COX-2, are responsible for naproxen and tramadol synergism in acute visceral pain.
9.2. INTERACTIONS BETWEEN CYCLOOXYGENASE INHIBITORS AND TRAMADOL IN ACUTE INFLAMMATORY PAIN

9.2.1. INTRODUCTION

Tissue inflammation produces spontaneous activity in otherwise silent small primary afferent axons and consequently evokes behavioral hyperalgesia. The peripheral hypersensitivity of the alter primary afferent can be explained in part by complex cascade of events involving initial release of SP and glutamate that occur in both central and periphery, and enhance local release of proinflammatory substances, such as bradykinin and prostaglandins (PGs), which activate and sensitize the peripheral nerve endings (Malmberg and Yaksh, 1995; Simmon et al., 2005). In particular, spinal phospholipases are activated leading to generation of spinal prostanoids by cyclooxygenase (COX) (Willingale et al., 1997; Eberberger et al., 1999; Yaksh et al., 1999).

It is well known that NSAIDs acts by inhibiting COX isoforms and thereby blocking generation of PGs. Indeed, combinations of NSAIDs with opioids are currently used in clinical practice to reduce opioid requirements (Burns et al., 1991; Kehlet and Dahl, 1993). The purpose is to improve analgesia without enhancing the side effects of each drug. Various clinical studies have demonstrated a 20–50% reduction in the opioid requirement when NSAIDs are added (Kehlet and Dahl, 1993). Experimental studies have reported a synergism between intravenous morphine and diclofenac, but only an additive interaction between morphine with propacetamol in an inflammatory pain model in rats (Fletcher et al., 1997). On the other hand, the morphine–ketorolac combination has shown a significant synergism in the formalin, visceral nociception and neuropathic pain tests (Malmberg and Yaksh, 1993; Maves et al., 1994; Lashbrook et al., 1999). Moreover, acetylsalicylic acid significantly increased the antinociceptive effect of morphine in the hot-plate and formalin tests (Sandrini et al., 1998), whereas that local administration of dipyprone increased the peripheral antinociceptive effect of morphine in the formalin test (Aguirre-Banuelos and Granados-Soto, 1999). Accumulating data demonstrates that combinations of opioid, particularly morphine and codeine with NSAIDs are not well tolerated and do not offer a superior alternative for pain control (Eckhardt et al., 1998; Chang et al., 2001). Moreover, the use of NSAIDs is limited by ceiling effects and by adverse
events, especially gastrointestinal and renal effects produced by the inhibition of COX-1 isoform (Peskar, 2001; Peng and Duggan, 2005).

Tramadol, a synthetic opioid, is a racemic mixture of two enantiomers with both opioid and non-opioid mechanisms that independently contribute to its analgesic effect (Raffa et al., 1993; Grond and Sablotzki, 2004). Moreover, it is associated with few opioid-related side effects, such as respiratory depression, sedation, and constipation, and is widely used for post-operative pain, refractory cancer pain, chronic inflammatory disorders and neuropathic pain (Lewis and Han, 1997). Recently, a clinical study has showed that the tramadol–diclofenac combination produced a better analgesic response than diclofenac alone (Wilder-Simth et al., 2003).

Despite enormous research on antinociceptive interactions, the information regarding the potential benefit of NSAID-opioid combinations yielding a rational basis for their use in clinical practice is still scarce. Thus, to gain more insight on the antinociceptive efficacy of opioid–NSAIDs combinations, the present study was designed to assess the antihyperalgesic effect of tramadol and NSAIDs with differential selectivity for COX isoforms and their possible synergistic interaction by isobolographic analysis in the rat inflammatory pain models.

9.2.2. MATERIALS AND METHODS

9.2.2.1. Experimental animals

As per 1.2.1.

Each animal was used for a single treatment and each group consisted of 6 or 8 animals.

9.2.2.2. Formalin-induced tonic pain

As per 1.2.2.2.

9.2.2.3. Carrageenan-induced mechanical hyperalgesia

Mechanical hyperalgesia (decreased threshold to mechanical stimuli) were evaluated in ipsilateral paw of rats. The withdrawal threshold was evaluated using an analgesymeter (Ugo Basile, Italy) by applying noxious pressure to hind paw and expressed in grams as per 1.2.2.3b.

The % reversal of hyperalgesia was calculated according to the following formula:

\[
\text{% Reversal} = \left( \frac{\text{Postdose threshold} - \text{Predose threshold}}{\text{Naive threshold} - \text{Predose threshold}} \right) \times 100
\]
In this assay, the ED$_{50}$ for each agent and their combinations was calculated at peak antihyperalgesic effect observed in dose-response studies.

9.2.2.4. Drugs and treatment schedule
The drugs used in the study were indomethacin, naproxen, valdecoxib, tramadol hydrochloride (all from Panacea Biotec Ltd., Lalru, India).

The solutions of all the drugs alone or in combination for intraperitoneal or per oral (p.o.) administration were freshly prepared by solubilizing them in distilled water with one or two drops of Tween 80 except tramadol, which was dissolved in distilled water only and administered 1 ml/100 g body weight 30 min before formalin or 2 h after carrageenan injection into hindpaw. Control animals received equivalent volume of saline intraperitoneally or orally.

9.2.2.4. Experimental design

9.2.2.4.1. Dose-response studies for NSAIDs and tramadol
Six to eight animals were used at each of at least four doses to determine a dose-response curve. Dose-response curves were constructed to assess antinociceptive activity of NSAIDs and tramadol administered intraperitoneally in the formalin test and orally in carrageenan-induced hyperalgesia. Several doses of naproxen (5, 10, 20, 50 or 100 mg/kg, i.p.), valdecoxib (1, 5, 10, 20 or 50 mg/kg, i.p.), and tramadol (1, 2, 5, 10 or 20 mg/kg, i.p.) were administered to plot dose-response curve in the formalin test whereas naproxen (1, 3, 10 or 30 mg/kg, p.o.), valdecoxib (0.3, 1, 3 or 10 mg/kg, p.o.), and tramadol (1, 3, 10 or 30 mg/kg, p.o.) were administered to plot dose-response curve in carrageenan-induced hyperalgesia. ED$_{50}$ (effective dose estimated to produce 50% MPE or reversal of hyperalgesia) with its 95% confidence intervals (CI) using standard linear regression analysis of log dose-response curve was calculated for each drug.

9.2.2.4.2. Drug-interaction studies
Isobolographic analysis was used as per 9.1.2.4.2.

The theoretical and experimental ED$_{50}$ values of the studied combination were also contrasted by calculating the interaction index ($\gamma$) as follows:

$$\gamma = \frac{\text{Experimental ED}_{50} \text{ of combination}}{\text{Theoretical ED}_{50} \text{ of combination}}$$

The interaction index indicates the magnitude of interaction that accounts for possible additive, synergistic/potentiation or antagonistic effect. Values near to 1 correspond to an additive interaction, values higher than 1 imply an antagonistic
interaction, and values lower than 1 indicates a synergistic interaction. When one of
the agents lacks efficacy and the interaction index values near to 1 imply no potential
interaction since there is any absolute difference between experimental and theoretical
ED50 values of combination.

9.2.2.4.3. Measurement of gastrointestinal side effects
NSAID-induced gastric damage in rats was evaluated following the procedure
described by Deciga-Campos et al. (2003). In fasted (24 h) rats, indomethacin (20
mg/kg) was given orally to produce 100% gastric ulcers. Similarly, naproxen (30
mg/kg), valdecoxib (10 mg/kg), tramadol (30 mg/kg), vehicle, the combination of
tramadol+naproxen (30 mg/kg + 30 mg/kg, respectively) and tramadol+valdecoxib
(30 mg/kg + 10 mg/kg, respectively) were administered orally at the same time to
seven groups (six to eight rats in each) of fasted rats; 2.5 h later, all the groups
received a second administration of the same doses. Five hours later, the rats were
killed and the stomach was excised along its smaller curvature, gently rinsed with
formal saline (2%), and the mucosa was examined for the presence of lesions i.e.
petechiae (erosion) or frank hemorrhagic lesions (ulcers). The severity of gastric
lesions induced by the drug treatments was calculated as the ratio between the number
of lesions (stomach ulcer or erosion) caused by a given treatment and the number of
lesions produced by indomethacin (100%). This value was considered to reflect drug-
induced gastrointestinal side effects and was used for comparison between groups.

9.2.2.5. Statistical analysis
As per 2.1.2.5 and 9.1.2.5.

9.2.3. RESULTS

9.2.3.1. Dose-response studies for NSAIDs and tramadol

9.2.3.1.1. Formalin-induced tonic pain
Intraplantar administration a 20 µl solution of 5% formalin into the hind paw of mice
induced a characteristic biphasic licking and biting response with an early and a late
phase. Valdecoxib (up to 50 mg/kg, i.p.) failed to have any effect on either phase of
the formalin assay after administration (Fig. 9.2.1). Naproxen did not alter the early
phase of the formalin response but it dose dependently (5 – 100 mg/kg, i.p.)
attenuated the development of the late phase as compared to control animals (Fig.
(Fig. 9.2.1 and 9.2.2). However, tramadol (1 – 20 mg/kg, i.p.) produced dose-
dependent antinociceptive effect in both the phases as there was significant reduction
in the number of nociceptive responses as compared to control animals in the formalin test (Fig. 9.2.1 and 9.2.2)

9.2.3.1.2. Carrageenan-induced mechanical hyperalgesia

Carrageenan administration into the hindpaw produced significant edema associated with hyperalgesia, as shown by a decreased paw withdrawal threshold in response to mechanical pressure 2 h after injection. Both naproxen (1.0 – 30 mg/kg, p.o.) and valdecoxib (0.3 – 10 mg/kg, p.o.) showed dose-dependent reversal of carrageenan-induced mechanical hyperalgesia with almost 78 and 92 % reversal observed 2 h after drug administration at 30 mg/kg of naproxen (Fig. 9.2.6) and 10 mg/kg of valdecoxib (Fig. 9.2.7), respectively. In this assay, valdecoxib was approximately 4 times more potent than naproxen (Table 9.2.1). Tramadol (1 – 30 mg/kg, p.o.) also produced dose-dependent reversal of hyperalgesia with peak reversal of hyperalgesia (75%) was observed between 1 – 2 h after drug administration at dose 30 mg/kg (Fig. 9.2.7). The corresponding ED$_{50}$ with its 95% confidence intervals for each NSAID and tramadol after oral administration in this assay were calculated 2 h after drug administration and are shown in Table 9.2.1.

9.2.3.2. Drug-interaction studies

9.2.3.2.1. Formalin-induced tonic pain

The combination of tramadol and naproxen showed dose dependent analgesic activity when administered intraperitoneally in the late phase of the formalin test (Fig. 9.2.3). Isobolographic analysis using fixed ratio (1:1) of the ED$_{50}$ fractions revealed a significant supra-additive interaction between naproxen and tramadol after intraperitoneal administration in the late phase the formalin test. The point for experimental ED$_{50}$ for this combination was obtained below the theoretical additive line indicating possible synergistic interaction (Fig. 9.2.4A). Further, the experimental ED$_{50}$ dose was significantly different from ($p < 0.05$) the calculated additive ED$_{50}$ dose thereby demonstrating a synergistic interaction. This was further evidenced by interaction index where $\gamma$ for this combination was far below 1 indicating supra-additive interaction only (Table 9.2.1).

Valdecoxib and tramadol were combined in the ratio of 1:1 of the ED$_{50}$ of tramadol to evaluate possible interaction between these agents on formalin-induced nociceptive behavior. The interaction between these agents was characterized using modified method as described by Porreca et al. (1990) as valdecoxib alone had no
analgesic effect in the formalin test. The analgesic effect (%MPE) of tramadol in the mixture (1:1) was same as that was observed with the tramadol alone in dose-response studies (Fig. 9.2.3). Isobolographic analysis for this combination revealed that valdecoxib did not contribute to antinociceptive effect in formalin-induced nociceptive responses instead it increased the bulk of the mixture (Fig. 9.2.4B). Further, there was no significant difference between the amounts of tramadol in experimental ED<sub>50</sub> value was compared with that of the theoretical ED<sub>50</sub> value (Table 9.2.1). In these interaction studies, each agent except tramadol alone did not alter nociceptive responses in the early phase of the formalin test (Fig. 9.2.5). Tramadol in combination with any of the NSAIDs markedly reduced nociceptive responses in the early phase, however, the sum of responses were not significantly different from that of tramadol alone indicating that antinociceptive effect in this phase was contributed by tramadol only (Fig. 9.2.5).

9.2.3.2.2. Carrageenan-induced mechanical hyperalgesia
Interaction studies between tramadol and NSAIDs on carrageenan-induced hyperalgesic response showed dose-dependent antihyperalgesic activity when evaluated 2 h after drug mixture administration (Fig. 9.2.8A and 8B). The corresponding ED<sub>50</sub> with its 95% confidence intervals for such combinations in this assay were calculated 2 h after drug mixture administration (Table 9.2.1). Isobolographic analysis, using fixed ratio (1:1) ED<sub>50</sub> fractions revealed a significant supra-additive or synergistic interaction between naproxen or valdecoxib and tramadol after oral administration in this nociceptive assay (Fig. 9.2.9A and 9B). The points for experimental ED<sub>50</sub> for these combinations were obtained markedly below the theoretical additive line indicating possible synergistic interaction. Further, the experimental ED<sub>50</sub> doses were significantly less (p < 0.05) than the calculated additive ED<sub>50</sub> doses thereby demonstrating a synergistic interaction. The interaction index (γ) for these combinations was far below 1 indicating supra-additive interaction only (Table 9.2.1).

The calculated theoretical ED<sub>50</sub> with 95% CI and the corresponding experimental ED<sub>50</sub> with 95% CI obtained for each agent and their combinations and interaction index for such combinations are given in Table 9.2.1.

9.2.3.2.3. Measurement of gastrointestinal side effects
The administration of tramadol (30 mg/kg, p.o.) did not produce ulcers or erosions. Its adverse effects were similar to those of vehicle. Indomethacin (20 mg/kg, p.o.) produced severe gastric lesions with prominent petechiae and frank hemorrhagic ulcers. However, naproxen (30 mg/kg, p.o.) generated a less number of petechiae and erosions as compared to indomethacin, which was considered to be the most disadvantageous in terms of the number and severity of the lesions caused in the stomach (i.e., ulcers or erosions) (100%). In contrast, valdecoxib (10 mg/kg, p.o.) did not produce any gastric erosions and ulcers. The combination of naproxen with tramadol generated less number of erosions and ulcers as compared to indomethacin. However, there is no significant difference in ulcer severity in the combination of naproxen with tramadol as compared to naproxen alone. The combination of valdecoxib with tramadol did not produce any gastric erosion and the effect was similar to those of vehicle or each drug alone (Fig. 9.2.10).

**Fig. 9.2.1.** Effect of intraperitoneally administered naproxen 100 mg/kg (Nap 100), valdecoxib 50 mg/kg (Val 50) and tramadol 200 mg/kg (Gab 200) on nociceptive behavior during the early phase (0 - 10 min) and the late phase (10 – 60 min) of the formalin test in mice. The data represent the mean ± S.E.M. of sum of formalin-induced licking and biting responses in seconds during the early and late phase. *P < 0.05 as compared to control animals (one way ANOVA followed by Dunnett’s test).
Fig. 9.2.2. Dose-response curves for intraperitoneal administration of naproxen (Nap; 5 – 100 mg/kg, i.p.), valdecoxib (Val; 1 – 50 mg/kg, i.p.), tramadol (Tra; 1 – 50 mg/kg, i.p.) during the late phase (10 – 60 min) of the formalin test in mice. Drugs were intraperitoneally administered 30 min before nociceptive assay. Each dose point on graph represents mean %MPE ± SEM.

Fig. 9.2.3. Dose-response curves for intraperitoneal administration of combination of naproxen and tramadol (Nap+Tra; 7.54 – 60.29 mg/kg, i.p.) or valdecoxib and tramadol (Val+Tra; 2.36 – 18.90 mg/kg, i.p.) during the late phase (10 – 60 min) of the formalin test in mice. Drugs in combination were intraperitoneally administered 30 min before nociceptive assay. Each dose point on graph represents mean %MPE ± SEM.
Fig. 9.2.4. Isobologram for interaction between intraperitoneally administered (A) naproxen and tramadol (1:1 of their ED$_{50}$ fractions) and (B) valdecoxib and tramadol (1:1 of ED$_{50}$ of tramadol) during the late phase (10 – 60 min) of the formalin test. The straight solid line is the theoretical additive line and the open point corresponds to theoretical ED$_{50}$ ± SEM and the filled point corresponds to experimental ED$_{50}$ ± SEM of the mixture.
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Fig. 9.2.5. Effect of intraperitoneal administration of naproxen (Nap; ED$_{50}$ = 50.84 mg/kg), tramadol (Tra; ED$_{50}$ = 9.45 mg/kg), their combination (Nap + Tra; 60.29 mg/kg), valdecoxib (Val; 9.45 mg/kg) and co-administration of 1:1 mixture of valdecoxib and tramadol (Val+Tra; 18.9 mg/kg) on formalin-induced early phase nociceptive responses. The data represent the mean ± S.E.M. * P < 0.05 as compared to vehicle-treated animals (one way ANOVA followed by Dunnett’s test).

Fig. 9.2.6. Dose-dependent antinociceptive effect of orally administered naproxen (1 – 30 mg/kg) against carrageenan-induced hyperalgesia in rats. The drug was administered was 2 h after subplantar injection of carrageenan (100 µg per paw). Values are mean ± S.E.M.
Fig. 9.2.7. Dose-dependent antinociceptive effect of orally administered (A) valdecoxib (0.3 – 10 mg/kg) and (B) tramadol (1 – 30 mg/kg) against carrageenan-induced hyperalgesia in rats. The drug was administered 2 h after subplantar injection of carrageenan (100 μg per paw). Values are mean ± S.E.M.
Fig. 9.2.8. Dose-dependent reversal of orally administered (A) naproxen (Nap; 1 – 30 mg/kg), tramadol (Tra; 1 – 30 mg/kg), and their combination (Nap+Tra; 1.7 – 13.57 mg/kg), (B) valdecoxib (0.3 – 10 mg/kg), tramadol (1 – 30 mg/kg), and their combination (Val+Tra; 1.14 – 9.08 mg/kg) against carrageenan-induced hyperalgesia in rats. The percent reversal of hyperalgesia was calculated 4 h after carrageenan administration by taking the values in the control group as 0% reversal. Values are mean ± S.E.M. *P < 0.05 vs vehicle control.
Fig. 9.2.9. Isobologram for interaction between orally administered (A) naproxen and tramadol (1:1 of their ED$_{50}$ fractions) and (B) valdecoxib and tramadol (1:1 of their ED$_{50}$ fractions) in the carrageenan-induced hyperalgesia in rats. The straight solid line is the theoretical additive line and the open point corresponds to theoretical ED$_{50}$ ± SEM and the filled point corresponds to experimental ED$_{50}$ ± SEM of the mixture.
The present study systematically examined the emerging aspect of opioid and non-opioid analgesia and the application of drug combinations for balanced and beneficial analgesia. Systemic administration of naproxen or tramadol alone resulted in the dose-dependent antinociception in the late phase of the formalin test. However, rofecoxib, a selective COX-2 inhibitor, lacked analgesic efficacy in this test. The co-administration of naproxen, a nonselective COX inhibitor, but not valdecoxib, a selective COX-2 inhibitor with tramadol showed dose-dependent antinociceptive activity in the late phase of the formalin test that produced a synergistic interaction up on isobolographic analysis. In contrast, there was no additive or potentiation interaction between valdecoxib and tramadol suggesting that the PGs derived from COX-1 but not by COX-2 play role in this interaction.
Table 9.2.1. Theoretical and experimental ED$_{50}$ values with 95% confidence intervals (CI) and interaction index for the antinociceptive effect of naproxen, valdecoxib, tramadol alone and their combination with tramadol.

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Formalin-induced nociception</th>
<th>Carrageenan-induced hyperalgesia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Theoretical (mg/kg; 95% CI)</td>
<td>Theoretical (mg/kg; 95% CI)</td>
</tr>
<tr>
<td></td>
<td>Experimental (mg/kg; 95% CI)</td>
<td>Experimental (mg/kg; 95% CI)</td>
</tr>
<tr>
<td></td>
<td>Interaction index (γ)</td>
<td>Interaction index (γ)</td>
</tr>
<tr>
<td>Naproxen</td>
<td>50.84 (42.54 – 60.76)</td>
<td>6.03 (4.3 – 8.46)</td>
</tr>
<tr>
<td>Valdecoxib</td>
<td>NA</td>
<td>1.54 (0.96 – 2.46)</td>
</tr>
<tr>
<td>Tramadol</td>
<td>9.45 (7.09 – 12.6)</td>
<td>7.54 (5.87 – 9.75)</td>
</tr>
<tr>
<td>Naproxen +</td>
<td>30.15 (25.76 – 35.28)</td>
<td>6.79 (5.47 – 8.41)</td>
</tr>
<tr>
<td>Tramadol #</td>
<td>10.04 (6.98 – 14.45)</td>
<td></td>
</tr>
<tr>
<td>Valdecoxib +</td>
<td>18.44 (16.91 – 20.10)</td>
<td>4.37 (3.27 – 5.8)</td>
</tr>
<tr>
<td>Tramadol</td>
<td>20.08 (13.96 – 28.9)</td>
<td>2.29 (1.34 – 3.71)</td>
</tr>
<tr>
<td>Valdecoxib +</td>
<td>(3.33 – 6.18)</td>
<td>0.50*</td>
</tr>
<tr>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

The drugs were administered intraperitoneally and orally for antinociceptive interaction in the formalin test and carrageenan-induced hyperalgesia, respectively.  # Proportion of effective analgesic agent in experimental ED$_{50}$ value of the mixture; NA: not achieved; * γ values are far below 1 indicating supra-additive interaction.
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On the other hand, oral administration of COX inhibitors or tramadol alone resulted in the dose-dependent antihyperalgesic effect. Oral co-administration of naproxen or valdecoxib with tramadol showed dose-dependent synergistic antinociceptive activity that was confirmed by isobolographic analysis and interaction index. In addition, the combination of COX inhibitors with tramadol is further benefited by the absence of severe gastrointestinal side effects. Therefore, the beneficial, but differential interaction between NSAIDs and tramadol in these two inflammatory pain tests suggesting that the PGs derived from COX-1 and/or COX-2 invariably participate along with opioidergic mechanisms and play an important role in this interaction.

Role of inhibition of COX isoforms in synergistic interaction

The current study demonstrates that systemically administered naproxen, but not valdecoxib produced dose-dependent antinociception in the formalin test. It is well known that the formalin test reproduces various aspects of acute inflammatory pain analogous to human postoperative pain. It is well known that formalin induces two phases of nociceptive responses with the first or acute phase lasts for about 5 min which is followed by a longer-lasting, more persistent phase (about 40 min) that is characterized by shaking or licking and biting behavior of the paw (Murray et al., 1988). More importantly, valdecoxib is less polar and readily crosses the blood-brain barrier (Merck and Co., issue 2004) and its central effects have been observed after systemic administration (Schwartz et al., 1999). So the lack of efficacy of this drug is not due to sufficient drug concentration at the vicinity of spinal cord, but it may be due to absence of adequate levels of COX-2 expression to release PGs or ineffective participation of COX-2. Indeed, the antinociceptive effect of selective COX-1 and nonselective COX inhibitors has been demonstrated in acute inflammatory pain models. In contrast, selective COX-2 inhibition failed to attenuate nociceptive responses in the formalin test (Ochi et al., 2000; Dirig et al., 1997; Jain et al., 2001). The present data suggest that inhibition of COX-1 rather than COX-2 may be involved in analgesic interaction and therefore, produced beneficial synergistic effect.

It is well known that intraplantar administration of carrageenan causes inflammation and tissue injury, which evokes persistent afferent traffic that initiates a spinal sensitization. Selective COX-2 inhibition showed marked reversal of hyperalgesia and produced antihyperalgesic activity more predominantly than that observed with selective COX-1 and nonselective COX inhibitors in carrageenan-
induced hyperalgesia (Zhang et al., 1997; Dirig et al., 1998; Yaksh et al., 2001). Accumulating data indicates for the presence of both the COX mRNA and protein constitutively and COX-2 mRNA is up-regulated 3 - 6 h following peripheral inflammation (Beiche et al., 1996; Hay et al., 1997; Itchitani et al., 1997). Therefore, it is possible that formalin-induced licking and biting for 60 min would not be sufficient to activate COX-2 mRNA and the generation of COX-2 and PGs. It is likely that COX-2 may not be associated with spinal prostanoid synthesis acutely or with facilitated nociception, which occurs within limited time frame of acute analgesic tests. These data along with the present observations implicate that inhibition of COX-1 in the formalin test and inhibition of both isozymes of COX in the carrageenan-induced hyperalgesia is required for alleviating hyperalgesia and their combination with tramadol could result in beneficial interaction by reducing prostaglandin-induced sensitization in the primary afferent neurons and at the spinal cord, although the participation of additional mechanisms cannot be ruled out.

**Role of tramadol in synergistic interaction**

It is well reported that peripheral opioid receptors play an important role in the spinal nociceptive processing. Opioid receptors are present at the primary afferent neurons and local administration of μ- and δ agonists suppress spontaneous activity following peripheral inflammation (Levine and Taiwo, 1989; Hong and Abbott, 1995). The possible opioidergic contribution to the synergism is discussed in the previous part of this chapter. Despite, a direct antinociceptive action of tramadol by other mechanism cannot be ruled out. In addition to opioidergic mechanism, it has been demonstrated that a marked antinociceptive synergy exists between the two racemic forms of tramadol through adrenergic and serotonergic modulation (Raffa et al., 1993). Thus, both opioid and non-opioid components of tramadol contribute to the synergistic interaction with naproxen and/or valdecoxib. Growing body of evidence indicates that μ-opioid receptor agonists have antinociceptive activity in the inflammatory pain tests after peripheral, spinal and systemic administration (Antonijevic et al., 1995; Granados-Soto et al., 1997; Shannon and Lutz, 2002; Malmberg and Yaksh, 1993; Fletcher et al., 1997; Deciga-Campos et al., 2003). It is presently known that the effect of opioids involves several mechanisms. It has been described that μ-opioid receptor agonists act to inhibit activation of adenylyl cyclase (Ingram and Williams, 1996) and tetrodotoxin-resistant Na+ channels on peripheral
afferent neurons produced by inflammatory mediators such as PGE2 and serotonin (Gold et al., 1996a, b). Accumulating data indicates that opioids also inhibit release of SP and CGRP from primary afferent neurons (Yaksh, 1988), and open ATP-sensitive K+ channels via Gi proteins resulting in hyperpolarization, reduction in firing of the primary afferent neuron and antinociception (Ocana et al., 1990; Lohmann and Welch, 1999; Rodrigues and Duarte, 2000). All or some of these mechanisms could be involved in the antinociceptive effect of tramadol at the peripheral, spinal or supraspinal level.

In order to find the advantages of combining NSAIDs with tramadol, the present study also investigated the presence of gastric injuries as a reflection of unwanted gastric side effects. The doses of naproxen (30 mg/kg), valdecoxib (10 mg/kg, p.o.), tramadol (30 mg/kg), and their combination at fixed doses were selected for the gastric effect of the drugs because these doses of each drug produced the maximum antinociceptive effect in carrageenan-induced hyperalgesia. The results showed that the adverse effects could be reduced; that is, the incidence of gastrointestinal adverse events (erosions) was absent with the combination of valdecoxib and tramadol than with or indomethacin alone, whereas the combination of naproxen and tramadol was also able to generate ulcers (low percent); these adverse effects were similar to that produced by naproxen alone. This is important because the combination did not produce a higher incidence of side effects than that produced by each drug alone; instead, the results of synergistic antinociceptive interaction reflect a potentiation type of interaction.

The present results suggest a pharmacodynamic interaction appears more plausible. The mechanism of the observed synergism could be due to the different sites of action of NSAIDs and tramadol as well as to the multiple mechanisms of antinociceptive action of both drugs. Previous studies have shown that the combination of μ-opioid like morphine with some NSAIDs can activate the serotonergic (Sandrini et al., 1998) and the opioid (Maves et al., 1994) systems. Accumulating data indicates that COX-2 and opioid receptors in both the spinal cord and periphery expressed constitutively and COX-2mRNA is up-regulated following peripheral inflammation (Beiche et al., 1996; Hay et al., 1997; Ichtitani et al., 1997). It has also been reported that peripheral inflammation causes a significant increase in μ opioid receptors in spinal cord (Goff et al., 1998; Mousa et al., 2002). The
participation of the nitric oxide-cGMP pathway and other mechanisms such as activation of opioid and prostanoid receptors cannot be excluded. Recent report indicates that COX-2 expression also increased in non-neuronal cells particularly parenchymal microglia and perivascular microglia/macrophages in the CNS (Ibuki et al., 2003). Thus, it is also possible that the nature of the interaction between tramadol and NSAIDs might occur at several levels both of neuronal and non-neuronal origin. Conceptually, by using a fixed-ratio strategy, isobolographic analysis demonstrated a significant synergistic interaction between tramadol and NSAIDs. These results confirm previous experiments showing that co-administration of opioids and NSAIDs to rats produce an increased peripheral (Aguirre-Banuelos and Granados-Soto, 1999), spinal (Malmberg and Yaksh, 1993) and systemic (Fletcher et al., 1997) antinociceptive effect compared with individual drugs.

It has been proposed that opioids produce analgesia within the midbrain periaqueductal grey by inhibiting GABAergic system on neurones, which form part of a descending antinociceptive pathway and microinjections of cyclooxygenase inhibitors into the periaqueductal grey produce analgesia (Tortorici and Vanegas, 1995). Previous studies have shown that the co-administration of opioids and NSAIDs produces increased antinociception or a reduction in the requirements of opioid agents (Kehlet and Dahl, 1993; Tallarida et al., 1999; Silvanto et al., 2002). This is supported by previous electrophysiological observations showing that opioids produce antinociception by suppressing the inhibitory influence of GABA on neurons constituting a descending antinociceptive pathway, and such effect is potentiated by COX-1 inhibitors (Vaughan et al., 1997; Vaughan, 1998). It has been proposed that this mechanism accounts for the antinociceptive activity of COX inhibitors in the periaqueductal gray, as well as for NSAID synergism with opioids (Vaughan et al., 1997; Christie et al., 1999). In addition to possible pharmacodynamic mechanism of the combination antinociception, NSAIDs may alter the pharmacokinetic properties of the tramadol and vice versa.

In summary, this study quantified the antinociceptive synergy between NSAIDs and tramadol in the acute inflammatory pain in the rat. The data demonstrates that co-administration of NSAIDs with tramadol results in synergistic antinociception during inflammatory pain and further the dose of each agent in the combination was significantly lower when the two agents were administered together. In addition, the data also demonstrates that the synergistic antinociceptive effects
were not accompanied by increased gastric side effects thus the combination of the NSAIDs used in the present study with tramadol may provide better analgesia and decrease or avoid adverse drug effects by reducing the analgesic dose requirement.