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Synergistic interaction between meloxicam and aminoguanidine in formalin-induced nociception in mice

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Abstract

Objectives: The objective of this study was to examine the nature of interaction between cyclooxygenase-2 inhibitor meloxicam and inducible nitric oxide synthase inhibitor aminoguanidine in formalin-induced nociception in mice and the possible therapeutic advantage.
Methods: Antinociceptive effect of meloxicam (1, 3, 10 and 30 mg/kg, oral) and aminoguanidine (10, 30, 100 and 300 mg/kg, oral) and their combinations was examined in formalin-induced paw licking model in mice. Analysis of variance and isobolographic method were employed to identify the nature of antinociceptive interaction.
Results: Higher doses of meloxicam (10 and 30 mg/kg) and aminoguanidine (100 and 300 mg/kg) produced significant reduction in paw licking time (antinociceptive) in late phase of formalin-induced nociception. Combination of sub-threshold dose of meloxicam (3 mg/kg) with increasing doses of aminoguanidine (10, 30, 100 and 300 mg/kg) resulted in synergistic antinociceptive effect. Similarly, co-administration of sub-threshold dose of aminoguanidine (30 mg/kg) with increasing doses of meloxicam (1, 3, 10 and 30 mg/kg) produced significant reduction in formalin-induced paw licking behaviour. The experimental ED_{50} for combination with their confidence limits are below the confidence interval of theoretical line of additive interaction, suggesting synergistic nature of interaction between meloxicam and aminoguanidine in isobolographic analysis.
Conclusion: Co-administration of meloxicam and aminoguanidine showed synergistic antinociceptive effect which might possibly reduce gastrointestinal toxicity associated with the use of meloxicam.

Keywords: Meloxicam; Aminoguanidine; Interaction; Nociception; iNOS; COX-2

1. Introduction

Among the most widely prescribed drugs worldwide, nonsteroidal anti-inflammatory drugs (NSAIDs) are effective for relieving pain and are associated with high incidence of gastro-intestinal (GI) adverse events. Both beneficial and harmful effects of NSAIDs result from inhibition of cyclooxygenase (COX) enzyme. Recognition of two distinct COX isoforms prompted the development of drugs that selectively block the activity of COX-2, thus providing pain relief and reducing inflammation while sparing COX-1, the enzyme apparently responsible for most protective prostaglandin synthesis in mucosa of stomach and duodenum. The results of preclinical and clinical studies indicate that COX-2 inhibitors exhibit high selectivity in inhibiting COX-2,

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provide excellent pain relief and cause significantly less gastrointestinal toxicity than do conventional NSAIDs. Although, they represent a significant advancement over nonselective NSAIDs, selective COX-2 inhibitors are not without limitations. They do not completely eliminate GI toxicity or renal side effect associated with the use of conventional NSAIDs.

Meloxicam is a enlocarboxamide and has been recognized as selective COX-2 inhibitor since 1994 (Simmons et al., 2004). Meloxicam has 100-fold selectivity for COX-2 in microsomal preparations of human recombinant enzymes (Pairet et al., 1998) and 10-fold selectivity in human whole blood (Warner et al., 1999). Meloxicam, developed as selective COX-2 inhibitor, to minimize the risk of GI toxicity, has been found to demonstrate ulcerogenic properties in rats (Gambero et al., 2005). Various reports also suggested varying degree of GI toxicity including perforation/bleeding with meloxicam in clinical cases (Layton et al., 2003; MacDonald et al., 2003; Laporte et al., 2004; Richy et al., 2004). Hepatotoxicity is rare complication of most NSAIDs, the high level of uses of NSAIDs means that these drugs cause liver disease. Though, there is no direct report of hepatotoxicity of meloxicam in animals, isolated information is available about its hepatotoxicity in human patients (Staerkel and Horsmans, 1999).

It has been demonstrated that carrageenan-induced hyperalgesia is associated with increased COX-2 expression in spinal cord and this can be inhibited by Dup-697, a selective COX-2 inhibitor (Hay and de-Belloroche, 1997). Anti-PGE_2 antibody and selective COX-2 inhibitor SC-58635 have effectively controlled oedema and hyperalgesia induced by carrageenan injection into foot pads of rats (Zhang et al., 1997). The role of COX-2 in formalin-induced acute inflammatory pain has been demonstrated by various workers (Yamamoto and Nozaki-Taguchi, 2002; Choi et al., 2003). COX-2 mRNA in spinal cord is increased rapidly from 0.5 h and reached maximum 2 h after formalin injection. However, COX-2 protein remained unaltered up to 4 h after 5% formalin injection (Tegeder et al., 2001).

To date, the exact role of inducible nitric oxide synthase (iNOS) in inflammatory pain remains controversial. However, majority of the studies demonstrate a role of iNOS in pain condition with inflammatory component and the potential value of iNOS inhibitor in such conditions (LaBuda et al., 2006). Preferential iNOS inhibitor aminoguanidine (Alderton et al., 2001) has also been found to suppress nociceptive behaviour during late phase of formalin-induced nociception (Doursout et al., 2003; Dudhgaonkar et al., 2004). Alterations in the expressions of iNOS was studied after hind paw injection of formalin and it was found that in hind paw, iNOS mRNA is increased from 4 h and maximum significant effect was observed at 48 h. However, iNOS protein in spinal cord remain unaltered up to 2 h after formalin injection and significantly reached at 48 h (Shi et al., 2005). Since the expression and activity of both iNOS and COX-2 is associated with inflammatory pain, it has been proposed that inhibition of both iNOS and COX-2 would provide most potent antinociceptive effect. Therefore, it is thought worthwhile to study the interaction of aminoguanidine with meloxicam by isobolographic study which will be useful strategy to reduce the dose of meloxicam with enhanced antinociceptive effect.

2. Materials and methods

2.1. Experimental animals

Adult male albino mice (20–25 g) were divided into different groups of 6 animals each. Animals were housed in a temperature controlled room with a standard light–dark cycle. Food and water were provided *ad libitum* before the start of the experiment which were withdrawn during experimentation. Experimental procedures were approved by the Institute Animals Ethics Committee and guidelines on the ethical standards for investigation of experimental pain in animals were followed (Zimmermann, 1983) and animals were euthanized by higher dose of thiopental.

2.2. Drugs and their administration

Meloxicam, a selective COX-2 inhibitor gifted by Intas Laboratories, Ahmedabad, India and aminoguanidine (as hydrochloride), a preferential iNOS inhibitor procured from Sigma, USA were used in the present study. Both the drugs were administered orally in the form of suspension in 1% polysorbate-80.

2.3. Test of antinociception

The effect of drugs was observed on pain produced in hind paw of mice with 20 μL of 2.5% formalin solution in glass distilled water, according to the method of Correa and Calixto (1993). One hour before the induction of pain, meloxicam (1, 3, 10 and 30 mg/kg) or aminoguanidine (10, 30, 100 and 300 mg/kg) was administered orally to mice. The effect of drugs at different doses was compared with that of vehicle-treated control. Interaction study was conducted by co-administering meloxicam (3 mg/kg) with different doses of aminogunidine (10, 30, 100 and 300 mg/kg). In a similar way, fixed doses of aminoguanidine (30 mg/kg) was co-administered with different doses of meloxicam (1, 3, 10 and 30 mg/kg). Mice were placed in a glass bell jar to record the time spent in licking the injected paw in late (15–30 min) phase after formalin injection. The results obtained from interaction studies were compared with

vehicle-treated control and individual drug-treated control groups.

2.4. Data analysis

The data obtained were converted to % maximum possible effect (MPE) by the following equation

$$\%\text{MPE} = 100 - \left(\frac{100 \times \text{Time spent in licking the formalin-injected paw by drug-treated mice}}{\text{Time spent in licking the formalin-injected paw by vehicle-treated control mice}}\right)$$

The log doses were plotted vs. % MPE, and regression of log dose response curve was used to calculate ED_{50} and its 95% confidence limit (CL). The interaction between meloxicam and aminoguanidine was examined by analysis of variance in dose–response relationship and the nature of interaction was further confirmed by isobologram (Gessner, 1988; Gennings et al., 1990; Nelson and Kursar, 1999).

In the isobolographic analysis, the ED_{50} of meloxicam was plotted on the ordinate and ED_{50} of aminoguanidine on the abscissa. A theoretical line of additive interaction was drawn by connecting the ED_{50} of meloxicam with that of aminoguanidine. The experimental ED_{50} of combination (meloxicam + aminoguanidine: point 'A' in Fig. 3) was then plotted on isobolograph. If SEM surrounding the combination data point (Point 'A' in Fig. 3) did not overlap the confidence interval of theoretical line of additive interaction, then statistically significant synergism or antagonism may be concluded; when combination data points come below or above the theoretical line of additive interaction, respectively. The intensity of synergistic or antagonistic interaction was represented by the potency ratio (Gessner, 1988; Nelson and Kursar, 1999). The potency ratio is the distance from origin to the observed or experimental ED_{50} for the combination (point 'A' in Fig. 3) divided by the distance from origin to the expected ED_{50} coordinates of the theoretical line for the additive interaction (point 'B' in Fig. 3). The point 'B' is the expected ED_{50} of the combination. The ratio of observed to expected distance represents the potency ratio. A potency ratio less than one indicates synergism and a ratio of more than one is indicative of antagonism.

3. Results

3.1. Antinociceptive effect of meloxicam and aminoguanidine alone and their combination

Meloxicam and aminoguanidine produced a statistically significant reduction in paw licking time (antinociception) in late phase of formalin-induced nociception (Fig. 1a and b). At higher doses (10 and 30 mg/kg), meloxicam produced statistically significant antinociceptive effect whereas, lower doses (1 and 3 mg/kg) did not produce a significant reduction in late phase of formalin-induced nociception, however, higher doses of aminoguanidine (100 and 300 mg/kg) showed significant decrease in paw licking time (Fig. 1).

On the basis of dose–response relationship of meloxicam and aminoguanidine administered alone, the sub-threshold dose of meloxicam (3 mg/kg) was combined with increasing doses of aminoguanidine (10–300 mg/kg), whereas sub-threshold dose of aminoguanidine (30 mg/kg) was combined with increasing doses of meloxicam (1–30 mg/kg). A dose is considered sub-threshold which did not produce significant (just lower than producing significant effect) reduction in paw licking behaviour in formalin-induced pain in

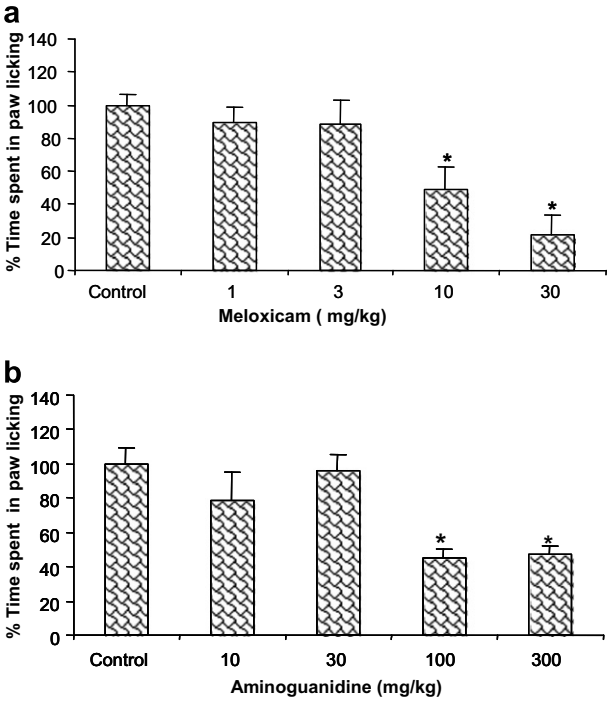

Fig. 1. Antinociceptive effect of meloxicam (a) and aminoguanidine hydrochloride (b) in formalin-induced paw licking test in mice. Meloxicam (a) and aminoguanidine hydrochloride (b) were administered orally 1 h prior to subplanter injection of formalin. Control animals were injected with an appropriate volume of vehicle. Results show mean ± SE. (vertical bars: % time in paw licking in late phase, 15-30 min). $^{*}p < 0.05$ when compared with that of control.

dose–response study. Co-administration of fixed dose of meloxicam (3 mg/kg) with different doses of aminoguanidine (30, 100 and 300 mg/kg) produced statistically significant reduction in formalin-induced late phase of paw licking (Fig. 2a). In a similar way, co-administration of fixed dose of aminoguanidine (30 mg/kg) with different doses of meloxicam (1, 3, 10 and 30 mg/kg) produced significant antinociceptive effect in the late phase of formalin-induced nociception (Fig. 2b).

3.2. Isobolographic analysis

The data for co-administration of meloxicam and aminoguanidine are presented as isobologram in Fig. 3. In Fig. 3, the doses of meloxicam are on the ordinate and those of aminoguanidine are on the abscissa of isobolograph, respectively. The ED_{50} values of meloxicam (9.33 ± 0.10 mg/kg) and aminoguanidine (306.19 ± 0.10 mg/kg) were plotted on ordinate and abscissa of isobolograph, respectively. These points were connected by solid theoretical line of additivity. The 95% CL for a theoretical additive interaction were 0.1 mg/kg for both meloxicam and aminoguanidine (not shown in Fig. because of low value). The ED_{50} (74.13 ± 0.03 mg/kg) for combination i.e. fixed dose of meloxicam (3 mg/kg) with different does of aminoguanidine (10, 30, 100 and 300 mg/kg) and ED_{50} (5.12 ± 0.07 mg/kg) for other combination i.e. fixed dose of aminoguanidine (30 mg/kg) with different doses of meloxicam (1, 3, 10 and 30 mg/kg) were then plotted on isobolograph (Fig. 3 point 'A'). Point 'A' is experimental or observed ED_{50}. Expected ED_{50} for combination (meloxicam + aminoguanidine; Point 'B' in Fig. 3) was plotted on isobolograph and the potency ratio (0.78) was calculated as described in Section 2 to know the intensity of interaction. The intensity of synergistic or antagonistic interaction was represented by potency ratio (Gessner, 1988; Nelson and Kursar, 1999). A potency ratio of less than 1 in this study reflects synergism.

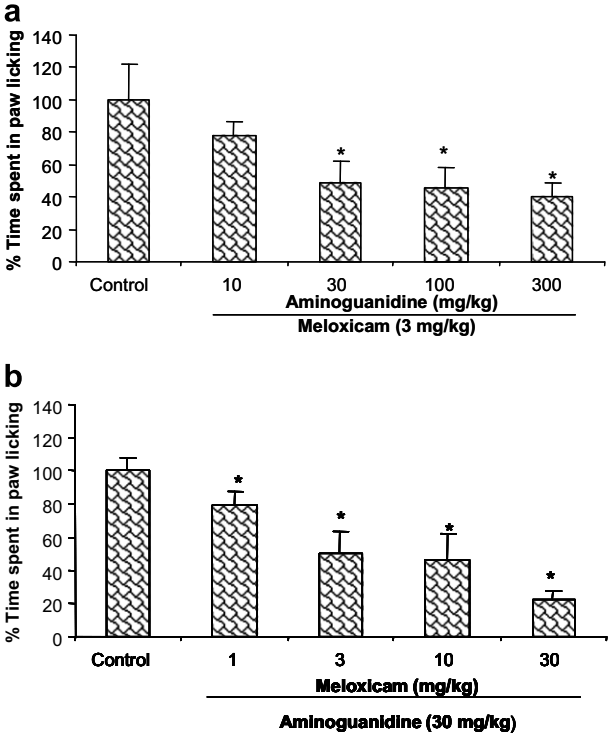

Fig. 2. Antinociceptive effect of meloxicam (3 mg/kg) combined with increasing doses of aminoguanidine hydrochloride (a) and aminoguanidine hydrochloride (30 mg/kg) combined with increasing doses of meloxicam (b) in formalin-induced paw licking test in mice. Drugs were administered orally 1 h prior to subplanter injection of formalin. Control animals were injected with an appropriate volume of vehicle. Results show mean ± SE. (vertical bars: % time in paw licking in late phase, 15-30 min). $^{*}p < 0.05$ when compared with that of control.

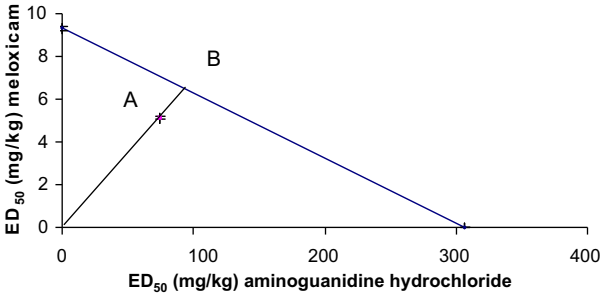

Fig. 3. Isobolographic analysis of an experiment indicating synergism between meloxicam and aminoguanidine hydrochloride. The diagonal line is the zero interaction isobole constructed from experiments with each compounds alone. Point 'A' below the zero interaction isobole is the concentration of combination produced 50% antinociceptive effect along with 95% confidence intervals. The ratio of line originating from origin to point 'A' and from origin to point 'B' is a measure of synergism. The 95% confidence intervals for additive points (ED_{50} values of meloxicam and aminoguanidine hydrochloride) could not be drawn because of low values.

4. Discussion

Meloxicam is a selective COX-2 inhibitor (Pairet et al., 1998; Warner et al., 1999; Simmons et al., 2004) and aminoguanidine is preferential iNOS inhibitor (Alderton et al., 2001). In the present study, meloxicam and aminoguanidine produced significant reduction in paw licking time in formalin-induced nociception, suggesting their antinociceptive action, as has been demonstrated in mice and rats in earlier studies (Santos et al., 1998; Pinardi et al., 2003; Miranda et al., 2004). Similar to meloxicam, aminoguanidine was also found to possess antinociceptive effect by us (Dudhgaonkar et al., 2004) and by other workers (Doursout et al., 2003). Thus, meloxicam and aminoguanidine might be useful in therapeutic management of pain.

Formalin-induced nociception is biphasic with an early neurogenic component followed by late tissue-mediated response (Wheeler-Aceto and Cowan, 1991).

The first phase of pain is attributed to direct activation of nociceptors and primary afferent C fibers by formalin, causing release of bradykinin and substance P (Shibata et al., 1989; Correa and Calixto, 1993). Second phase of nociception is believed to be due to inflammatory reaction caused by tissue injury leading to release of histamine, serotonin, prostaglandins and excitatory amino-acids (Coderre and Melzack, 1992; Damas and Liegeois, 1999). Thus, formalin test is a useful method for examining nociception and its modulation by pharmacological means. Previously, it has been demonstrated that nitric oxide is responsible for nociceptive behaviour in second phase of formalin-induced nociception in mice (Moore et al., 1993). The second phase is inhibited by both COX-2 inhibitor meloxicam and iNOS inhibitor aminoguanidine and the present results substantiate earlier observations (Doursout et al., 2003; Dudhgaonkar et al., 2004).

Acute tissue damage is often accompanied by development of hyperalgesia and allodynia (Andrew and Greenspan, 1999). Both peripheral mechanism at the site of injury and central processes particularly in the spinal cord contribute to this phenomenon. PGs (Yaksh and Malmberg, 1993) as well as NO (Lawand et al., 1997) are produced in response to tissue damage peripherally and centrally. PGs are thought to play an important role in nociceptive processing, both peripherally and centrally in spinal cord (Scheuren et al., 1997). COX-2 has been demonstrated to be major source of PGE_2 in many pain models, and COX-2 selective inhibitors are potent antihyperalgesic substances. In animal models of inflammatory pain, such as carrageenan-or zymosan-evoked thermal hyperalgesia, COX-2 inhibitors reduced markedly the nociceptive response (Zhang et al., 1997; Niederberger et al., 2003). It has been suggested that COX-2 is expressed in CNS including spinal cord and COX-2 plays an important role in spinal nociceptive transmission during formalin test (central response), but not during hot plate test (local response) in rats (Yamamoto and Nozaki-Taguchi, 2002). COX-2 mRNA is constitutively expressed in spinal cord of normal rat and may be involved with the processing of nociceptive stimuli by releasing PGE_2 (Beiche et al., 1996; Yaksh and Svensson, 2001). The antihyperalgesic action of NSAIDs is mediated by inhibition of constitutional COX-2 but not COX-1 (Yaksh and Svensson, 2001). Meloxicam, when given systemically, suppressed inflammatory pain response locally (Laird et al., 1997) without affecting the central pain transmission. In contrast to this study, meloxicam given peripherally reduces prolonged stimulation-evoked after discharges of dorsal horn neuron, suggesting COX-2 may be involved in mediating and/or modulating excitatory effect of nociceptive input to dorsal horn neurons (Pitcher and Henry, 2002). Thus, meloxicam produces its antinociceptive effect by inhibiting COX-2 peripherally and at spinal level. The central effect of meloxicam is further substantiated by its ability to cross the blood brain barrier (Mohn et al., 2001). Though there is isolated information about the absence of COX-2 labeling in dorsal root ganglion and also in neurons in adjuvant-induced model of monoarthritis in rats (Chopra et al., 2000), there are ample evidences for the significance of COX-2 expression in pain (Ballou et al., 2000; Yaksh et al., 2001; Yamamoto and Nozaki-Taguchi, 2002). However, other mechanism of meloxicam by opening of large-and-small conductance Ca^{2+}-activated K^{+}-channels can not be rule out (Ortiz et al., 2005).

PGs are generally accepted to play a dominant role in nociceptive sensitization (Bley et al., 1998), the role of NO is less clear. Some authors claim an antinociceptive action of NO (Goettl and Larson, 1996; Hamalainen and Lovick, 1997), while most favour a pronociceptive activity (Malmberg and Yaksh, 1993; Chen and Levine, 1999). NO is involved in the transmission and modulation of nociceptive information at periphery, spinal cord and supraspinal level (Moore et al., 1993). Studies on the peripheral role of NO pathway involved in the mechanism of hyperalgesia have been limited. Although NOS inhibitors apparently have little or no effect on nociceptive transmission under normal conditions (Meller and Gebhart, 1993), there are ample evidences that peripheral inflammation or CNS injury increases NOS activity that may underline abnormal pain related sensation (Meller et al., 1992; Semos and Headley, 1994). iNOS is generally considered not to be constitutively expressed in CNS (Meller et al., 1994; Barker et al., 1998) but significant amounts of iNOS mRNA were detected 30 and 60 min in spinal cord after zymosan injection (Guhring et al., 2000). However, iNOS mRNA were absent under control conditions and in iNOS−/− mice after zymosan injection. Further, iNOS expression following formalin injection in paw has been studied and 1-fold increase in the expression of protein was observed in paw at 4 h and in spinal cord at 2 h (Shi et al., 2005). Aminoguanidine, a preferential iNOS inhibitor, resulted in dose-dependent inhibition of persistent thermal pain but not mechanical hyperalgesia, produced by intraplanter zymosan (Meller et al., 1994) suggesting a role of iNOS expressed by glia in mechanism of hyperalgesia in spinal cord of rats. However, aminoguanidine has been demonstrated not to cross the blood brain barrier (Mahar Doan et al., 2000; Mohn et al., 2001) suggesting its peripheral antinociceptive action. Peripheral antinociceptive action is substantiated by decrease in paw oedema volume in response to formalin (Doursout et al., 2003) and carrageenan-induced hyperalgesia in rats (Allawi et al., 1994) suggesting its antinociceptive activity in formalin model is secondary to anti-inflammatory effect, inhibiting expression/activity of iNOS and/or oxyradicals and peroxynitrite scavenging action (Yildiz et al., 1998).

One of the objectives of the present study was to analyze the effect of combination of meloxicam and aminoguanidine on formalin-induced nociception and also to analyze the nature of interaction between both the drugs. Simultaneous inhibition of iNOS by aminoguanidine and COX-2 by meloxicam treatment in mice resulted in significantly greater antinociceptive effect in late phase (15–30 min) of formalin-induced paw licking response, than the administration of either drug alone. The degree of potentiation observed was too great to be accounted by simple additive effect, thereby suggesting a synergistic interaction between meloxicam and aminoguanidine. A similar synergistic antinociceptive effect has been reported in our earlier study between rofecoxib and aminoguanidine (Dudhgaonkar et al., 2004). The present study also suggests that the interaction can be exploited in other inflammatory pain conditions where iNOS and COX-2 are upregulated and for individuals sensitive to rofecoxib.

The analysis of drug interaction is a complex task and analysis that compares the effect of drug combinations to those predicted by an additive model, e.g. isobolographic analysis (Loewe, 1953), has been useful and allows precise definition of the terms such as additivity, antagonism and synergism. Variable dose-ratio method was employed for studying drug interaction between meloxicam and aminoguanidine, in which particular dose is held constant and varying amount of second drug is given in combination to yield ED_{50} values and confidence intervals. Isobologram using variable dose-ratio experiments have been employed by various workers for drug interaction studies (Foltin et al., 1983; Carter et al., 1998). In the present study, isobolographic analysis suggests that the interaction represents a highly significant synergism and fulfills all the three criterion required for synergism (Gessner, 1988; Nelson and Kursar, 1999).

The mechanism of interaction between aminoguanidine and meloxicam can not be established from the present data. It was previously reported that iNOS and COX-2 pathways interact closely and iNOS stimulates COX-2 activity, possibly via reaction with heme component which binds to the active site of COX-2 enzyme (Salvemini et al., 1993; Kim et al., 2005). Inhibition of iNOS by aminoguanidine inhibits the NO formation, which in turn fails to stimulate the COX-2 activity and decreases both NO and PG formation, thus, resulting in potentiated/synergistic effect. This is in addition to direct inhibition of COX-2 by meloxicam and iNOS by aminoguanidine. It has been stated earlier that meloxicam produces its antinociceptive effect by peripheral (Laird et al., 1997) and central (Pitcher and Henry, 2002) mechanism by inhibiting COX-2. Further meloxicam is able to cross the blood brain barrier (Mohn et al., 2001) but aminoguanidine has been found to possess only peripheral antinociceptive effect by inhibiting iNOS (Doursout et al., 2003) at the site of injury, besides antioxidant and peroxynitrite scavenging effect (Yildiz et al., 1998). Therefore, differences in the mechanism of action of meloxicam and aminoguanidine in nociceptive pathway may account for their synergistic antinociceptive effect when given in combination.

Though, there is limited information about GI toxicity of meloxicam in experimental animals (Villegas et al., 2002; Gambero et al., 2005), it has been shown to possess varying degree of GI toxicity in clinical cases (Layton et al., 2003; MacDonald et al., 2003; Laporte et al., 2004; Richy et al., 2004).There is no direct evidence from the present study that aminoguanidine has reduced GI toxicity of meloxicam, but aminoguanidine has been found to be gastroprotective in adjuvant-induced arthritic rats exposed to indomethacin (Kato et al., 1999) and acetic acid-induced ulcers (Akiba et al., 1998). In conclusion, the use of aminoguanidine will help in reducing the dose of meloxicam, showing synergistic antinociceptive effect and might reduce GI toxicity.

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