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Differential effect of zileuton, a 5-lipoxygenase inhibitor, against nociceptive paradigms in mice and rats

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Abstract

Pain is commonly associated with inflammation. Several mediators including prostaglandins have been implicated in pain and inflammation. However, the recent reports indicated the role of leukotrienes as signaling molecules in pain. The present study was aimed to evaluate the effect of 5-LOX inhibitor, zileuton in nociceptive paradigms including inflammatory pain. Acetic acid-induced writhing, tail flick and hot plate tests to assess pain response were used. The effect on carrageenan-induced mechanical hyperalgesia, and acetic acid-induced vascular permeability was also determined. Zileuton (ED_{50} = 31.81 mg/kg p.o.), zafirlukast (ED_{50} = 6.19 mg/kg p.o.), montelukast (ED_{50} = 7.17 mg/kg p.o.) inhibited acetic acid-induced writhing in mice. Further, zileuton and ZK 158252, leukotriene B_4 receptor antagonist did not alter basal response against tail flick and hot plate assays. Acetic acid-induced vascular permeability was significantly inhibited by zileuton. Oral administration of zileuton showed efficacy against carrageenan-induced mechanical hyperalgesia and also reversed histological changes in paw biopsies. These data suggest that zileuton, a 5-LOX inhibitor, exhibited antinociceptive effect in paradigms of inflammatory pain.

Keywords: 5-LOX: 5-Lipoxygenase; LTs: leukotrienes; Nociception; Inflammatory pain

1. Introduction

Tissue injury results in inflammatory pain. Various inflammatory mediators are involved in initiating and sustaining pain/inflammatory cascade. The role of prostaglandins (PGs) in mediating nociception and inflammation is well understood and, thus, the use of non-steroidal anti-inflammatory drugs (NSAIDs) as analgesics and anti-inflammatory agents (Vane and Blotting, 1995). PGs are generated during metabolism of arachidonic acid (AA) by cyclooxygenase (COX) pathway. However, AA is also metabolized via lipooxygenase (LOX) pathway, leading to the generation of leukotrienes (LTs) (Funk, 2001). Of all the LOX isoforms, 5-lipoxygenase (5-LOX) catalyzes the initial steps in leukotriene biosynthesis (Carter et al., 1991). These leukotrienes can elicit most of the symptoms associated with inflammatory events including pain. Leukotriene B_4 (LTB_4) has been detected in samples of patients complaining hyperalgesia during masseter muscle and radicular pain (Kawakami et al., 2001). Hyperalgesia occurs following LTB_4 challenge through different routes (Levine et al., 1984). Furthermore, LTB_4 decreases the mechanical and thermal threshold of nociceptors (Kawakami et al., 2001). Jain et al. (2001a) reported the role of cysteinyl leukotrienes (Cyst LTs) in animal models of nociception. Recently, Aley and Levine (2003) reported the role of lipoxygenase metabolites in prostaglandin and epinephrine-mediated mechanical hyperalgesia. Singh et al. (2004a) reported that PGs and LTs have complementary effects in the development and persistence of inflammatory pain.

The present study was aimed to evaluate the effect of zileuton, a 5-LOX inhibitor in tail flick assay, hot plate test and animal model of algogen (acetic acid)-induced visceral-somatic pain. The effect on carrageenan-induced mechanical hyperalgesia and vascular permeability was also determined.

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2. Materials and methods

2.1. Animals

Swiss mice, 22–25 g and Wistar rats, 180–200 g (inbred in Central Animal House, Panacea Biotec Ltd., Lalru) of either sex were used. They were housed in plastic cages at temperature of 25±0.5 °C and 12 h light (6:00 a.m. to 6:00 p.m.): 12 h dark (6:00 p.m. to 6:00 a.m.) cycle with humidity 50±5% RH. Animals were given food (pellet chow, Ashirwad, Chandigarh, India) and water ad libitum. Experiments were carried out between 0900 and 1500 h. The experimental protocols were approved by the Institutional Animal Ethics Committee.

2.2. Drugs and treatment regimen

Zileuton (Archechem, Mumbai, India), zafirlukast, montelukast sodium, thymol blue, rofecoxib (Panacea Biotec, Ltd., New Delhi, India), acetic acid (SD fine chemicals, India), capsaicin and carrageenan (Sigma, St. Louis, USA), and ZK158252 [L-ANT; a leukotriene BLT (B_4 leukotriene receptor) antagonist, generously gifted by Schering AG, Germany] were used.

Zileuton, zafirlukast, and rofecoxib were suspended in Tween 80, montelukast sodium dissolved in saline and administered per orally (10 ml/kg). Rofecoxib was used as a critical control for writhing assay. The compound L-ANT was dissolved in 99.5% ethanol and sonicated on ice for 5 min. The solution was treated with equimolar concentration of sodium hydroxide to convert it into sodium salt. The final concentration was made by dilution with 0.9% NaCl and administered intraperitoneally (i.p.) in a fixed dose of 1 μg per mouse or rat. All the drugs were administered 30 min before the nociceptive challenge. This dose selection was based on earlier studies (effect of ZK158252 on rat paw biopsy, unpublished data). Control groups received saline or respective vehicle.

2.3. Analgesiometric tests

2.3.1. Writhing test (acetic acid-induced writhing assay)

A 1% v/v acetic acid solution (10 ml/kg) was used to produce writhing in mice. The number of writhes [constriction of abdomen, turning of trunk (twist), and extension of hind limbs] due to acetic acid was expressed as painful response. The number of writhes per animal was counted during a 20-min session, beginning 3 min after the acetic acid injection (Singh et al., 2002).

2.3.2. Tail flick test in mice

The nociceptive response was assessed by tail withdrawal (flicking) response from the radiant heat source (Analgesiometer IMCORP, India) in the animal. The baseline tail withdrawal of mice was adjusted 3–4 s to allow measurement of any antinociception. A cut-off time of 10 s was observed (Singh et al., 2001).

2.3.3. Hot plate test in mice

In this test, animals were individually placed on a hot plate (Eddy's Hot Plate) with the temperature adjusted to 55°C. The latency to the first sign of paw licking or jump response to avoid thermal pain was taken as an index of pain threshold. A cut-off time of 15 s was observed (Singh et al., 2001).

Each animal was exposed to both the test procedures one after another, i.e., the animal was first subjected to tail-flick test and 10 min later to the hot plate test.

Number of writhes and tail flick or hot plate response (latency) are represented as mean±S.E.M.

The ED_{50} for the individual drug was calculated from log dose-probit analysis of quantal dose–response curves. For ED_{50} calculations, the number of writhes (a graded response) was converted to a maximum possible effect (%MPE), which is a quantal response. The probit value for %MPE by each dose was firstly obtained from the probit table. A plot between the probit value and log dose was created and a best-fit line was generated. By intrapolation, log dose producing 50% effect was obtained. Finally, the antilog of the log dose thus generated the ED_{50} value.

2.3.4. Carrageenan-induced inflammatory nociception (hyperalgesia) in rats

The inflammatory nociception (hyperalgesia) was produced by injecting freshly prepared carrageenan type-IV (1% w/v, 0.1 ml/paw) in the right hind paw of rats. The control group received 0.1 ml normal saline injected intraplantarly. The response to inflammatory nociception was determined by measuring paw withdrawal latency of carrageenan- or saline-injected paw when exposed to mechanical stimuli (Randall Selitto). Briefly, an increasing pressure of 0–1000 g was applied to paw of individual rat and squeaking/withdrawal and/or struggling response was taken as the nociceptive threshold. Nociceptive threshold was observed for both carrageenan- and saline-treated group for 4 h after carrageenan administration (Patil et al., 2003). Basal nociceptive threshold to mechanical stimuli was recorded before any treatment. The mechanical threshold was expressed in pressure (mean±S.E.M.) applied in grams.

2.4. Histological examination

For histological examinations, biopsies of paws were taken 4 h following intraplantar injection of carrageenan, tissue from the footpads of rat hind paw being removed with scalpel. The tissue slices were fixed in Dietric solution (14.25% ethanol, 1.85% formaldehyde, 1% acetic acid) for 1 week at room temperature, dehydrated by graded ethanol, and embedded in wax. Sections (7 μm) were deparaffinized, stained with eosin and hematoxylin, and observed using a

microscope (Nikon Eclipse E600). A camera (Nikon DMX 1200F) mounted on the microscope projected the image on the monitor.

2.5. Acetic acid-induced vascular permeability in mice

The modified method of Olajide et al. (2003) was used. Thirty minutes after the adminstration of drugs, each animal was injected with 0.25 ml of 6% v/v solution of acetic acid intraperitoneally. Immediately after acetic acid injection, 10 ml/kg of 5% thymol blue dye was injected intravenously into the tail vein of the mice. Thirty minutes following thymol blue injection, the mice were sacrificed by cervical dislocation, and the viscera exposed. The animals were held by a flap of the abdominal wall and the viscera irrigated with 3 ml of saline over a Petri dish. The exudates were then filtered and made up to 5 ml. Thereafter, 0.1 ml of 0.1 M NaOH solution was added to each tube containing the fluid exudates to clear turbidity due to protein. The amount of dye leaking out of the capillaries was measured at a wavelength of 484 nm in a Perkin Elmer spectrophotometer. Actual concentrations were obtained from a calibration curve plotted with a blank thymol blue. The amount of dye leaking out was expressed in micrograms (mean ± S.E.M).

The dose of zileuton and zafirlukast used in vascular permeability is their ED_{50} dose against acetic acid-induced writhing.

2.6. Statistical analysis

The statistical significance in the responses of the treatment groups in comparison to control groups was determined by single-factor ANOVA followed by post hoc Dunnett's test. Values $p<0.05$ were considered to be significant.

3. Results

3.1. Analgesiometric tests

3.1.1. Acetic acid-induced writhing

5-LOX inhibitor, zileuton and cysteinyl receptor antagonists, zafirlukast and montelukast were evaluated in analgesiometric test. Oral administration of zileuton, (5–40 mg/kg), zafirlukast and montelukast (2–20 mg/kg) produced significant ($p<0.05$) analgesic effect when compared to vehicle control against acetic acid-induced abdominal constrictions (Fig. 1a–c). ED_{50} with 95% confidence limits are presented in Table 1. Further, treatment with a selective leukotriene BLT receptor antagonist, ZK 158252 (1 μg/mouse) did not reduce the number of writhes (42.3 ± 1.02) vs. control (44.5 ± 0.98).

Rofecoxib (per oral) used as a critical control did not show any antinociceptive effect against acetic acid-induced writhing (control: 37.2 ± 2.15; rofecoxib 5 mg/kg: 38.33 ± 1.72; rofecoxib 10 mg/kg: 36.5 ± 1.5; rofecoxib 20 mg/kg: 35.66 ± 2.1; rofecoxib 40 mg/kg: 36.33 ± 1.89). This is in concordance to earlier report by Jain et al. (2001b), where

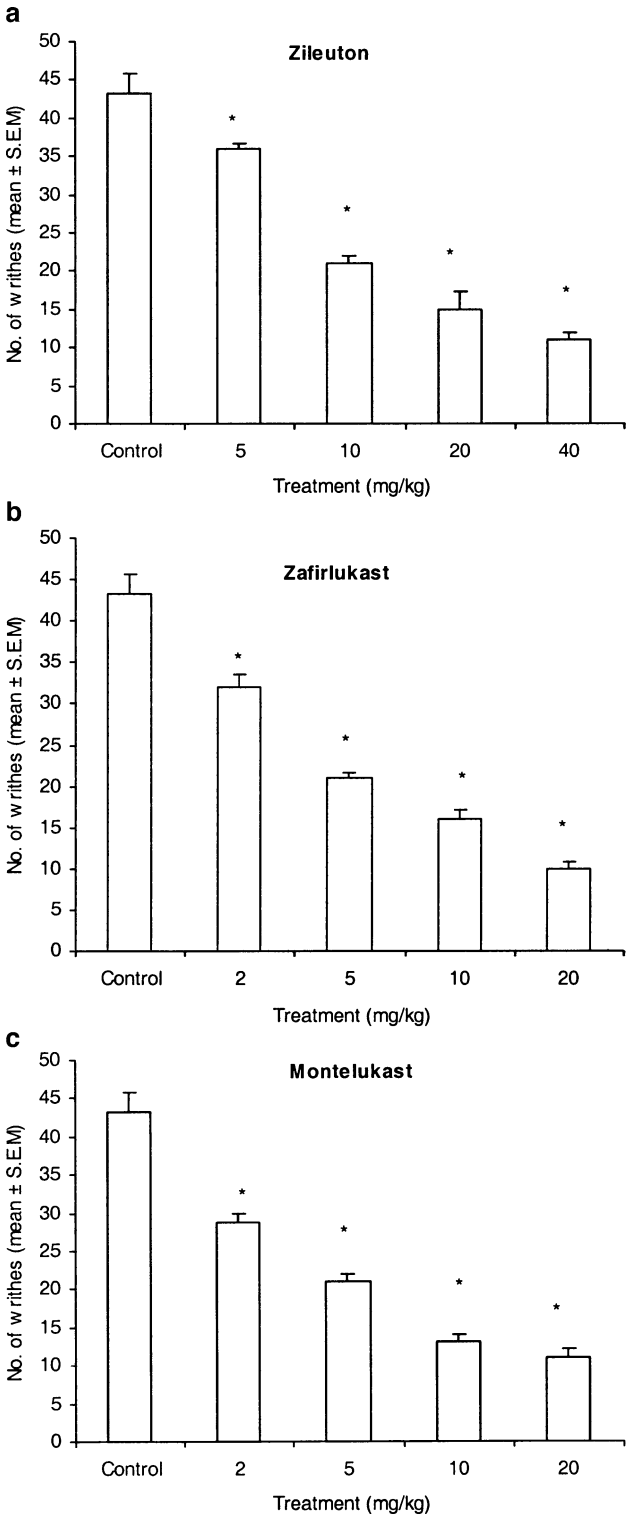

Fig. 1. Effect of (a) zileuton (5–40 mg/kg p.o.) (b) zafirlukast (2–20 mg/kg p.o.) and (c) montelukast (2–20 mg/kg p.o.) against acetic acid-induced abdominal constrictions in mice. Data presented as mean ± S.E.M. ($n=6$–7). *$p<0.05$ as compared to control (saline or respective vehicle treated).

Table 1
ED_{50} values with 95% confidence limits (CL) for antinociceptive effect against acetic acid-induced writhing in mice

Treatment	ED_{50} mg/kg p.o. (95%CL)
Zileuton	31.81 (20.42–43.19)
Zafirlukast	6.19 (1.1–11.274)
Montelukast	7.17 (4.83–9.5)

ED_{50} was calculated from log dose-probit analysis of quantal dose–response curves (SigmaStat Statistical software version 2 [Jandel Scientific, Erkrath, Germany]).

rofecoxib did not show any effect in acetic acid-induced writhing.

3.1.2. Hot plate and tail flick assay

Zileuton in the doses tested (10, 20, and 40 mg/kg p.o.) did not alter the acute thermal nociceptive response in the tail flick or hot plate assays. Further, 30-min prior administration of leukotriene BLT receptor antagonist ZK158252 (1 μg/mouse) also did not alter the basal response in both tail flick or hot plate assays (Fig. 2a and b).

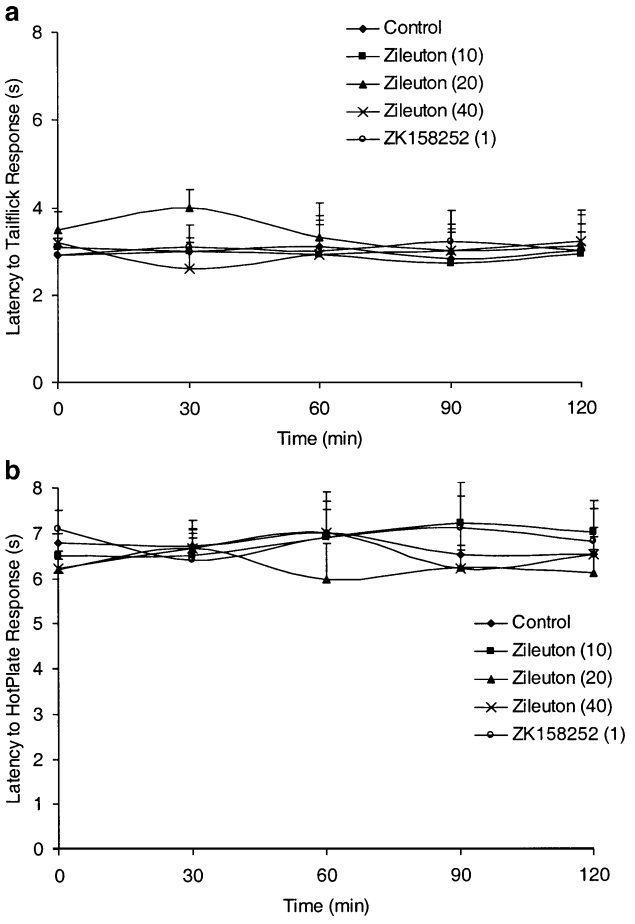

Fig. 2. Time course (a) tail flick (b) hot plate response of zileuton (10–40 mg/kg p.o.) and ZK 158252, a leukotriene BLT receptor antagonist in mice. Data presented as mean ± S.E.M. (n = 6–7). Control: saline or respective vehicle.

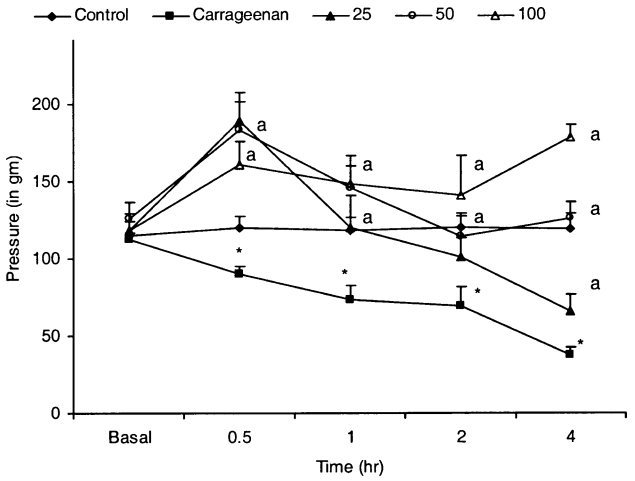

Fig. 3. Effect of zileuton (25–100 mg/kg p.o.) against carrageenan-induced mechanical hyperalgesia (Randall Selitto) in rats. Data presented as mean ± S.E.M. (n = 5–6). *p < 0.05 as compared to saline control. [a]p < 0.05 as compared to carrageenan group.

3.1.3. Carrageenan-induced inflammatory nociception (hyperalgesia): Effect of oral administration of zileuton

The rats injected with intraplantar carrageenan depicted hyperalgesia throughout the observation period. The rats injected with intraplantar saline did not show any change in

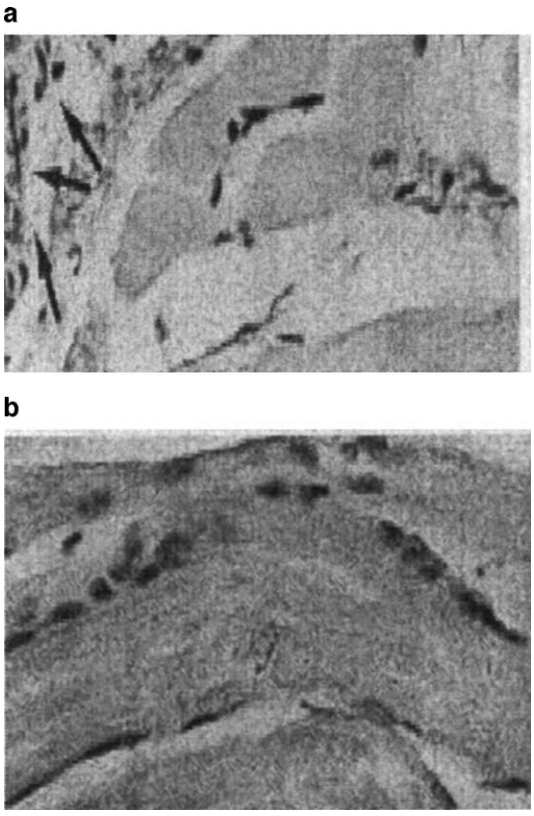

Fig. 4. Representative paw sections from (a) carrageenan-treated rats. Carrageenan treatment caused marked inflammatory changes, including pronounced cellular infiltration (arrows) (125×). (b) Carrageenan-treated rats that received zileuton (100 mg/kg). Zileuton reduced inflammatory changes (125×).

Table 2
Effect of various treatments at ED_{50} doses against acetic acid-induced vascular permeability

Treatment	Concentration of dye leaked (μg)	% inhibition
Saline	7.33±0.33	–
Acetic acid 6% v/v	18.33±2.41*	–
Zileuton (31.81 mg/kg p.o.)	12.00±2.08**	21.79
Zafirlukast (6.19 mg/kg, p.o.)	11.33±0.88**	26.08

Concentration of dye leaked expressed as μg (mean±S.E.M.).
* $p<0.05$ as compared to saline.
** $p<0.05$ as compared to acetic acid.

nociceptive threshold. Oral administration of zileuton (25–100 mg/kg) showed an anti-hyperalgesic effect as compared to the carrageenan effect per se (Fig. 3). Zileuton (100 mg/kg p.o.) per se did not alter basal response (data not shown).

3.1.4. Histological studies

Upon histological examination, the paws revealed pathological changes that can be correlated to decreased nociceptive threshold. The paw biopsies showed marked inflammatory changes after carrageenan administration, including pronounced cellular infiltration (Fig. 4a). Treatment with zileuton (100 mg/kg) significantly reduced the pathological changes in the tissues (Fig. 4b).

3.2. Acetic acid-induced vascular permeability

The efficacy of the drugs against exudation of fluid from blood vessels (vascular permeability) was tested by quantifying the amount of dye (thymol blue) leaked in peritoneum during acetic acid-induced increased vascular permeability. The intraperitoneal administration of acetic acid (6% v/v) and intravenous administration of thymol blue (5% w/v) led to a significant ($p<0.05$) amount of dye that leaked in peritoneal cavity as compared to saline-treated animals. The amount of dye that leaked into the peritoneum was significantly inhibited by pretreatment (30 min) of zileuton and zafirlukast (Table 2).

4. Discussion

In the present study, 5-LOX inhibitor and cysteinyl receptor antagonist inhibited number of writhes and, at their ED_{50} dose, exhibited inhibitory effect against increased vascular permeability in acetic acid-induced peritonitis. However, zileuton did not show any anti-nociceptive effect in tail flick and hot plate test in mice. Also, oral administration of zileuton alleviated mechanical hyperalgesia in rats.

Distinct measures of nociception were used to assess the anti-nociceptive effect of zileuton. Tail flick is an acute spinally mediated reflex to noxious thermal stimuli. The fast rising pain in the tail flick gives rise to rapid tail withdrawal at the lowest possible threshold for pain before the pain reaches a high level. Hot plate test involving paw licking and jump response is mediated through supraspinal centers. Writhing test produces steady and prolonged pain associated with tissue damage. (Zimmermann, 1979; Singh et al., 2001). Carrageenan-induced inflammatory pain, a well-characterized model, involves inflammatory mediators like histamine, leukotrienes, platelet activating factor, and cyclo-oxygenase (COX) products (Peskar et al., 1991).

It is widely recognized that in states of pain following tissue injury, prostaglandins generated locally act to sensitize peripheral nociceptors to noxious stimuli and subsequently release other mediators in spinal cord resulting in hyperalgesia (Collier and Schneider, 1972; Davis et al., 1993; Rueff and Dray, 1993). There is evidence that leukotrienes (LTs) at the peripheral and spinal level also exert similar effect. Intraplantar injection of leukotriene receptor agonist (LTB_4) and 8*R*-15(*S*)-dihydroxy-eicosa-5-*cis*-9,11,13-*trans*-tetrenoic acid (8R-15-diHETE), which are metabolites derived from 5 and 15 lipoxygenase pathways, respectively, evokes profound hyperalgesic response (Rakham and Ford Hutchinson, 1983; Levine et al., 1985; Martin et al., 1987, 1988). According to electrophysiological studies, LTB_4 sensitizes the C and Aδ nociceptors to thermal, mechanical and chemical stimuli (Martin et al., 1987, 1988). Polymodal C and Aδ fibers have been reported in gut and footpad of rodents (Marry, 2003). Zileuton exhibited antinociceptive effect against acetic acid writhing but did not alter the basal response of tail flick and hot plate assay in mice. Further, leukotriene BLT receptor antagonist, ZK 158252 also did not alter nociceptive response in tail flick, hot plate and writhing assay. ZK158252 is a competitive and specific LTB_4 receptor antagonist and does not bind to other LT receptors. Cysteinyl receptor antagonists, zafirlukast and montelukast also demonstrated anti-nociceptive effect in writhing assay. LTB_4, is a potent chemotactic factor (Wallace and Ma, 2001). Therefore, an indirect mechanism is likely to govern the role of LTB_4 in inflammatory pain. Indeed, LTB_4 has been shown to activate polymorphonuclear leukocytes to induce release of 8R-15-diHETE that contributes to peripheral and spinal nociception (Levine et al., 1986). Martin et al. (1987) reported that spinal injection of 8R-15-diHETE elicited hyperalgesia. Cysteinyl leukotrienes induce receptor-mediated stimulation of smooth muscle contraction, cause plasma exudation, and effect microvasculature (Wallace and Ma, 2001). Recently Gilbert et al. (2003) reported that cysteinyl receptor antagonist suppressed inflammatory pain but did not affect nociceptive response in tail flick and hot plate assay. This suggests that both LTB_4 and cysteinyl leukotrienes are involved in inflammatory pain contributing through different mechanism and zileuton influence inflammatory nociceptive mechanisms.

Furthermore, in acetic acid-induced vascular permeability, the efficacy of zileuton, a 5-LOX inhibitor, was similar to that of zafirlukast, a cysteinyl receptor antagonist. Thus, overall inhibition of 5-LOX pathway did not differ from that

of cysteinyl receptor blockade. This indistinguishable effect mechanistically suggests a pronounced effect of cysteinyl leukotrienes over LTB_4. LTB_4 is a potent chemotaxin with little or marginal effect on vascular permeability. In contrast, peptide leukotrienes cause an increase in the permeability of vascular endothelium (Wallace and Ma, 2001).

The present study thus suggested a role of leukotrienes in paradigms of acute nociception in addition to their earlier reported participation in chronic and subchronic painful conditions (Feitosa et al., 2002; Cunha et al., 2003).

However, the previous work with MK 886, a 5-lipoxygenase activating protein inhibitor (FLAP), did not observe antinociception in acute nociception (Griswold et al., 1991). FLAP inhibitors prevent cellular transfer of arachidonic acid to 5-LOX. In contrast to 5-LOX inhibitors and cysteinyl receptor antagonists, FLAP inhibitors are only effective in intact cells and not in situations where cell disruption occurs (viz., acetic acid-induced writhing which involves disruption of macrophages and release of pain mediators). They are effective under conditions of limited substrate availability. Also, it is reported that immediate enhanced local arachidonic acid release can occur in in vivo acute nociceptive paradigms (acetic acid-induced writhing) that could in turn reduce the local efficacy of FLAP inhibitors. On the other hand, under chronic conditions, the availability of reduced amount of arachidonic acid due to its metabolism might be providing a limited substrate for FLAP inhibitors to show their efficacy (Steinhilber, 1999).

Contrarily, 5-LOX inhibitors like zileuton interfere with the activation of the active catalytic site of 5-LOX and cysteinyl receptor antagonist act at their receptors. Both the mechanisms are independent of substrate (arachidonic acid) concentration (Steinhilber, 1999). Thus, it seems that blocking active site of 5-LOX or the receptors down the FLAP pathway might account for their efficacy in acute pain models.

Further, in the present study, zileuton also reduced the carrageenan-induced hyperalgesia in rats and decreased the histological changes in rat foot pad biopsies. Earlier Tonussi and Ferreira (1999) and Jain et al. (2001a) demonstrated reversal of carrageenan-induced hyperalgesia and inflammation with pretreatment of 5-LOX inhibitor or cysteinyl receptor antagonist, respectively. Similar to gut, polymodal nociceptor fibers are also present in rat footpad (Marry, 2003). Also, inflammatory changes with carrageenan administration involve activation of neutrophils and mast cells (Peskar et al., 1991). Neutrophils appears to be predominant source of powerful chemotactic LTs (LTB_4) and mast cell is the major source of peptide–leukotrienes (Wallace and Ma, 2001). Therefore, it appears that zileuton ameliorates inflammatory pain in carrageenan-treated rats by inhibiting the activation of peripheral nociceptors and through down-regulating the recruitment of inflammatory cells that produce leukotrienes. Indeed, zileuton is shown to reduce inflammation via reduction in myeloperoxidase activity and leukocyte recruitment (Singh et al., 2004b).

However, it is likely that zileuton also act at spinal level to reduce inflammatory hyperalgesia by attenuating sensitization response of leukotrienes. Spinal injection of LTB_4 or 15-HPETE is reported to augment the inflammatory pain response in mice (Trang et al., 2004). Leukotrienes causes sensitization of spinal neurons to the pro-nociceptive mediators released following activation of peripheral nociceptors during inflammatory pain (Porro and Cavazzuti, 1993).

In conclusion, the present study demonstrated that zileuton, a 5-LOX inhibitor, exhibited antinociceptive effect in paradigms of inflammatory pain.

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