Effect of licofelone against NSAIDs-induced gastrointestinal ulceration and inflammation

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The present study was aimed to evaluate the effect of licofelone, a dual inhibitor of cyclooxygenase1/2-5-lipoxygenase against indomethacin-induced gastric damage in rats and mice in order to assess the role of leukotrienes if any, in non-steroidal anti-inflammatory drugs (NSAIDs)-induced gastrointestinal inflammation. Acute pretreatment with licofelone reversed the indomethacin-induced gastric ulceration, neutrophil adhesion in mesentery venules, neutrophil count in blood, lipid peroxides and vascularity in the stomachs of mice and rats. Further, chronic pretreatment of licofelone also prevented indomethacin-induced gastric morphological changes and cellular infiltration in mesentery venules. Moreover, acute administration of indomethacin elevated leukotriene B4 levels in gastric mucosa, which was reversed by pretreatment with licofelone. The results suggest that licofelone offered gastroprotection against NSAIDs-induced gastropathy through its effect on leukotrienes and by inhibiting extravasation of neutrophils.

Keywords: Gastric ulceration, NSAIDs, Licofelone, LTB4, LOX, Neutrophils

Gastrointestinal (GI) damage induced by nonsteroidal anti-inflammatory drugs (NSAIDs) is one of the most frequently associated adverse effects. These changes are presumably due to inhibition of cyclooxygenase (COX), the enzyme responsible for conversion of arachidonic acid (AA) to prostaglandins (PGs) that are needed to maintain the integrity of gastric mucosa. However, the postulate that COX inhibition by NSAIDs diverts AA metabolism to 5-lipoxygenase (5-LOX) pathway, suggests the possible role of leukotrienes (LTs) in vascular changes and mucosal damage associated with the use of NSAIDs. Thus, dual inhibitors of COX/LOX pathway, which would be gastrofriendly whilst retaining anti-inflammatory effect, were developed. However, role of LTs in NSAIDs-induced gastropathy is debatable. Both, supporting and opposing reports on the effect of 5-LOX inhibitor and leukotriene receptor antagonists against NSAIDs-induced gastric damage are available. LTs cause chemotaxis, degranulation of neutrophils, increase vascular permeability and affect vascular tone. Neutrophils have been implicated as mediators of the epithelial cell and microvascular dysfunction associated with several models of gastrointestinal mucosal injury including that induced by NSAIDs. The circulating activated neutrophils appeared to be important in the development of gastric erosions, and changes in vascular integrity following NSAIDs treatment. Indeed, depletion of blood neutrophils or inhibition of their adherence is reported to prevent NSAID-gastropathy. Also, the role of neutrophil derived oxygen free radicals in NSAIDs-induced disruption of gastric membrane integrity is documented.

Licofelone (ML3000), a dual inhibitor of COX/5-LOX, has been reported to possess a potent antiinflammatory effect and a favorable gastrotolerability properties. The present study has been aimed to evaluate effect of licofelone, dual COX/LOX inhibitor against the events and on the effectors associated with NSAIDs-induced gastric damage in rats and mice.

Materials and Methods

Animals—Swiss mice (22-25 g), Wistar rats (180-200 g) (Central Animal House, Panacea Biotec Ltd., Lalru) of either sex were used. They were housed in plastic cages at room temperature (25±0.5°C) and 12:12 hr L:D cycle. Animals were given food and water ad libitum. Experiments were carried out between 0900 and 1500 hr. The experimental protocols were approved by the Institutional Animal Ethics Committee.

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Drugs and regimen—Licofelone, indomethacin, and naproxen (Panacea Biotec Ltd., New Delhi, India), were obtained in house. All drugs were suspended in Tween 80 and administered orally in the dose volume of 10 ml/kg. Schematic drawings representing the drug regimen in early and late events of indomethacin-induced gastroinflammation is given in Fig 1a and b. The dose selection was based on earlier studies.\(^9,12\)

**Visual gastric lesions**—Gastric score was calculated by method of Chan et al.\(^14\). After 4 hr of dosing, animals were sacrificed and stomach was excised along its greater curvature. After rinsing with normal saline, the mucosa was examined for the presence of petechiae or frank hemorrhage lesions. Petechiae were assigned a score of 1, and lesions were scored according to their length (a score of 5 for lesions greater than 3 mm; a score of 10 for lesions greater than 3 mm). The sum of total scores was used for comparison. All treatment groups were coded to prevent measurement bias.

**Intravital microscopic measurement of neutrophil adhesion in the rat mesenteric venules**—After 60 min of single acute/last chronic dose, the rats were anesthetized with thiopental sodium (25 mg/kg, ip) and were placed on a heated (37°C) platform used to adapt a microscope for intravital microscopy. A midline laparotomy was performed and a segment of mid-jejunum was exteriorized and placed over an optically clear saline-coated cover slip in such a way that a clear area of mesentery was within the focal area. The tissue was covered with heated (37°C) phosphate buffered saline (PBS) and saline moistened gauze was placed over the exposed intestine to prevent the tissue from dehydrating and to hold the tissue in the position. Single unbranched post-capillary venules approximately 25-35 µm in diameter were used for these studies, since they are transparent enough to observe blood flow and leukocyte adhering to the endothelial wall of the vessel.\(^15\). The preparation was allowed to stabilize for 8-10 min and mesenteric circulation was observed using an microscope (Nikon Eclipse E600). A camera (Nikon DMX 1200F) mounted on the microscope projected the image on the monitor. The area of normal blood flow and that with leukocyte adherence in mesentery was traced and photographed.

**Measurement of lipid peroxides and LTB\(_4\) in rat gastric mucosa**—Animals were administered vehicle or test compounds once and killed by cervical dislocation 60 min post-treatment. The gastric mucosa was removed from each stomach and placed in a glass tissue homogenizer. Tissue homogenate was prepared in a ratio of 1g of wet tissue to 9ml 1.15% KCl for lipid peroxides and in methanol for LTB\(_4\) estimation. Different group of animals were used for lipid peroxide and LTB\(_4\) measurements. The lipid peroxides (thiobarbituric acid reactive substances; TBARS) were measured by method of Wills.\(^16\) For LTB\(_4\) measurements the methanol homogenate was centrifuged (-13°C, 11,000 rpm, 20 min), and supernatant separated. The LTB\(_4\) content was determined using LCMS/MS (API 3000: PE SCIEX, using turbo ion spray as ion source. Column C18/5cm/Novapak, mobile phase: 0.001% ammonium acetate and acetonitrile in ratio 65:35, flow rate 0.2ml/min, injection volume: 50 µl). A linearity up to 500 pg concentration was observed. Neutrophil count in blood was carried out at time interval parallel to intravital microscopy. The neutrophil count in blood was determined using automatic counter (Swelab, Sweden).

**Gastric vascular permeability in mice**—The effect on vascularity was assessed by the formation of vascular casts incorporating supra allura red in mouse

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**Fig. 1**—Schematic drawings representing the experimental design and drug regimen to study (a) early events (b) late events associated with indomethacin-induced gastric damage.
Briefly, mice were fasted for 4 hr and administered indomethacin (10 mg/kg, po) or licofelone (10 mg/kg, po)/indomethacin (10 mg/kg, po). Indomethacin was administered 30 min after licofelone. After 30 min of indomethacin, mice were anesthetized with thiopental sodium (25 mg/kg, i.p) and peripheral vasodilation was achieved by placing in an heated jacket at 40°C for 10 min. The induction of peripheral vasodilation in the mice is important in overcoming alterations in peripheral vasomotor tone and tissue perfusion related to anesthesia and ambient temperature that may invalidate the results.

The cast was formed by iv injection of 1 ml of 5% supra allura red in 10% gelatin at 40°C into the warmed mice. The carcasses were chilled and the stomach isolated. The tissue was oven dried at 56°C for 48 hr and weighed. To overcome dye bleaching by the digestion of tissue in sodium hydroxide, the dried tissue was instead papain digested for 24 hr at 56°C in 0.9ml of digestive buffer (dithiothreitol 2 mM, disodium hydrogen orthophosphate 20 mM, EDTA 1mM, papain 12 U/ml) according to method of Farndale et al.

The dye was then dissolved by the addition of 0.1ml of 5M sodium hydroxide, and the digest were centrifuged at 2000 g for 10 min and filtered through a 0.45 μm nitrocellulose filter. The dye content of the filtered samples was spectrophotometrically determined at 504 nm. The result was expressed as either mg of dye content per sample or vascularity (V.I) as μg dye/mg dry weight of tissue.

Statistical analysis—Data presented as mean ± SE was analyzed using one-way ANOVA followed by post hoc Dunnett’s test. P<0.05 was accepted as the level of significant difference compared to time matched controls.

**Results**

**Effect of chronic administration of indomethacin, and its modification by licofelone of gross morphology of stomach and neutrophil adhesion in the rat mesenteric venules**—Chronic administration of indomethacin (2 mg/kg, po) for 4 consecutive days was associated with mortality (40%) and intestinal perforations (100%) in surviving rats. Licofelone (10 mg/kg, po) administered daily 30 min prior to indomethacin prevented any death and completely inhibited the intestinal perforation. In earlier studies, licofelone (10mg/kg, po) per se is reported to be free from any gastric side effects in rats. Further, rats chronically treated with indomethacin showed a marked cellular infiltration with in the mesenteric venules.

**Intravital microscopic evaluation of effects of COX, or dual COX/5-LOX inhibitor on the neutrophil adhesion in the rat mesenteric venules**—Single oral administration of indomethacin (100mg/kg), produced significant number of gastric lesions at 4hr after dosing. In comparison, 30 min prior administration of licofelone (30 mg/kg, po) was able to significantly reduce the indomethacin-induced gastric lesion score.

In another group, 1 hr after oral administration of indomethacin (100 mg/kg, po) neutrophil adherence to mesentery venules was microscopically recorded. Indomethacin induced a significant adherence of neutrophils when compared to the control group.

![Fig. 2](#)

**Fig. 2**—Intravital microscope photography of rat mesenteric venules (40x) taken after 4 days treatment with indomethacin (2 mg/kg). [◆ cellular infiltration].

![Fig. 3](#)

**Fig. 3**—Mean gastric lesion scores in rats following acute administration of indomethacin, licofelone or pretreatment with licofelone. *P<0.001* as compared to vehicle control; *as compared to indomethacin per se treatment. Vertical lines represent mean ± SE (n=5-6).
Licofelone pretreated animals showed response similar to that of control group (Fig 4c). Licofelone per se did not induce any neutrophil adhesion administered up to 100 mg/kg, po. Pretreatment with another NSAID, naproxen (100 mg/kg, po) also did not produce any significant increase in adhesion when compared with indomethacin treatment alone (data not shown).

Pretreatment with licofelone (30 mg/kg, po) also significantly inhibited the indomethacin-induced increase in blood neutrophil count (1.45 ± 0.72 × 10^3/µl vs. 4.72 ± 0.98 × 10^3/µl in indomethacin group). 

Table I—Effect of indomethacin, licofelone and licofelone/indomethacin on gastric LTB₄ levels

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>LTB₄ ng/g tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>25.9 ± 3.0</td>
</tr>
<tr>
<td>Indomethacin (100)</td>
<td>40.2 ± 2.1     *</td>
</tr>
<tr>
<td>Licofelone (30)</td>
<td>23.2 ± 4.1</td>
</tr>
<tr>
<td>Licofelone (30)/Indomethacin (100)</td>
<td>28.0 ± 4.21</td>
</tr>
</tbody>
</table>

P values: <0.05 * as compared to vehicle control; * as compared to indomethacin

The level of thiobarbituric acid reactive substances (TBARS, index of lipid peroxidation) of gastric mucosa was 0.529 ± 0.05 nmol/mg protein in indomethacin group vs. 0.303 ± 0.03 nmol/mg protein in control group. Licofelone per se did not alter the TBARS level (0.295 ± 0.04 nmol/mg protein). Pretreatment with licofelone significantly reduced the generation of TBARS (0.319 ± 0.02 nmol/mg protein vs. 0.529 ± 0.05 nmol/mg protein in indomethacin group) (Figs 5,6).

Effect of orally administered indomethacin, or licofelone on LTB₄ concentration and lipid peroxide in gastric mucosa—LTB₄ levels were elevated after indomethacin (100 mg/kg, po) administration, but not in licofelone (30 mg/kg, po) or licofelone pretreated group (Table 1).

Effect of licofelone pretreatment against indomethacin-induced vascular permeability—The vascularity (VI) of stomach derived from supra allura red administered with 10% gelatin (iv), was assessed after acute administration of indomethacin (10 mg/kg, po), and pretreatment with licofelone (10 mg/kg, po). Indomethacin resulted in significant (P<0.05) increase in VI when treated to control animals which was significantly reversed by pretreatment with licofelone. Licofelone per se did not affect the VI of mice stomach (Table 2).

Discussion

In the present study pretreatment with licofelone reversed the indomethacin-induced morphological changes, gastric ulceration, neutrophil adhesion in mesentery venules. Prior administration of licofelone also caused a significant reduction of blood neutrophil count, and lipid peroxides in rats. In addition, a reduction in mice stomach vascularity was observed.

Further, indomethacin treatment caused an elevation in LTB₄ levels in gastric mucosa, which was prevented with licofelone pretreatment. It is suggested that inhibition of COX enzyme by NSAIIDs leads to increased flux of AA through lipoxygenase pathway.
with a concomitant increase in LTs (LTB₄, and peptidoleukotrienes: LTC₄) production. LTB₄, a very potent chemotaxin can promote leukocyte recruitment by upregulating expression of β2 integrins on these cells.

Licofelone, a dual inhibitor is reported to suppress in vitro both 5-LOX (IC₅₀ = 0.18μM) and COX (IC₅₀ = 0.21 μM) activity, reduce LTB₄ levels in rat paw, and did not increase the gastric mucosal damage in aspirin treated rats. In the present study licofelone also attenuated indomethacin-induced increased gastric vascularity in mice. Licofelone is reported to inhibit LTC₄ formation in polymorphonuclear cells (IC₅₀ = 3.8 μM). Peptido-leukotrienes are known to increase the permeability of the vascular endothelium and increase P-selectin expression on these cells thereby promoting the rolling of leukocytes.

This indicated that licofelone conferred gastroprotection by inhibiting the indomethacin-induced increase in 5-LOX effectors. Since neutrophils are the major source of LTB₄ and extravasated neutrophils are implicated in the pathogenesis of gastric damage-induced by NSAIDs, effect of licofelone on neutrophils was also studied.

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Table 2—The effect of indomethacin, and pretreatment with licofelone on the tissue vascularity derived from supra allura red dye in mice gastric mucosa

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>Concentration of dye (μg)</th>
<th>Vascularity index (V.I)</th>
<th>Amount of dye (μg/mg tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>0.975 ± 0.08</td>
<td>0.0285 ± 0.003</td>
<td></td>
</tr>
<tr>
<td>Indomethacin (10)</td>
<td>4.56 ± 0.23*</td>
<td>0.071 ± 0.003*</td>
<td></td>
</tr>
<tr>
<td>Licofelone (10)</td>
<td>1.20 ± 0.05</td>
<td>0.030 ± 0.005</td>
<td></td>
</tr>
<tr>
<td>Licofelone (10)*</td>
<td>2.8 ± 0.49</td>
<td>0.037 ± 0.004*</td>
<td></td>
</tr>
</tbody>
</table>

*P values: <0.05* as compared to vehicle control; *as compared to indomethacin

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Fig. 5—Effect of different treatments on thiobarbituric acid reactive substances (TBARS) in rat gastric mucosa. P<0.001* as compared to vehicle control; P=0.005 *as compared to indomethacin per se treatment. Vertical lines represent mean ± SE (n=5-6).

Fig. 6—LCMS/MS chromatogram of (a) leukotriene B₄ (RT 10.15 min, Mass selection 337.5 to 249.5) (b), leukotriene internal standard (RT 9.36 min, Mass selection 411.1 to 380.1).
The effect on neutrophils was observed parallel to the effect on LTB₄ levels. Indomethacin caused significant neutrophil accumulation in postcapillary mesenteric venules, and increased neutrophil count in blood, which was completely inhibited following pretreatment with licofelone. Neutrophils by several possible mechanism contribute to NSAID-induced gastric ulceration. Neutrophils generated free radicals significantly contribute by (i) direct tissue necrotic effect 25, 26 (ii) and indirectly via oxygen free radicals. These free radicals influence the inactivation of endothelium-derived relaxing factor and alters the resistance of gastric mucosa to damage 27. Also, neutrophils can release protease and lipid mediators that contribute to gastrointestinal ulceration, changes in mucosal permeability and affect the vascular tone and permeability, respectively 28. In the present study, licofelone while inhibiting the indomethacin-induced neutrophil adhesion also significantly reduced the corresponding increase in TBARS levels. LTB₄ can stimulate the release of reactive oxygen metabolites from neutrophils. Lipid peroxidation (TBARS) degrades polyunsaturated fatty acids of the cellular membranes with subsequent disruption of membrane integrity and its role in pathogenesis of indomethacin-induced gastric ulceration is reported 29, 30. Indeed, licofelone is shown to inhibit generation of reactive oxygen species, and release of elastase by polymorphonuclear cells 31.

Thus, the results of the present study further indicates the role of 5-LOX metabolites in NSAID-induced gastroinflammation and suggests that licofelone counteracted the indomethacin-induced gastropathy by a combination of its effect on LTB₄ levels, inhibition of neutrophil adherence in postcapillary mesenteric venules and through decrease in related production of free radicals that disrupts integrity of stomach mucosa.

However, the role of LTs in the pathogenesis of NSAIDs-induced GI damage is controversial. There are evidences both in support and contrary to this hypothesis. Various studies have demonstrated no inhibition while others have demonstrated the amelioration of NSAIDs-induced gastric irritant effect by selective 5-LOX inhibitor and dual inhibitors 27. Inhibition of ulcerogenic events by licofelone within the dose range of its reported 5-LOX activity is suggestive of an important balance between cyclooxygenase and 5 lipoxygenase products. A marginal shift towards 5-LOX metabolite can induce diapedesis of inflammatory cells during NSAID therapy. However, as observed here and earlier by Wallace et al. 13 that licofelone per se did not change gastric leukotriene contents raises the possibilities that 5-LOX could have other likely implication viz. (i) formation of oxygenation products, and/or (ii) direct oxygenation of membrane phospholipids without participation of phospholipase during gastric injury. Recently, Smolka et al. 32 reported that licofelone inhibits gastric H, K ATPase enzyme and interleukin-8 secretion. This could also be an important mechanism in licofelone-persuaded amelioration of NSAIDs-induced gastropathy.

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References

8 Poff C D & Balazy M, Drugs that target lipxygenases and leukotrienes as emerging therapy for asthma and cancer, Current Drug Targets: Inflammation Allergy, 3 (2004) 19.
9 Wallace J H, Keenan C M & Granger D N, Gastric ulceration induced by Nonsteroidal antiinflammatory drugs is a neutrophil-dependent process, Am J Physiol, 259 (1990) G462.


