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# Licofelone, a novel 5-LOX/COX-inhibitor, attenuates leukocyte rolling and adhesion on endothelium under flow

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**Abstract**

The main mechanism of action of non-steroidal anti-inflammatory drugs (NSAIDs) is the inhibition of cycloxygenases COX-1 and COX-2. During recent years, combined 5-LOX/COX-inhibition, interfering with the biosynthesis of both prostaglandins and leukotrienes (LTs), has emerged as a possibility to avoid side effects related to COX-inhibition. The aim of the present study was to investigate if there is a contribution of mechanisms other than the reduction of inflammatory prostaglandins and leukotrienes to the anti-inflammatory effect of the LOX/COX inhibitor licofelone. In a flow chamber assay, licofelone (10–30 μM) dose-dependently decreased both the rolling and adhesion of leukocytes on endothelial cells (EC). In contrast, no effects were found after treatment of EC with the unselective COX-1/COX-2 inhibitor indomethacin (30 μM), the potent and selective 5-LOX inhibitor, ZD-2138 (30 μM), the mainly COX-2 inhibitor aceclofenac (30 μM), the selective COX-2 inhibitor celecoxib (30 μM) and the combination of ZD-2138 with the selective COX-2 inhibitor celecoxib (30 μM). In the presence of licofelone (30 μM) the expression of E-selectin mRNA in cytokine-stimulated EC was attenuated, whereas no NSAID (30 μM) tested showed any effect on E-selectin expression. Moreover, licofelone treatment (30 μM) attenuated expression of VCAM-1 and ICAM-1 on inflammatory EC. The effect of licofelone on leukocyte recruitment was also evaluated in vivo. Using a mouse peritonitis model it was found that leukocyte accumulation was markedly reduced in licofelone treated animals (100 mg/kg) compared to untreated mice. Thus, the novel 5-LOX/COX inhibitor licofelone possesses anti-inflammatory activity that, in addition to COX/LOX inhibition, involves effects on leukocyte-endothelial interactions.


*Keywords:* Cyclooxygenase; 5-Lipoxygenase; 5-LOX/COX inhibition; Leukocyte; Selectin; Adhesion; Rolling; Endothelium

## 1. Introduction

Non-steroidal antiinflammatory drugs (NSAIDs) are widely used for the treatment of pain, fever and inflammation. The main mechanism of action of these drugs is the inhibition of the cyclooxygenase enzymes (COX1-2) [1,2] which catalyse the conversion of arachidonic acid to prostaglandins.

The major side effects associated with all the currently available NSAIDs are gastrointestinal (GI) hemorrhage, ulceration and kidney failure [3], which are caused by interference with the physiological properties of prostaglandins. As a consequence, several approaches have been undertaken to reduce the unwanted side effects of NSAIDs. One such involves the selective inhibition of COX-2. However, clinical studies have suggested that selective COX-2 inhibitors could cause typical COX-mediated side effects such as gastrointestinal injury, increased systemic blood pressure and hypersensitivity [4]. Merck & Co. Inc. announced a voluntary withdrawal of Vioxx (rofecoxib) that was approved by FDA in May 1999 from the US and worldwide market due to safety concerns of an increased risk of cardiovascular events (including heart attack and stroke) in patients on Vioxx (http://www.fda.gov/cder/drug/infopage/vioxx/default.htm).

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During recent years, combined 5-LOX/COX-inhibition, interfering both with the production of prostaglandins and the biosynthesis of leukotrienes (LTs), has emerged as another possibility to avoid side effects related to COX-inhibition [5]. One compound in the 5-LOX/COX series of NSAIDs is licofelone ([2,2-dimethyl-6-(4-chlorophenyl)-7-phenyl-2,3-dihydro-1H-pyrrolizine-5-yl]-acetic acid (379.89, previously named ML3000). It is presently evaluated in Phase III clinical trials for the treatment of signs and symptoms of osteoarthritis. Its anti-inflammatory and analgesic activity is comparable to that of conventional NSAIDs and selective COX-2 inhibitors but with an improved gastrointestinal safety profile [6–8]. Thus, 5-LOX/COX-inhibitors like licofelone appear to be potent, effective and safe under clinical conditions.

During the last decade, several groups have described a number of non-prostaglandin-mediated anti-inflammatory effects of NSAIDs, suggesting that COX inhibition does not represent the only mechanism behind the anti-inflammatory action of this group of therapeutic agents. In this regard, it has been proposed that several NSAIDs are directly or indirectly able to interfere with the function of adhesion molecules that participate in the recruitment of leukocytes to inflammatory sites [9,10].

Here, we investigate the effect of licofelone and other NSAIDs on leukocyte rolling and adhesion on inflamed endothelium. We demonstrate that licofelone influences the recruitment of leukocytes to inflammatory sites more potently than conventional NSAIDs. The data suggest that inhibition of cell adhesion molecule (CAM) expression and function is important for the clinical effect of licofelone.

## 2. Materials and methods

### 2.1. Materials

The following cell culture media and reagents were purchased from Sigma (München, Germany): Medium 199, fetal bovine serum, penicillin-streptomycin solution, amphothericin B solution, L-glutamine solution, trypsin–EDTA solution, Dulbecco's buffered saline, recombinant human tumor necrosis factor alpha (rhTNF-α), trypan blue solution 0.4%, indomethacin. Aceclofenac was acquired from Council of Europe, European Pharmacopoeia. Licofelone was a generous gift from Merckle (Blaubeuren, Germany).

Cell culture flasks were obtained from Becton Dickinson (Heidelberg, Germany). Dispase was purchased from Roche (Mannheim, Germany), Accutase from PAA (Linz, Austria).

### 2.2. Endothelial cells

Primary cultures of human umbilical vein endothelial cells (HUVEC) were obtained from freshly collected umbilical cords as previously described [11]. Briefly, the umbilical vein was incubated with 0.1% dispase. After washing, HUVEC were cultured in endothelial basal medium with endothelial cell growth supplement (PromoCell, Heidelberg, Germany) at 37 °C in a humidified atmosphere containing 5% $CO_2$. The second passages were cultured in endothelial basal medium only. Cells were grown to confluence in six-well culture plates ($1 \times 10^5$ cells/well), and used for stimulation.

Bovine aortic endothelial cells (BAEC) were isolated und cultured as previously described [12]. In brief, bovine aortas were incubated with a solution containing 0.25% collagenase for 10 min, flushed with PBS and detached BAEC were collected. Harvested cells were cultured in medium containing RPMI 1640 and M199 (1:1), penicillin (100 U/ml) and streptomycin (100 μg/ml) in 20% FCS.

### 2.3. Leukocytes

Fresh peripheral blood was isolated from healthy donors who had refrained from taking medications for at least 2 weeks. Neutrophils (PMN) were isolated using standard dextran sedimentation and gradient separation on Histopaque-1077 (Sigma Chemical Co., München, Germany). This procedure yields a PMN population that is 95–98% viable (trypan blue exclusion) and 98% pure (acetic acid–crystal violet staining).

Mono Mac 6 cells (MM6) were maintained in RPMI 1640-Medium supplemented with 10% FCS, 100 mg/ml streptomycin, 100 U/ml penicillin, 1 mM sodium pyruvate, 1 × nonessential amino acids (MEM non-essential amino acid solution, Sigma), 1 mM oxalacetic acid and 10 μg/ml human insulin [13]. All cultures were seeded at a density of $2 \times 10^5$ cells/ml.

### 2.4. Flow chamber

Laminar flow chamber assays were performed on BAEC and HUVEC grown to confluence at 5% $CO_2$ and 37 °C in six-well microtiter plates. The cells were preincubated for 4 h with medium alone (control) or with medium containing 10 ng/ml TNF-α/IL-1β (R&D Systems) in the presence/absence of licofelone dissolved in DMSO (10–100 μM final concentration) or ZD-2138, aceclofenac, indometacin and celecoxib dissolved in DMSO (30 μM final concentration). After incubation, the endothelial cells were washed with PBS and a flat-polished flow chamber (Glycotech) was placed on the endothelial monolayer. MM6 cells, suspended at $1.0 \times 10^6$ cells/ml in RPMI medium plus 1% FCS, were perfused through the flow chamber at a constant shear stress of 1 dyne/cm$^2$ for 4 min. Rolling and adherent leukocytes were visualized and counted in each well in four separate randomly chosen fields within the first minute using an inverted phase contrast microscope connected to a video camera (Kappa CF 15/2) and a video recorder (Panasonic SVHS TL700).

### 2.5. RT-PCR

Cellular RNA was extracted from BAEC using the High Pure RNA Isolation Kit system™ (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. The BAEC were preincubated for 4 h with medium alone (control) or with medium containing 10 ng/ml TNF-α/IL-1β (R&D Systems) in the presence/absence of test substances dissolved in DMSO (30 μM final concentration). The RNA samples were treated prior to reverse transcription with RNase-free DNAse I (Promega). Briefly, 1.5 mg of total RNA, 3 μl of 5× first strand buffer [250 mM Tris–HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$] (Life Technologies), 1 U of RNAse-free DNAse (Promega) and DEPC-treated water were combined in a final reaction volume of 15 μl. After incubation at 37 °C for 15 min, the reaction was stopped by adding 1.5 μl of 25 mM EDTA (Life Technologies). The samples were heated to 75 °C for 10 min and kept on ice until further processing. Subsequently, 25 μl of a master mix containing 50 units/ml Taq DNA Polymerase buffer, 400 mM dNTP and 3 mM $MgCl_2$, 5 μl Forward Primer 10 μM (final concentration), 5 μl Reverse Primer 10 μM (final concentration), 5 μl RNA Template, 10 μl purified water and 1 μl (5 U) AMV Reverse Transcriptase. Reverse transcription was carried out in a total reaction volume of 50 μl at 48 °C for 45 min. PCR amplification of specific cDNA sequences within the mixture was performed in parallel reactions. Gene-specific oligonucleotide primers based on sequences published in G7enBank human DNA database were purchased from CyperGene AB (Huddinge, Sweden). The following primer sequences were used: for E-selectin (5′–3′) sense CATGGACCTGGATAGGGACC, antisense GCAGCTGCTGGCAGGAGAAA and for β-actin (5′–3′) sense CCTAGGCACCAGGGCGTAAT, for antisense TTGGCCTTAGGGTTCAGGGG. The second PCR amplification step was done in an iCycler (Biorad, Germany). Following an initial denaturation step at 94 °C for 2 min, amplifications were cycled for 30 s at 94 °C, 1 min at 50 °C, and 2 min at 68 °C. The last cycle was followed by a final extension step at 68 °C for 7 min. PCR products were separated by electrophoresis through 1.5% agarose gel. The expression of β-actin was used as control and to control for contamination with genomic DNA, experiments were performed omitting the enzyme during the reverse transcription step.

The relative amounts of mRNA were determined by Quantity One (Biorad, Germany) visually by densitometric scanning of gels using a computer-based system for data analysis (Gel-Doc 2000; BioRad, USA).

### 2.6. Fluorescence activated cell sorter (FACS) analysis

#### 2.6.1. ICAM-1 and VCAM-1 expression on HUVEC

HUVEC were preincubated for 4 h with medium alone (control) or with medium containing LPS (1 μg/ml) (R&D Systems) or TNF-α/IL-1β (10 ng/ml) in the presence/absence of licofelone dissolved in DMSO (10–100 μM final concentration). After incubation, the cells were washed twice with $Ca^{2+}$/$Mg^{2+}$-free Dulbecco's phosphate-buffered saline (PBS) and incubated for 4 min with PBS/2 mM Trypsin at 37 °C. Cells were recovered from the plate and washed once with PBS and incubated with PE-conjugated anti-CD106, anti-CD54 or isotype-matched $IgG_1$ (purchased from Dianova, Hamburg, Germany) at a 1:50 dilution in PBS/0.5% bovine serum albumin for 30 min at 4 °C. Cells were washed twice in PBS and analyzed using a BD FACS Calibur and Cell Quest software.

### 2.7. Mouse peritonitis

Male C57BL/6 mice were subjected to intraperitoneal injection of 3 ml TNF-α and IL-1β (20 ng/ml in HBSS) with or without simultaneous i.p. treatment with licofelone (100 mg/kg). After 6 h, leukocytes were harvested from the peritoneum with 3 ml cold HBSS containing 5 mM EDTA. Samples were centrifuged (2000 rpm for 5 min), labeled with 5 μl FITC conjugated rat anti-mouse macrophage/monocyte antibody (MOMA-2, ImmunoKontact), anti-mouse PMN antibody (Anti-PMN, ImmunoKontact) and anti-mouse T-lymphocyte antibody (Anti-CD2, ImmunoKontact) for 20 min at 4 °C, washed twice and resuspended in HBSS. The fluorescence intensity of leukocytes was analyzed by FACS. Gating for leukocyte subpopulations was based on forward and side scatter parameters and FL-1. Relative mean fluorescence intensity was calculated by subtracting the specific staining intensity by that obtained with control.

### 2.8. Statistics

Data are presented as mean ± S.E.M. Statistical analysis was done by one-way analysis of variance (ANOVA) followed by Holm–Sidak test; a $P$ value of 0.05 or less was considered significant.

## 3. Results

### 3.1. Effect of licofelone on leukocyte rolling and adhesion on cultured endothelium

The effect of licofelone on leukocyte rolling and adhesion on endothelium was investigated in a flow chamber assay. Rolling and adhesion of leukocytes was significantly increased after 4 h incubation of BAEC with a combination of IL-1β and TNF-α (10 ng/ml). Under these conditions, licofelone in a concentration-dependent manner decreased both rolling and adhesion on EC (Fig. 1a). At the dose of 30 μM, licofelone caused a 46% reduction in leukocyte rolling and a 56% inhibition of leukocyte firm adhesion

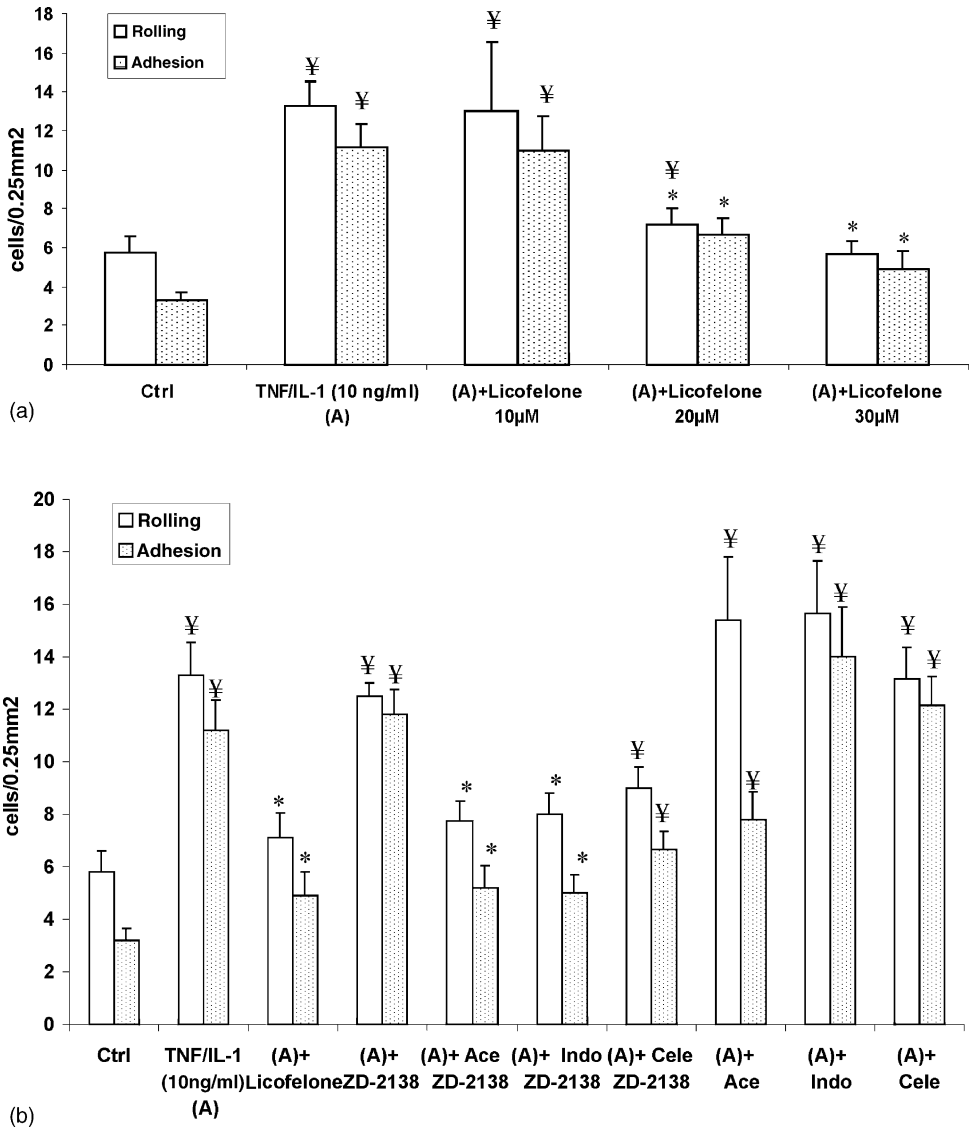


Fig. 1. (a) Effect on rolling and adhesion of MM6 to BAEC after pre-treatment with vehicle or 10 ng/ml TNF-α/IL-1β in the presence/absence of different concentrations of licofelone (10, 20 and 30 μM). The cells were incubated with vehicle (DMSO) as negative control or treated with 10 ng/ml TNF-α/IL-1β for 4 h in the absence (positive control) or the presence of licofelone in the indicated concentration. MM6 cells were perfused in the flow chamber for 4 min and adherent cells were counted. Values are mean ± S.E.M. of 10 experiments ($^{*}P < 0.05$ statistical significant vs. stimulated BAEC, $^{¥}P < 0.05$ statistical significant vs. non-stimulated BAEC). (b) Effect of different compounds or their combinations on rolling and adhesion of MM6 to BAEC. The cells were incubated with vehicle (negative control) or treated with TNF-α/IL-1β (10 ng/ml) for 4 h in the absence (positive control) or the presence of the indicated substance(s). MM6 cells were perfused in the flow chamber for 4 min and adherent cells were counted. Values are mean ± S.E.M. of 10 experiments ($^{*}P < 0.05$ statistical significant vs. stimulated BAEC, $^{¥}P < 0.05$ statistical significant vs. non-stimulated BAEC).

($n = 10$, $P < 0.005$). Similar inhibitory effects by licofelone were obtained when HUVEC and PMN were used instead of BAEC and MM6 (data not shown).

In contrast, no effects were found after treatment with the unselective COX-1/2 inhibitor indomethacin (30 μM) ($n = 5$), the potent and selective 5-LOX inhibitor ZD-2138 (30 μM) ($n = 6$), the COX-2 preferential NSAID aceclofenac (30 μM) ($n = 10$), the selective COX-2 inhibitor celecoxib (30 μM) ($n = 12$) and the combination of ZD-2138 with the selective COX-2 inhibitor celecoxib (30 μM) ($n = 6$) (Fig. 1b). However, the combination of ZD-2138 (30 μM) with aceclofenac (30 μM) caused a 41% reduction in rolling and a 54% reduction in adhesion ($n = 6$, $P < 0.005$) versus IL-1β and TNF-α treatment alone (10 ng/ml). Combinations of ZD-2138 (30 μM) with the unselective COX-1/2 inhibitor indomethacin (30 μM) resulted in a similar decrease. Taken together these findings suggest that combined 5-LOX/COX inhibition was necessary for inhibition of rolling and adhesion induced by inflammatory cytokines.

### 3.2. Effect of licofelone on endothelial CAM expression

In order to investigate if the antiadhesive effect of licofelone was mediated by changes in expression of

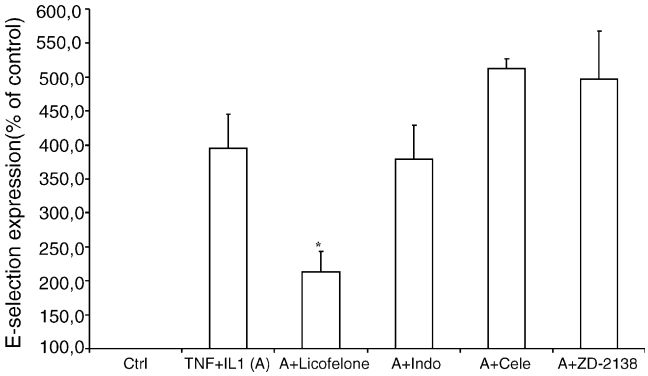


Fig. 2. Effect of NSAIDs (30 μM) on cytokine-induced (TNF-α/IL-1β; 10 ng/ml) expression of E-selectin in BAEC evaluated by RT-PCR semi-quantification (background-subtracted). Amplified sequences were resolved on gel and visualized by ethidium bromide staining, β-actin was used as internal control. The histogram shows the result of the quantification of three independent experiments. Densitometric analysis was performed with the Biorad Quantity One software. Values are mean ± S.E.M. ($^{*}P < 0.05$ vs. stimulated BAEC).

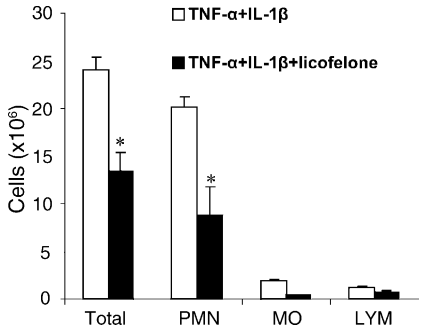


Fig. 4. Effect of licofelone on leukocyte recruitment in a mouse peritonitis model. Leukocyte extravasation into the peritoneal cavity was analyzed in mice after intraperitonical treatment with TNF-α and IL-1β (10 ng/ml) for 6 h (black bars). One group of animals simultaneously received licofelone (100 mg/kg) intraperitonically (white bars). Leukocytes harvested from the peritoneal cavity after 6 h were stained with sub-group specific PE-conjugated antibodies and differentiated according to forward and side scatter parameters and fluorescence intensity. Values are mean ± S.E.M. of four independent experiments ($^{*}P < 0.05$ vs. control).

adhesion molecules we assessed the mRNA levels for E-selectin in BAEC after cytokine stimulation with or without simultaneous treatment with licofelone. mRNA for E-selectin was significantly upregulated by cytokine treatment. Simultaneous treatment with licofelone (30 μM), but not other NSAIDs, significantly attenuated the expression of E-selectin mRNA ($n = 5, P < 0.05$, Fig. 2).

Because licofelone reduced the increase in expression of CAM mRNA in EC, we investigated the effect of licofelone on CAM expression on protein level. HUVEC treated with LPS (1 μg/ml) responded with strong expression of VCAM-1 ($n = 4$, $P < 0.002$) and ICAM-1 ($n = 4$, $P < 0.02$) (Fig. 3), which was attenuated by licofelone (30 μM) by 80% and 40%, but not other NSAIDs (data not shown), respectively. Similar results were found after treatment with IL-1β/TNF-α (10 ng/ml) and licofelone (30 μM) (data not shown). To exclude the possibility that inhibition of ICAM-1 and VCAM-1-expression seen with licofelone might have resulted from a cytotoxic effect of this compound on HUVEC we investigated viability by trypan blue exclusion. After 6 h, trypan blue exclusion was 96 ± 4% and 94 ± 2%, indicating that loss in cell viability does not account for changes in CAM expression on EC.

### 3.3. Effect of licofelone on leukocyte recruitment in vivo

The effect of licofelone on leukocyte recruitment in vivo was tested in a mouse peritonitis model. Leukocyte extravasation was induced by i.p. treatment with TNF-α and IL-1β (20 ng/ml). As seen in Fig. 4 combined treatment with licofelone (100 mg/kg) reduced the total cell count in the peritoneum, the majority of which were PMNs, from $24 \times 10^6$ cells in cytokine-treated mice to $13 \times 10^6$ cells (∼55%) ($n = 4$, $P < 0.05$). Only few monocytes and lymphocytes had extravasated in response to cytokine stimulation.

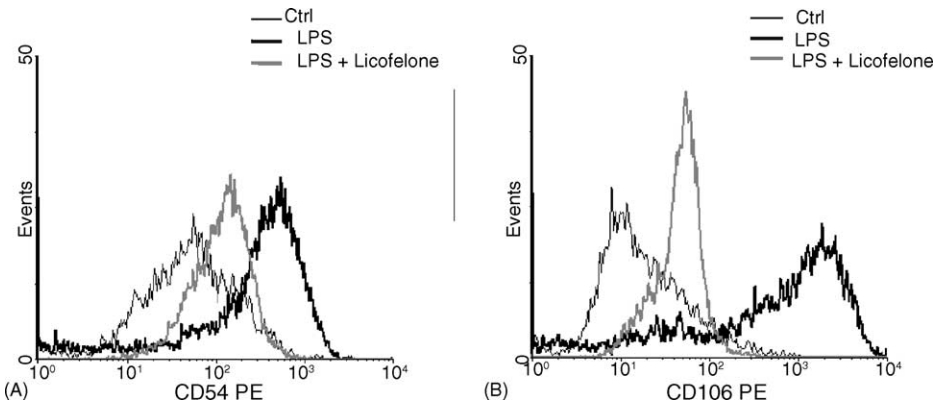


Fig. 3. Effect of licofelone on ICAM-1 and VCAM-1 expression on HUVEC. Representative FACS histograms are shown. (A) An overlay histogram for ICAM-1 (CD54); thin black line represents control, bold black represents LPS (1 μg/ml) stimulated expression of CD54 while bold grey line represents treatment with a combination of LPS (1 μg/ml) and licofelone (30 μM). (B) An overlay histogram for VCAM-1; thin black line represents control, bold black line represents LPS (1 μg/ml) stimulated expression of VCAM-1 (CD106), bold grey line represents a combination of LPS (1 μg/ml) treatment with licofelone (30 μM).

## 4. Discussion

NSAIDs are heterogeneous compounds that inhibit prostaglandin synthesis. However, it has been described that aspirin, as well as other NSAIDs, have additional important anti-inflammatory mechanisms. In the present study, we investigated the effect of a new combined 5-LOX/COX-inhibitor, licofelone, on inflammation-mediated recruitment of leukocytes in vitro and in vivo.

We found that licofelone, compared to other NSAIDs possesses a unique ability to inhibit leukocyte rolling and adhesion to the endothelium. Similar effects were observed only by the combination of the 5-LOX inhibitor ZD-2138 and the non-selective COX inhibitor aceclofenac or the combination of ZD-2138 and the unselective COX-1/2 inhibitor indomethacin. Thus, these experiments suggest that combined inhibition of 5-LOX and COX, conveniently achieved with licofelone, potently attenuates cytokine-induced leukocyte-endothelial interactions. The fact that both rolling and adhesion were affected suggests that licofelone may interfere with regulation of adhesion molecule expression on EC induced by cytokines. Indeed, transcriptional activity of E-selectin was attenuated by licofelone compared to the effects achieved by commonly used NSAIDs. This is in line with previous observations that licofelone is able to reduce E-selection expression on protein level [14].

Moreover, inflammatory expression of ICAM-1 and VCAM-1 was also inhibited by licofelone whereas other COX inhibitors were without effect (data not shown). Because 5-LOX is most probably not expressed by vascular endothelial cells like HUVEC or BAEC but only at minimal levels in pulmonary artery endothelial cells [15–17], the data indicate that the effects on CAM expression in EC are not related to its primary pharmacological target. These findings correspond to the studies of Patel et al. [18] who investigated the effects of (1) the 5-LOX inhibitor zileuton and (2) 5-LOX gene knockout (5-LOX(−/−)) mice on renal dysfunction and injury caused by renal ischemia/reperfusion (I/R) of the kidney in mice. Administration of zileuton before I/R significantly reduced the degree of renal dysfunction (urea, creatinine) and injury (AST, histology). In addition, zileuton reduced the expression of ICAM-1 and the associated PMN infiltration caused by I/R of the mouse kidney. Compared with wild-type mice, the degree of renal dysfunction, injury, and inflammation caused by I/R in 5-LOX (−/−) mice was also significantly reduced.

However, the precise mechanism of action of zileuton and licofelone remains to be elucidated. Especially the effect of licofelone on the proinflammatory transcription factor nuclear factor-κB (NF-κB) is interesting in this regard because NF-κB activation represents an essential pathway for the induction of numerous proinflammatory genes in the vascular wall, including CAMs and cytokines.

Herein we demonstrate that licofelone impedes leukocyte adhesion through interfering with CAM expression at the endothelial level. Previously, it has been shown that the compound attenuates chemoattractant-induced Mac-1 expression on leukocytes and reduces binding to endothelial counter-receptors [19]. These effects may both contribute to the markedly reduced leukocyte accumulation found after licofelone treatment in the mouse peritonitis model in vivo. Importantly, this in vivo effect was achieved at a clinically relevant dose of licofelone [20].

In conclusion, we have shown that the novel 5-LOX/COX inhibitor licofelone possesses unique anti-inflammatory effects compared to other NSAIDs. These effects appear to be mediated not only by its known pharmacological properties but also by as yet undetermined mechanisms of action. Thus, our results together with previously reported data indicate an interesting coexistence, within the same molecule, of a broad spectrum of anti-inflammatory pharmacological properties that may increase its therapeutic value in clinically related diseases.

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