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Licofelone, a dual lipoxygenase–cyclooxygenase inhibitor, downregulates polymorphonuclear leukocyte and platelet function

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Abstract

Polymorphonuclear leukocytes are strongly implicated in the pathogenesis of inflammatory disease. Polymorphonuclear leukocyte recruitment at sites of inflammation, mainly sustained by the β2-integrins, is followed by the synthesis and release of inflammatory mediators, such as leukotrienes, proteolytic enzymes and reactive oxygen species. Functional and metabolic interactions between polymorphonuclear leukocytes and platelets can contribute to and exacerbate the process. The effects of the dual 5-lipoxygenase and cyclooxygenase inhibitor licofelone ([2,2-dimethyl-6-(4-chlorophenyl)-7-phenyl-2,3-dihydro-1*H*-pyrrolizine-5-yl]-acetic acid) were studied on arachidonic acid transcellular metabolism occurring between polymorphonuclear leukocytes and platelets. The formation of leukotriene C_4, a leukotriene A_4-derived metabolite, by mixed polymorphonuclear leukocyte/platelet suspensions stimulated with 10 μM A23187 was inhibited by licofelone with an IC_{50} of 3.8 ± 0.07 μM. The formation of 5,12-di-hydroxy-eicosatetraenoic acid (HETE) was abolished at concentrations ≥10 μM. Licofelone also inhibited the generation of reactive oxygen species by polymorphonuclear leukocytes stimulated with 1 μM *n*-formyl-methionyl-leucyl-phenylalanine (fMLP), 10 nM complement fraction 5a (C5a) and 1 μM platelet activating factor (PAF) with IC_{50}s of 24.4 ± 0.6, 11.0 ± 1.5 and 11.7 ± 1.2 μM; elastase release induced by the three agonists was inhibited with IC_{50}s of 12.2 ± 2.2, 23.5 ± 8 and 2.6 ± 1 μM, respectively. Homotypic polymorphonuclear leukocyte aggregation induced by fMLP, C5A and PAF was inhibited by licofelone with IC_{50}s of 23.7 ± 4.8, 15.6 ± 3.4 and 15.4 ± 4 μM, respectively. The present study extends the anti-lipoxygenase and anti-cyclooxygenase activities of licofelone to the production of arachidonic acid metabolites generated as a consequence of polymorphonuclear leukocyte-platelet transcellular metabolism and to polymorphonuclear leukocyte responses relevant to the pathogenesis of inflammation. The coexistence within the same molecule of a wide spectrum of anti-inflammatory properties is of interest.

Keywords: Inflammation; Polymorphonuclear leukocyte, human; 5-Lipoxygenase; Cyclooxygenase; Transcellular metabolism; Adhesive interaction

1. Introduction

Polymorphonuclear leukocytes are heavily involved in the evolution and progression of inflammatory diseases. Bacterial peptides, chemokines, lipid mediators and complement fractions, generated at or near the site of the lesion, promote polymorphonuclear leukocyte recruitment in the microcirculation, an essential early event for subsequent polymorphonuclear leukocyte migration through the vessel wall and accumulation at sites of damaged or inflamed tissues. Polymorphonuclear leukocyte recruitment is fully accomplished through homotypic and heterotypic cell–cell adhesive interactions, sustained by adhesion receptors, including the β_2-integrin Mac-1 (CD11b/CD18) (Tonnesen et al., 1989). This molecule is in part constitutively expressed on the surface of polymorphonuclear leukocytes and is also stored in secretory granules. Upon activation by inflammatory stimuli, rapid translocation of Mac-1 from intracellular pools to the cell surface occurs (upregulation) and, in parallel, conformational changes take place within the molecule to allow competent binding to its counter-receptors on polymorphonuclear leukocytes (homotypic cell adhesion), as well as on platelets and on the vascular cell surface (heterotypic cell adhesion) (Arnaout, 1990).

Activated polymorphonuclear leukocytes synthesize and release a variety of mediators that contribute in a concerted manner to the evolution and propagation of the

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inflammatory reaction (Henderson, 1994; Doring, 1994; Cuzzocrea et al., 2001). Through the 5-lipoxygenase pathway, polymorphonuclear leukocytes directly metabolise arachidonic acid to leukotriene B_4, the most potent chemotactic agent for leukocytes (Lewis et al., 1990). Arachidonic acid transcellular metabolism represents an important aspect of platelet/polymorphonuclear leukocyte cross-talk, relevant in the pathogenesis of inflammatory disease (Cerletti et al., 1999). In vitro, activated platelets significantly increase polymorphonuclear leukocyte leukotriene B_4 synthesis, and polymorphonuclear leukocytes increase platelet thromboxane B_2 synthesis, by providing each other with free arachidonic acid (Marcus et al., 1982; Palmantier and Borgeat, 1991; Maugeri et al., 1992). Vasoactive peptido-leukotrienes, such as leukotriene C_4, can be synthesised from arachidonic acid by white cell populations, with the exception of polymorphonuclear leukocytes. However, platelets contribute to leukotriene C_4 production by metabolising polymorphonuclear leukocyte-derived leukotriene A_4 through their own glutathione-*S*-transferase (Lewis et al., 1990). Finally, 5,12-di-hydroxy-eicosatetraenoic acid (HETE) is formed by leukocytes and platelets in mixed suspension, probably by oxygenation of 5-HETE by platelet 12-lipoxygenase, or of 12-HETE by leukocyte 5-lipoxygenase. Its biological activities have hardly been investigated. However, consistent data exist on the ability of this metabolite to inhibit platelet activation, probably by interacting at the thromboxane A_2/prostaglandin H_2 receptor, resulting in feedback regulation of platelet function (Croset and Lagarde, 1983; Mais et al., 1990).

Polymorphonuclear leukocytes are also the major source of oxygen radical species and of matrix-degrading proteases, such as elastase, that are rapidly produced and secreted in the extracellular milieu during the respiratory burst and granule secretion. These products mediate cellular injury by initiating lipid peroxidation, altering vascular permeability and activating vascular and circulating cells (Totani et al., 1998; Finkel, 1998; Vaday and Lider, 2000).

Licofelone ([2,2-dimethyl-6-(4-chlorophenyl)-7-phenyl-2,3-dihydro-1*H*-pyrrolizine-5-yl]-acetic acid, MW 379.89, previously named ML3000) is a dual inhibitor of cyclooxygenase and 5-lipoxygenase in bovine and human platelets and granulocytes in vitro (Laufer et al., 1994a). It was specifically developed in a search for compounds with similar analgesic and anti-inflammatory activity, but higher gastrointestinal safety than classical non-steroidal anti-inflammatory drugs (NSAIDs). In several experimental animal models, the compound has shown anti-inflammatory, analgesic, antipyretic and antiasthmatic effects (Laufer et al., 1994b, 1995; Abraham et al., 1997) at dosages (10–100 mg kg^{-1}) that do not cause any gastrointestinal damage (Wallace et al., 1994; Laufer et al., 1994c) or general pharmacological effects (Algate et al., 1995). Licofelone is currently under clinical evaluation for the treatment of osteoarthritis.

The present study confirms the anti-lipoxygenase activity of licofelone and extends its inhibitory effect to the production of 5-lipoxygenase metabolites generated as a consequence of platelet–polymorphonuclear leukocyte transcellular metabolism. Moreover, this drug was able to block superoxide anion generation, lysosomal granule secretion and Mac-1-mediated homotypic polymorphonuclear leukocyte aggregation, three relevant polymorphonuclear leukocyte responses to inflammatory agonists. These data provide new insight into the anti-inflammatory action of licofelone.

2. Materials and methods

2.1. Materials

Licofelone was kindly provided by Merckle (Ulm, Germany); (*R*)-2[4-(quinolin-2-yl-methoxy)phenyl]-2-cyclopentyl acetic acid (BAY X1005) and diclofenac were kindly provided by Bayer (Wuppertal, Germany) and by Alfa Wassermann (Alanno, Italy), respectively.

Ferric cytochrome *c*, *n*-formyl-methionyl-leucyl-phenylalanine (fMLP), calcium ionophore A23187, complement fraction 5a (C5a), prostaglandin E_1, cytochalasin B, HEPES, EGTA, *N*-succinyl-Ala-Ala-Val-*p*-nitroanilide and fluorescein isothiocyanate (FITC)-conjugated mouse monoclonal antibodies against Mac-1 -clone 44, immunoglobulin (Ig) G1- and against E-selectin (clone 1.2B6, IgG1) were purchased from Sigma (St. Louis, MO, USA); Platelet activating factor (PAF) was purchased from Calbiochem (Calbiochem–Novabiochem, La Jolla, CA, USA); hydroethidine was purchased from Molecular Probes Europe (Leiden, The Netherlands). Paraformaldehyde was from Fluka (Milano, Italy); dextran T500 and Ficoll-Hypaque were from Pharmacia Fine Chemicals (Uppsala, Sweden).

Licofelone and A23187 were dissolved in dimethyl sulfoxide (DMSO); BAY X1005 was dissolved in ethanol; diclofenac was dissolved in bidistilled water; fMLP and C5a were dissolved in saline; PAF was dissolved in 0.15 M Tris–HCl buffer containing 37 mM bovine albumin. Addition of the organic solvents, DMSO or ethanol to the polymorphonuclear leukocyte suspension (up to 0.5% of the suspension volume) did not affect any of the polymorphonuclear leukocyte functions studied.

Solvents for high-performance liquid chromatography (HPLC) were all pure grade.

2.2. Methods

Blood (anticoagulated with 3.8% of trisodium citrate, 9:1, v/v) was collected from the antecubital vein of adult healthy volunteers who had not received any medication for at least 2 weeks under informed consent.

Platelets and polymorphonuclear leukocytes were isolated from whole blood using standard procedures (Evangelista et al., 1991). Briefly, platelet-rich plasma was

prepared by centrifuging whole blood at 250 × *g* for 25 min. Platelets were separated from platelet-rich plasma by centrifugation in the presence of 2 μM prostaglandin E_1, and the pellet was then washed with 10 ml of HEPES–Tyrode buffer (129 mM NaCl, 9.9 mM $NaHCO_3$, 2.8 mM KCl, 0.8 mM KH_2PO_4, 5.6 mM dextrose, 10 mM HEPES) containing 2 μM prostaglandin E_1 and 5 mM EGTA.

Polymorphonuclear leukocytes were isolated from the remaining blood by dextran sedimentation followed by Ficoll-Hypaque gradient and hypotonic lysis of erythrocytes.

All the procedures were performed at room temperature and at 4 °C for platelets and for polymorphonuclear leukocyte isolation, respectively.

Platelets and polymorphonuclear leukocytes were resuspended in HEPES–Tyrode buffer (pH 7.4), containing 1 mM $CaCl_2$ and 1 mM of $MgCl_2$.

2.3. Lipoxygenase and cyclooxygenase metabolism in mixed cell suspension

Polymorphonuclear leukocyte and platelet coincubation, extraction of leukotrienes and HPLC analysis were performed using previously reported procedures (Celardo et al., 1994; Evangelista et al., 1999).

Briefly, mixed cell suspensions of platelets and polymorphonuclear leukocytes (1×10^8 and 0.5×10^7, respectively, in 1 ml) were preincubated in siliconised glass cuvettes with DMSO (control, 0.5% final concentration) or with different concentrations of licofelone for 15 min at room temperature. Samples were thereafter placed in a lumiaggregometer (Platelet Ionized Calcium Aggregometer, Chronolog, Mascia Brunelli, Milan, Italy) at 37 °C under constant stirring at 1000 rpm, achieved with an iron bar (4 mm long) rotating under a magnetic field and stimulated with 10 μM calcium ionophore A23187 for 5 min at 37 °C.

The reaction was stopped by addition of two volumes of ice-cold methanol and the sample was extracted on Sep-Pak C_{18} cartridges (Waters Spa, Vimodrone, Italy) pretreated with 10 ml each of methanol, water and 0.1% ethylendiaminetetraacetic acid. Prostaglandin B_2 was added as internal standard before extraction with *n*-hexane, followed by methanol, 5 ml each.

2.4. HPLC analysis

The Sep-Pak eluate was dehydrated under nitrogen and extracts were reconstituted in the mobile phase, consisting of methanol, acetonitrile and acetic acid (0.1% in deionised water, adjusted to pH 5.6 with NH_4OH) in the ratio 60:5:35, v/v/v. Reconstituted samples were thereafter injected into an automated Reversed Phase-HPLC system (RP-HPLC System Gold Nouveau, Beckman Instruments, San Ramon, USA), consisting of a dual-pump programmable solvent module and Diode Array detector, equipped with a personal system Windows 98.

A 4-μm Superspher Hibar LichroCart column (244 × 4.0 mm internal diameter, Merck, Darmstad, Germany) was used.

Leukotrienes were eluted at a flow rate of 0.5 ml min^{-1} and identified on the basis of retention time and UV spectra at 280 nm of authentic standards. Quantification was performed by using a plot of the ratio between the peak area of the analyte and the peak area of the internal standard versus known concentrations of the analyte in a reference curve.

Data are reported as percentages of leukotrienes produced by mixed cell suspensions stimulated with A23187 in the presence of the drug versus control mixed cell suspensions stimulated in the presence of the vehicle, DMSO (% of control).

Thromboxane B_2, the stable analogue of platelet thromboxane A_2, was measured by radioimmunoassay, using a specific antibody (kindly provided by Dr. Giovanni Ciabattoni, University of Chieti, Chieti, Italy).

2.5. Superoxide anion production

Superoxide anion production was determined by measuring the reduction of ferric cytochrome *c* (Bazzoni et al., 1991). Polymorphonuclear leukocytes, in the presence of 2.5 μg/ml cytochalasin B and 20 nmol ferric cytochrome *c*, were preincubated in a 96-well microtiter plate with DMSO (control) or with different concentrations of licofelone for 10 min at room temperature, before stimulation with 1 μM fMLP, 10 nM C5a, or 1 μM PAF for 40 min. Basal superoxide anion production from unstimulated PMN was assessed in samples incubated with DMSO, cytochalasin B and ferric cytochrome *c*, but without stimulus. Thereafter, absorbance was measured simultaneously at 550 and 540 nm with a Multiskan spectrophotometer (Titertek, Flow Laboratories, Great Britain), and the resulting ratio was expressed as nanomoles of ferric cytochrome *c* reduced by 10^6 PMN in 40 min.

2.6. Polymorphonuclear leukocyte homotypic aggregation

Samples of washed polymorphonuclear leukocytes (500 μl, 1×10^7 ml^{-1}) in HEPES–Tyrode buffer containing $CaCl_2$ 1 mM were incubated with the vehicle DMSO or with different concentrations of licofelone for 10 min at room temperature. Cytochalasin B (2.5 μg/ml) was then added and the cell suspension was stimulated with 1 μM fMLP, 10 nM C5a, or 1 μM PAF as above described. Aggregation was recorded for 3 min as the change in light transmission. Fifty-microliter aliquots of the polymorphonuclear leukocyte suspension were then fixed with paraformaldehyde (1% f.c.) and single, nonaggregated polymorphonuclear leukocytes were counted under contrast light microscopy. The numbers of single polymorphonuclear leukocytes counted before and after stimulation were used to calculate the percentage of aggregated polymorphonuclear leukocytes. The remaining polymorphonuclear

leukocyte suspension was cooled at 0 °C and centrifuged for 3 min at 14,000 rpm in an Eppendorf centrifuge; the supernatant was stored at −20 °C for measurement of elastase release.

2.7. Polymorphonuclear leukocyte cellular elastase release

Elastase activity, released into supernatants from polymorphonuclear leukocytes activated with fMLP, C5a, or PAF in the presence of cytochalasin B (2.5 μg ml^{-1}), was assayed spectrophotometrically by monitoring the rate of release of *p*-nitroanilide from the specific chromogenic substrate *N*-succinyl-Ala-Ala-Val-*p*-nitroanilide at 410 nm in 5 min (Evangelista et al., 1991). Elastase concentration was extrapolated from a standard curve of known concentrations of purified human elastase.

2.8. Cytofluorimetric analysis of Mac-1 expression and activation

Samples of washed polymorphonuclear leukocytes (500 μl, 1×10^7 ml^{-1}) in HEPES–Tyrode buffer containing $CaCl_2$ 1 mM were incubated with the vehicle DMSO or with different concentrations of licofelone for 10 min at room temperature, and then stimulated for 3 min with 1 μM fMLP, 10 nM of C5a, or 1 μM PAF at 37 °C under constant stirring at 1000 rpm in the lumiaggregometer. After stimulation, 100-μl aliquots were incubated with a FITC-conjugated anti-Mac-1 mouse monoclonal antibody for 30 min at room temperature. Incubation was stopped by addition of 1 ml of ice-cold HEPES–Tyrode buffer. The FITC-conjugated anti-E-selectin antibody was used as negative control staining. Polymorphonuclear leukocyte fluorescence was analysed by using a FACStar flow cytometer (Becton Dickinson) with an argon-ion laser operated at 400 mW output power as previously described (Evangelista et al., 1996). Acquisition and processing of data from 5000 cells were carried out with a Consort 30 Program (Becton Dickinson) on a Hewlett Packard 300 computer.

Labelling with anti-Mac-1 antibody was evaluated as mean fluorescence intensity in arbitrary units.

2.9. Cell viability

Trypan blue exclusion was monitored to assess polymorphonuclear leukocyte viability over a 30-min period of treatment with DMSO (control) or with the highest concentration of licofelone (100 μM). After 30 min, trypan blue exclusion was 95 ± 3% and 94 ± 2% (mean ± S.E.M., $n = 3$) in control and in licofelone-treated cells, respectively.

2.10. Statistical analysis

Data are presented as means ± S.E.M. for the indicated number of independently performed experiments. Statistical analysis was done by one-way analysis of variance (ANOVA) followed by Dunnett's test; a P value of 0.05 or less was considered significant. IC_{50} values (i.e. the concentration of licofelone required to produce 50% of inhibition of maximal response) were calculated using a Mac Allfit computer program (Consorzio Mario Negri Sud, Santa Maria Imbaro, Italy).

3. Results

3.1. Effect of licofelone on arachidonate metabolism in mixed polymorphonuclear leukocyte-platelet suspensions

The production of arachidonic acid metabolites of lipoxygenase and cyclooxygenase was evaluated in mixed platelet–polymorphonuclear leukocyte suspensions challenged with the calcium ionophore A23187; this is the stimulus of choice for a higher and more reproducible synthesis of arachidonic acid metabolites in mixed cell suspensions (Evangelista et al., 1999). While leukotriene B_4 and 6- plus 12-*trans* leukotriene B_4 are the polymorphonuclear leukocyte intracellular enzymatic and the extracellular non-enzymatic metabolites of leukotriene A_4, respectively, leukotriene C_4 is the transcellular metabolite synthesised by platelets from leukocyte-derived leukotriene A_4 through their own glutathione-*S*-transferase (Lewis et al., 1990). Finally, 5,12-diHETE is the mixed product of both polymorphonuclear leukocyte 5-lipoxygenase and platelet 12-lipoxygenase (Marcus et al., 1982).

Leukotriene B_4 (135 ± 11 ng) and 6- plus 12-*trans* leukotriene B_4 (87 ± 4 ng) (means ± S.E.M., $n = 5$) were generated by A23187-stimulated mixed cell suspensions of polymorphonuclear leukocytes (0.5×10^7) and platelets (1×10^8). The production of both was inhibited by licofelone in a concentration-dependent manner with IC_{50}s of 6.7 ± 0.9 and 3.9 ± 0.8 μM, respectively, in agreement with the reported anti-lipoxygenase activity of the drug (Laufer et al., 1994a).

Mixed cell suspensions stimulated by A23187 also produced 275 ± 52 ng of leukotriene C_4 and 213 ± 41 ng of 5,12-diHETE (means ± S.E.M., $n = 5$); in contrast, neither polymorphonuclear leukocyte nor platelet suspensions stimulated separately under comparable conditions produced detectable amounts (1 ng/ml) of leukotriene C_4 or 5,12-diHETE.

The formation of leukotriene C_4 was inhibited by licofelone with an IC_{50} of 3.8 ± 0.07 μM. In contrast, the formation of 5,12-diHETE was not inhibited by licofelone at concentrations up to 5 μM, but was abolished at a drug concentration of 10 μM (Figs. 1 and 2). These results suggest that the activity of 5-lipoxygenase, which catalyses the formation of leukotriene A_4 from 5-hydroperoxy-eicosatetraenoic acid (HPETE), is inhibited by licofelone at lower concentrations than the step going from the latter to

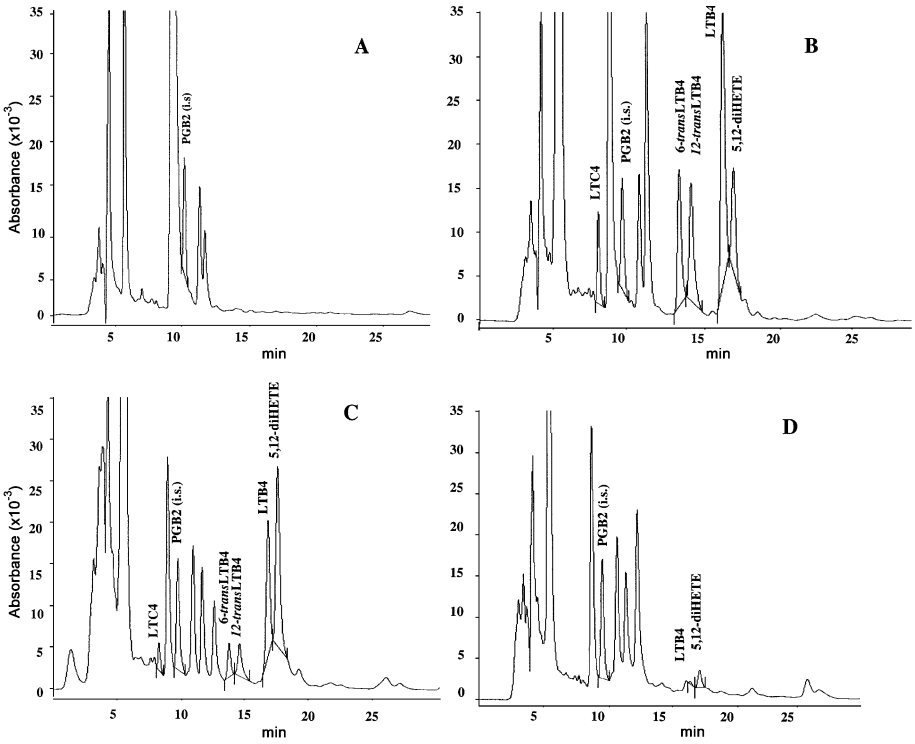

Fig. 1. Chromatograms of HPLC analysis of lipoxygenase metabolites formed by mixed polymorphonuclear leukocyte-platelet suspensions. (A) Unstimulated sample; (B) stimulated with 10 μM A23187, in the presence of the vehicle DMSO; (C) and (D) stimulated in the presence of 5 and 10 μM licofelone, respectively. PGB_2 = Prostaglandin B_2; i.s. = internal standard. Representative of five experiments.

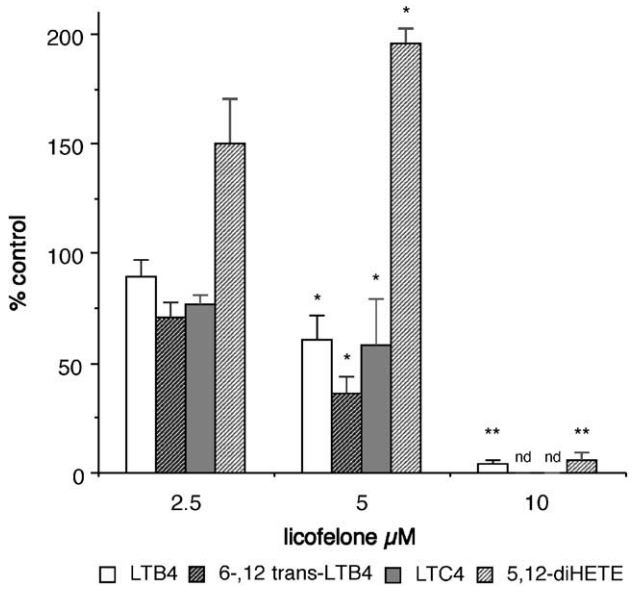

Fig. 2. Effect of licofelone on the production of lipoxygenase metabolites in mixed polymorphonuclear leukocyte-platelet suspensions. Polymorphonuclear leukocytes and platelets (0.5×10^7 and 1×10^8, respectively in 1 ml sample) were preincubated with DMSO (0.5% final concentration, control) or with licofelone for 15 min at room temperature. Samples were thereafter placed in the aggregometer and stimulated with 10 μM A23187 for 5 min at 37 °C under stirring (means ± S.E.M., $n = 5$). * $P < 0.05$, significantly different as compared to control by ANOVA followed by Dunnett's test; nd, not detectable.

leukotriene A_4. Under these conditions, 5-HPETE accumulates and more 5,12-diHETE can be formed. At higher concentrations of licofelone, the activity of both 5-lipoxygenase steps is comparably hampered and the formation of all metabolites is blocked.

The effect elicited by licofelone in the high-concentration range is comparable to that of a well-characterised 5-lipoxygenase inhibitor, BAY X1005, which inhibits 5-lipoxygenase translocation from the cytosol to the membrane (Hatzelmann et al., 1993) and thereby reduces the formation of all metabolites at comparable concentrations (IC_{50} 1.1 ± 0.8 μM for leukotriene B_4 and leukotriene C_4, and 0.74 ± 0.8 μM for 6- plus 12-*trans* leukotriene B_4).

The NSAID diclofenac (Todd and Sorkin, 1988) had no effect on leukotriene production in mixed cell suspensions at concentrations as high as 100 μM (not shown).

Thromboxane B_2 production by stimulated mixed polymorphonuclear leukocyte-platelet suspensions was reduced by licofelone in a concentration-dependent manner (3.4 ± 1.5% of control, mean ± S.E.M., $n = 3$) and was almost completely abolished at 10 μM.

3.2. Effect of licofelone on reactive oxygen species produced by stimulated polymorphonuclear leukocytes

Superoxide dismutase-inhibitable ferric cytochrome *c* reduction was used to specifically assess the effect of

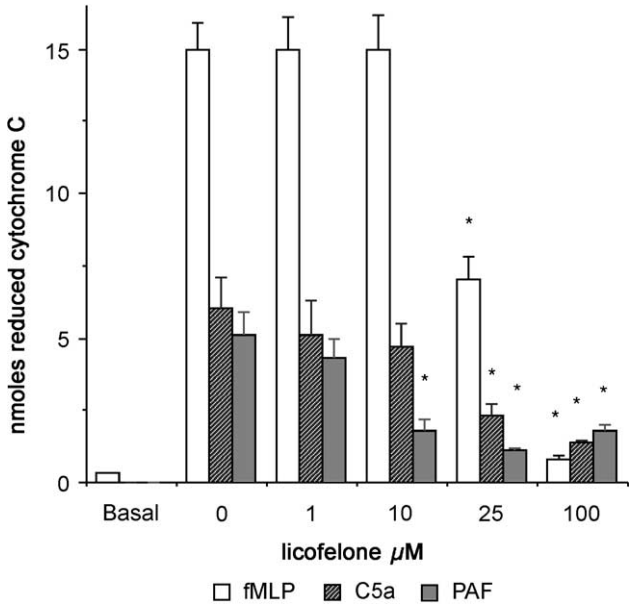

Fig. 3. Effect of licofelone on superoxide anion produced by stimulated polymorphonuclear leukocytes. Polymorphonuclear leukocytes were pre-incubated without or with licofelone for 10 min at room temperature in the presence of 2.5 μg/ml cytochalasin B and 20 nmol of ferric cytochrome *c* before stimulation with 1 μM fMLP, 10 nM C5a, or 1 μM PAF for 40 min. Basal value was obtained in the absence of stimulus. Values are reported as nmol of ferric cytochrome *c* reduced by 1×10^6 polymorphonuclear leukocytes in 40 min of stimulation (means ± S.E.M., $n = 4$). * $P < 0.05$, significantly different as compared to control, by ANOVA followed by Dunnett's test.

licofelone on superoxide anion production by stimulated polymorphonuclear leukocytes. Basal superoxide anion production by unstimulated polymorphonuclear leukocytes (1×10^6, in 40 min) generated 0.3 ± 0.06 nmol of reduced cytochrome *c* (mean ± S.E.M., $n = 4$). This value rose to 15 ± 0.9, 6 ± 1 and 5.1 ± 0.8 ($n = 4$) upon stimulation with 1 μM fMLP, 10 nM C5a and 1 μM PAF, respectively. Licofelone inhibited the generation of superoxide anion induced by the three stimuli in a concentration-dependent manner (Fig. 3), with IC_{50}s of 24.4 ± 0.6, 11 ± 1.5 and 11.7 ± 1.2 μM, respectively.

BAY X1005 and diclofenac were also able to reduce superoxide anion production by fMLP-stimulated polymorphonuclear leukocytes, but at higher concentrations. The calculated IC_{50}s were 45.5 ± 7.4 and 60.5 ± 13.0 μM for BAY X1005 and diclofenac, respectively.

Superoxide anion production induced by C5a was only affected by BAY X1005 and diclofenac at the highest concentration tested. The amount of reduced cytochrome *c* dropped from 6.4 ± 0.1 to 3.2 ± 0.08 nmol ($P < 0.05$, $n = 3$), and from 8.6 ± 1.1 to 4 ± 0.3 nmol ($P < 0.05$, $n = 3$) in the presence of 100 μM of BAY X1005 and diclofenac, respectively.

PAF-induced superoxide anion production was not affected either by BAY X1005 or by diclofenac.

3.3. Effect of licofelone on polymorphonuclear leukocyte elastase release

Unstimulated polymorphonuclear leukocytes (500 μl at 1×10^7 ml^{-1}) preincubated with DMSO for 8 min at room temperature and for further 5 min under stirring at 1000 rpm at 37 °C released elastase activity corresponding to 16.7 ± 1.3 nM enzyme (mean ± S.E.M., $n = 6$). Stimulation with 1 μM fMLP, 10 nM C5a, or 1 μM PAF (controls) caused a massive release of the enzyme. Preincubation with licofelone significantly inhibited elastase release induced by all three agonists in a concentration-dependent manner, reaching values close to that for unstimulated polymorphonuclear leukocytes at 100 μM licofelone (Fig. 4). The calculated IC_{50}s of licofelone for elastase release induced by fMLP, C5a and PAF were 12.2 ± 2.2 ($n = 7$), 23.5 ± 8 ($n = 3$) and 2.6 ± 1 ($n = 4$) μM, respectively.

At concentrations higher than those of licofelone, BAY X1005 and diclofenac also hampered elastase release by stimulated polymorphonuclear leukocytes. FMLP-induced elastase release was inhibited by the drug with IC_{50}s of 24.7 ± 7.0 and 50.0 ± 0.7 μM ($n = 3$) for BAY X1005 and diclofenac, respectively.

Elastase release induced by C5a was not significantly reduced from 260 ± 39 to 218 ± 59 nM ($n = 3$), and from

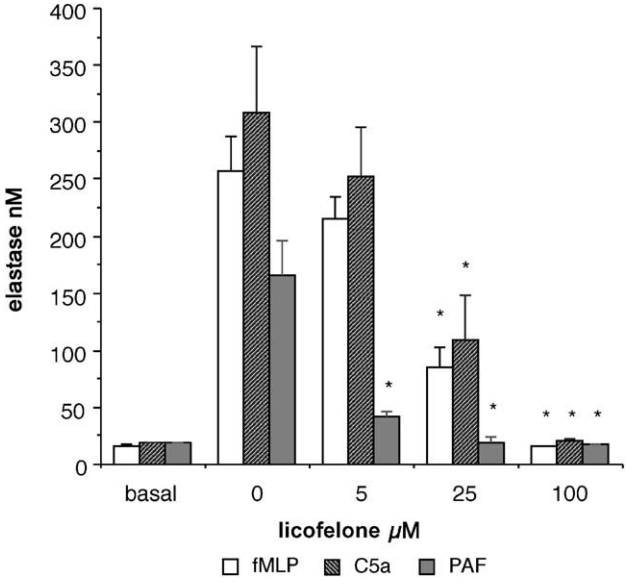

Fig. 4. Effect of licofelone on elastase activity released by stimulated polymorphonuclear leukocytes. Polymorphonuclear leukocytes (1×10^7/ml, 500 μl) were preincubated without or with licofelone for 10 min at room temperature in the presence of 2.5 μg/ml cytochalasin B, before stimulation with 1 μM fMLP, 10 nM C5a, or 1 μM PAF at 37 °C under stirring. After 3 min, samples were centrifuged and supernatants were used to measure enzyme release, as described under Methods. Basal value was obtained in the absence of stimulus. Elastase is reported as nM enzyme released in the supernatant from 0.5×10^7 cells (means ± S.E.M., $n = 4–7$). * $P < 0.05$, significantly different as compared to control by ANOVA followed by Dunnett's test.

313 ± 60 to 167 ± 33 nM (n = 3) by 100 μM of BAY X1005 and diclofenac, respectively.

The elastase release induced by PAF was inhibited by BAY X1005 and diclofenac with IC_{50}s of 66 ± 7 (n = 4) and 98.6 ± 4 (n = 4), μM, respectively.

3.4. Effect of licofelone on polymorphonuclear leukocyte homotypic aggregation

Stimulation of polymorphonuclear leukocyte suspensions for 3 min under constant stirring at 1000 rpm induced the formation of homotypic aggregates, monitored as an increase in light transmission in a lumiaggregometer and evaluated by counting single, nonaggregated polymorphonuclear leukocytes under an optical microscope, after fixation of 50-μl aliquots of the same polymorphonuclear leukocyte suspension with 2% paraformaldehyde (1:1, v/v). Single polymorphonuclear leukocyte counts before and after stimulation were used to calculate the percentage of aggregated polymorphonuclear leukocytes. After 3 min of stimulation with 1 μM fMLP, 10 nM C5a, or 1 μM PAF, the percentages of aggregated polymorphonuclear leukocytes were 83 ± 2.3 (mean ± S.E.M., n = 8), 67 ± 5 (n = 9) and 51 ± 6 (n = 6), respectively. Licofelone reduced the increase in light transmission induced by stimulation of polymorphonuclear leukocytes in a concentration-dependent manner and decreased in parallel the percentage of aggregated polymorphonuclear leukocytes (Fig. 5). At licofelone ≥ 50 μM, most polymorphonuclear leukocytes observed by optical microscopy appeared as single and round shaped, similar to those observed in unstimulated samples. The calculated IC_{50}s of licofelone for polymorphonuclear leukocyte homotypic aggregation induced by fMLP, C5a and PAF were 23.7 ± 4.8 (n = 8), 15.6 ± 3.4 (n = 9) and 15.4 ± 4 (n = 6) μM, respectively.

Neither BAY X1005 nor diclofenac affected polymorphonuclear leukocyte homotypic aggregation induced by fMLP, even at the highest concentration tested (100 μM).

Polymorphonuclear leukocyte aggregation induced by C5a and PAF was barely affected by BAY X1005 and diclofenac. At 100 μM, BAY X1005 reduced the percentage of C5a-aggregated polymorphonuclear leukocytes from 61 ± 6% to 39 ± 5.6% of total (P < 0.05, n = 7), and PAF-aggregated polymorphonuclear leukocytes from 50.7 ± 5% to 23 ± 8% of total (P < 0.05, n = 6).

Diclofenac (100 μM) reduced C5a-aggregated polymorphonuclear leukocytes from 69 ± 6% to 35 ± 8% of total (P < 0.05, n = 6), and PAF-aggregated polymorphonuclear leukocytes from 50 ± 4% to 28 ± 4 % of total (P < 0.05, n = 6).

3.5. Effect of licofelone on the expression of the β2-integrin Mac-1

The expression of Mac-1 was evaluated as mean fluorescence intensity and reported as the percentage of the basal level of unstimulated polymorphonuclear leukocytes. Stimulation with fMLP, C5a, or PAF increased the surface expression of Mac-1 to 138 ± 31% (n = 5), 143 ± 12% (n = 3) and 160 ± 20% (n = 3) over unstimulated levels, respectively. Preincubation of polymorphonuclear leukocytes with increasing concentrations of licofelone resulted in a concentration-dependent inhibition of fMLP-induced Mac-1 expression, with a calculated IC_{50} of 30 ± 7.7 μM. Licofelone did not affect the upregulation of Mac-1 induced by C5a or PAF. Neither the inhibitor of 5-lipoxygenase, BAY X1005, nor the NSAID diclofenac affected Mac-1 expression, even at the highest concentration tested (100 μM, not shown).

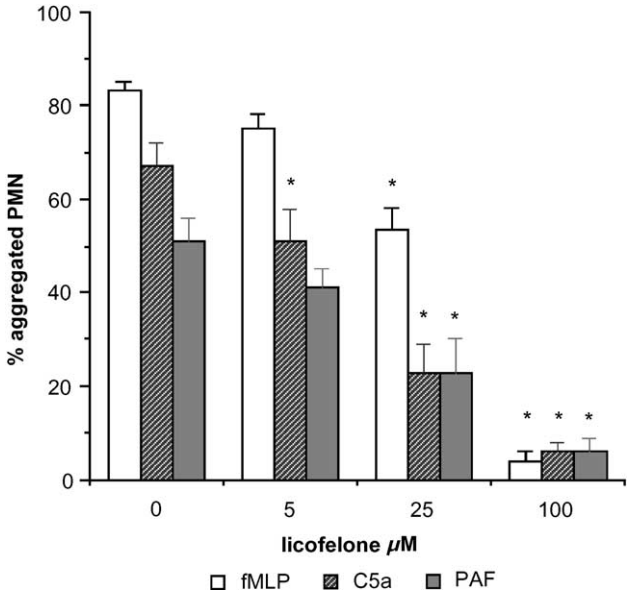

Fig. 5. Effect of licofelone on homotypic aggregation of polymorphonuclear leukocytes. Polymorphonuclear leukocytes (1×10^7/ml, 500 μl), preincubated without or with licofelone for 10 min at room temperature, were stimulated with 1 μM fMLP, 10 nM C5a, or 1 μM PAF for 3 min at 37 °C under stirring. Thereafter, 50-μl aliquots were fixed in 2% paraformaldehyde (1:1, v/v) and single, nonaggregated polymorphonuclear leukocytes were counted under an optical microscope. The number of single polymorphonuclear leukocytes counted before and after stimulation was used to calculate the percentage of aggregated polymorphonuclear leukocytes (means ± S.E.M., n = 6–9). PMN = polymorphonuclear leukocytes. * P < 0.05, significantly different as compared to control by ANOVA followed by Dunnett's test.

4. Discussion

In the present study, we describe newly observed inhibitory activities of licofelone, a pyrrolizine derivative previously characterised as a dual inhibitor of both cyclooxygenase and 5-lipoxygenase (Laufer et al., 1994a,b), on polymorphonuclear leukocyte function relevant to the pathogenesis of inflammatory processes. Indeed, we have found that licofelone not only prevents the synthesis of leukotriene B_4, a product of polymorphonuclear leukocyte lipoxygenase

activity, and of thromboxane B_2, a product of platelet cyclooxygenase, but also leukotriene C_4 and 5,12-diHETE, the products of transcellular metabolism of 5-lipoxygenase products between polymorphonuclear leukocytes and platelets (Marcus et al., 1982; Lewis et al., 1990).

Previous studies reported that licofelone inhibits leukotriene B_4 production by leukocytes, indicating the ability of the drug to interfere with the enzymatic activity of 5-lipoxygenase inside the cell (Laufer et al., 1994a). Here, we confirm and extend this observation, showing that this compound blocks the production of arachidonate metabolites specifically formed in a mixed cell system in which polymorphonuclear leukocytes cooperate with platelets in the generation of these inflammatory mediators (Brady and Serhan, 1992; Maugeri et al., 1994). At concentrations lower than 10 μM, licofelone inhibited the formation of leukotriene B_4 and the non-enzymatic derivatives 6- plus 12-*trans* leukotriene B_4, as well as the formation of the leukotriene A_4-derived transcellular metabolite leukotriene C_4; the drug enhanced the production of the mixed 5- and 12-lipoxygenase product, 5,12-diHETE. These results indicate that, over the low-concentration range, the drug selectively blocks the 5-lipoxygenase activity that converts 5-HPETE to leukotriene A_4, favoring the formation of 5-HPETE. At higher concentrations, in contrast, licofelone inhibits all 5-lipoxygenase activities.

In the second part of the study, licofelone was also found to inhibit in a similar manner polymorphonuclear leukocyte generation of superoxide anions, release of elastase and homotypic aggregation induced by fMLP, C5a and PAF.

When the effect of licofelone on the surface expression of Mac-1 was investigated, we found that the drug inhibited fMLP- but not C5a- or PAF-induced Mac-1 upregulation.

Homotypic aggregation of polymorphonuclear leukocytes is mainly mediated by the β_2-integrin Mac-1, whose function is regulated by the cell at two different levels. First, the surface expression of Mac-1 can be rapidly increased following exocytosis of Mac-1-storing granules. Second, like other β_2-integrins, Mac-1 requires functional upregulation mediated by so-called "inside-out" signalling, triggered by aggregating stimuli.

Taken together, our data indicate that licofelone mainly interferes with the "inside-out" signalling that allows competent binding of Mac-1 to its counterreceptors, which is necessary for efficient cell–cell adhesion to occur.

The mechanisms by which licofelone prevents polymorphonuclear leukocyte activation remain to be clarified. Leukotriene B_4 has been shown to mediate elastase release from azurophilic granules of polymorphonuclear leukocytes stimulated with A23187, but not with fMLP (Hatzelmann et al., 1994), suggesting that blockade of leukotriene B_4 synthesis does not necessarily account for the inhibition of azurophilic granule secretion.

In our system, the 5-lipoxygenase inhibitor BAY X1005 and the NSAID diclofenac did not affect, or inhibited to a lesser extent and at higher concentrations than licofelone, polymorphonuclear leukocyte activation triggered by all three stimuli. Their IC_{50}s, however, were much higher than those required to inhibit 5-lipoxygenase and cyclooxygenase enzyme, which were in the submicromolar range.

This is in agreement with previous observations of anti-inflammatory activities of 5-lipoxygenase inhibitors and of NSAIDs, as well as of dual lipoxygenase and cyclooxygenase inhibitors (Werner et al., 1993; Diaz-Gonzalez et al., 1995; Zhou et al., 1996; Pillinger et al., 1998), which are independent of their effect on the lipoxygenase- and cyclooxygenase-mediated pathway.

In conclusion, our results suggest that licofelone may interfere with the signalling pathway switched on by the G-protein-coupled receptors that lead to full polymorphonuclear leukocyte activation, independently of its anti-lipoxygenase and anti-cyclooxygenase activities.

An important question is whether the concentrations of the drug effective in vitro can be achieved in vivo. Pharmacokinetic data for rats indicate dose-dependent drug absorption and elimination (Deigner et al., 1995): peak plasma levels of about 17, 33 and 70 μg/ml were achieved within 2–3 h after single oral doses of 10, 30 and 100 mg/kg, respectively, of licofelone, corresponding to plasma concentrations ranging between 44 and 185 μM, higher than those effective in inhibiting polymorphonuclear leukocyte function in our experimental models (20–50 μM) in vitro. Thus, the inhibitory effects of licofelone on polymorphonuclear leukocyte function, observed in vitro, may also be achieved in vivo.

Although further studies are needed to clarify the cellular target(s) that explain the 5-lipoxygenase- and cyclooxygenase-independent effects of licofelone, our results indicate the interesting coexistence, within the same molecule, of a wide spectrum of anti-inflammatory pharmacological properties.

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