Licofelone Suppresses Prostaglandin E₂ Formation by Interference with the Inducible Microsomal Prostaglandin E₂ Synthase-1

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ABSTRACT

The anti-inflammatory drug licofelone [2-[(6-(4-chlorophenyl)-2,2-dimethyl-7-phenyl-2,3-dihydro-1H-pyrrolizin-5-yl) acetic acid], currently undergoing phase III trials for osteoarthritis, inhibits the prostaglandin (PG) and leukotriene biosynthetic pathway. Licofelone was reported to suppress the formation of PGE₂ in various cell-based test systems, but the underlying molecular mechanisms are not entirely clear. Here, we examined the direct interference of licofelone with enzymes participating in PGE₂ biosynthesis, that is, cyclooxygenase (COX)-1 and COX-2 as well as microsomal PGE₂ synthase (mPGES)-1. Licofelone concentration-dependently inhibited isolated COX-1 (IC₅₀ = 0.8 μM), whereas isolated COX-2 was less affected (IC₅₀ > 30 μM). However, licofelone efficiently blocked the conversion of PGH₂ to PGE₂ mediated by mPGES-1 (IC₅₀ = 6 μM) derived from microsomes of interleukin-1β-treated A549 cells, being about equipotent to 3-[1-(4-chlorobenzyl)-3-t-butyl-thio-5-isopropylindol-2-yl]-2,2-dimethylpropanoic acid (MK-886), a well recognized mPGES-1 inhibitor. In intact interleukin-1β-treated A549 cells, licofelone potently (IC₅₀ < 1 μM) blocked formation of PGE₂ in response to calcimycin (A23187) plus exogenous arachidonic acid, but the concomitant generation of 6-keto PGF₁α, used as a biomarker for COX-2 activity, was not inhibited. We conclude that licofelone suppresses inflammatory PGE₂ formation preferentially by inhibiting mPGES-1 at concentrations that do not affect COX-2, implying an attractive and thus far unique molecular pharmacological dynamics as inhibitor of COX-1, the 5-lipoxygenase pathway, and of mPGES-1.

Prostaglandins (PGs) and leukotrienes are powerful bioactive lipid mediators that are involved not only in numerous homeostatic biological functions but also in inflammation (Funk, 2001). The biosynthesis of PGs is initialized by COX isoenzymes, namely, COX-1, a constitutively expressed enzyme in numerous cell types thought to provide PGs mainly for physiological functions; and COX-2, an inducible isoform in inflammatory cells, primarily producing PGs relevant for inflammation, fever, and pain (Hawkey, 1999). After conversion of arachidonic acid to PGH₂ by COX enzymes, PGH₂ is subsequently isomerized by three different PGE₂ synthases to PGE₂. Whereas the cytosolic PGE₂ synthase (cPGES) and the membrane-bound PGE₂ synthase (mPGES)-2 are constitutive enzymes, the mPGES-1 is an inducible isoform (Samuelsson et al., 2007). Cotransfection experiments of COX-1/2 with PGES isoforms imply that select molecular interactions between COX and PGES isoforms cause preferential functional coupling (Murakami et al., 2000; Samuelsson et al., 2007). Thus, cPGES uses PGH₂ produced by COX-1, whereas mPGES-1 receives PGH₂ from COX-2.

PGE₂ plays a major role in the pathophysiology of inflammation, pain, and pyresis, but it also regulates physiological functions in the gastrointestinal tract, the kidney, and in the immune and nervous system (Smith, 1989). The nonsteroidal anti-inflammatory drugs (NSAIDs) reduce PGE₂ biosynthesis by inhibiting both COX isoenzymes, and they are potent

ABBRVIATIONS: PG, prostaglandin; COX, cyclooxygenase; cPGES, cytosolic prostaglandin E₂ synthase; mPGES, microsomal prostaglandin E₂ synthase; NSAID, nonsteroidal anti-inflammatory drug; FLAP, 5-lipoxygenase-activating protein; MK-886, 3-[1-(4-chlorobenzyl)-3-t-butyl-thio-5-isopropylindol-2-yl]-2,2-dimethylpropanoic acid; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; RP, reversed phase; HPLC, high-performance liquid chromatography; A23187, calcimycin; ELISA, enzyme-linked immunosorbent assay; 12-HHT, 12(8,10-10-trans-heptadecatrienoic acid; ANOVA, analysis of variance; HSD, honestly significant difference; BAY 1005, 2-(4-(quinolin-2-yl-methoxy)phenyl)-2-cyclopentylacetic acid; Indo, indomethacin; Cele, celecoxib; CV 4151, (E)-7-phenyl-(7-(3-pyriddy)-6-heptenoic acid.  

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suppressors of inflammation, fever, and pain (Funk, 2001). Chronic use of these drugs is associated with severe side effects, mainly gastrointestinal injury and renal irritations, apparently due to suppression of COX-1-derived PGE_{2} (Rainsford, 2007). COX-2-selective inhibitors were designed to minimize gastrointestinal complications of traditional NSAIDs, but recent clinical studies indicated small but significantly increased risks for cardiovascular events (McGettigan and Henry, 2006).

Licofelone (1H-[4-cho[0.1cm]lorophenyl]-2,2-dimethyl-7-phenyl-2,3-dihydro-1H-pyrrolizin-5-y] acetic acid; Fig. 1A) is an anti-inflammatory drug that inhibits the COX and 5-lipoxygenase pathway and is currently undergoing phase III trials for osteoarthritis (for review, see Celotti and Lafer, 2001; Kulkarni and Singh, 2007). The effectiveness of licofelone has been demonstrated in animal models as well as in studies on humans, and it is attributed mainly to the efficient suppression of PGE_{2} formation (Kulkarni and Singh, 2007). It is interesting to note that in contrast to NSAIDs and selective COX-2 inhibitors, licofelone shows an improved gastrointestinal and potentially cardiovascular safety (Bias et al., 2004; Rotondo et al., 2006; Vidal et al., 2007). This effect of licofelone might be attributable to the accompanied suppression of leukotrienes (Celotti and Lafer, 2001), which significantly contribute to gastric epithelial injury as well as to atherogenesis (Peters-Golden and Henderson, 2007).

Although the effectiveness and tolerability of licofelone as an anti-inflammatory drug are well documented, sparse data on its molecular mode(s) of action are available. We recently analyzed the molecular mechanism of the inhibition of the biosynthesis of 5-lipoxygenase products by licofelone, and we found that licofelone-mediated suppression of leukotriene synthesis (IC_{50} = 1.7 μM) is mainly related to its interference with 5-lipoxygenase (Fischer et al., 2007). MK-886, a well known FLAP inhibitor, was shown to inhibit mPGES-1 activity (Claveau et al., 2003). Because we hypothesized similarities between licofelone and MK-886, both in terms of structural conformation and mechanism of leukotriene synthesis inhibition (Fischer et al., 2007), we addressed the mode of action of licofelone in the suppression of PGE_{2} formation. Our data indicate that the potent inhibition of COX-2-mediated PGE_{2} formation by licofelone is seemingly a cause of interference with mPGES-1, rather than with COX-2.

**Materials and Methods**

**Materials.** Licofelone, bovine insulin, and anti-6-keto PGF_{1α} antibody were generous gifts by Merckle GmbH (Ulm, Germany), Sanofi-Aventis (Frankfurt, Germany), and Dr. T. Dingermann (University of Frankfurt, Frankfurt, Germany), respectively. The thromboxane synthase inhibitor CV4151 was synthesized according to Kato et al. (1985). Other materials and their sources are as follows: DMEM/high-glucose (4.5 g/l) medium, penicillin, streptomycin, trypsin/EDTA solution (PAA Laboratories GmbH, Coelbe, Germany); PGE_{2} (Larodan, Malmö, Sweden); 11β-PGE_{2}, PGB_{1}, MK-886, 6-keto PGF_{1α}, human recombinant COX-2, ovine-isolated COX-1 (Cayman Chemical, Ann Arbor, MI); [5,6,8,9,11,12,14,15-3H]arachidonic acid ([3H]arachidonic acid) (BioTrend Chemicals GmbH, Cologne, Germany); and Ultima Gold XR (PerkinElmer Life and Analytical Sciences, Boston, MA). All other chemicals were obtained from Sigma Chemie (Deisenhofen, Germany), unless stated otherwise.

**Cells and Cell Viability Assay.** Platelets were freshly isolated from leukocyte concentrates obtained at the Blood Center of the University Hospital Tuebingen (Tuebingen, Germany) as described previously (Albert et al., 2002). In brief, venous blood was taken from healthy adult donors that did not take any medication for at least 7 days and leukocyte concentrates were prepared by centrifugation (4000 g; 20 min; 20°C). Platelets were immediately isolated by dextransedimentation and centrifugation on Nycodenz cushions (PAA Laboratories GmbH). The supernatants were mixed with PBS, pH 5.9 [3:2 (v/v)], and then centrifuged (2100 g) for 15 min at room temperature. The pelleted platelets were resuspended in PBS, pH 5.9 and 0.9% NaCl [1:1 (v/v)]. Washed platelets were finally resuspended in PBS, pH 7.4, and 1 mM CaCl_{2}. For incubations with solubilized compounds, methanol or DMSO was used as vehicle, never exceeding 1% (v/v).

AS49 cells were cultured in DMEM/high glucose (4.5 g/l) medium supplemented with heat-inactivated fetal calf serum [10% (v/v), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C and 5% CO_{2}. After 3 days, confluent cells were detached using 1 × trypsin/EDTA solution and reseeded at 2 × 10^{6} cells in 20 ml of medium. Cell

![Fig. 1. Effects of licofelone on the activity of isolated COX enzymes.](image-url)
viability was measured using the colorimetric 3-(4,5-dimethylthia-
zol-2-yl)-2,5-diphenyltetrazolium dye reduction assay. A549 cells (4 × 10^6 cells in 100 μl of medium) were plated into a 96-well microplate and incubated at 37°C and 5% CO₂ for 16 h. Then, 10 μM licofelone or solvent (DMSO) was added, and the samples were incubated for another 5 h. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphe-
nylterazolium (20 μl, 5 mg/ml) was added, and the incubations were continued for 4 h. The formazan product was solubilized with SDS [10% (w/v) in 20 mM HCl], and the absorbance of each sample was measured at 595 nm relative to that of vehicle (DMSO)-treated control cells using a multiwell scanning spectrophotometer (Victor^4 plate reader; PerkinElmer, Rodgau-Jügesheim, Germany). Licofe-
lone did not significantly reduce cell viability within 5 h (data not shown), excluding possible acute cytotoxic effects of the compound in the cellular assays.

**Determination of mPGES-1 in A549 Cells and Isolation of Micro-

omes.** Preparation of A549 cells was described as performed previ-
ously (Jakobsson et al., 1999). In brief, cells (2 × 10^6 cells in 20 ml of medium) were plated in 175-cm² flasks and incubated for 16 h at 37°C and 5% CO₂. Subsequently, the culture medium was replaced by fresh DMEM/high glucose (4.5 g/l) medium containing fetal calf serum [2% (v/v)]. To induce mPGES-1 expression, 1 ng/ml interleu-
kin-1β was added, and the cells were incubated for another 72 h. Thereafter, cells were detached with 1 × trypsin/EDTA, washed with PBS, and frozen in liquid nitrogen. Ice-cold homogenization buffer (0.1 M potassium phosphate buffer pH 7.4, 1 mM phenylmethylsul-
fonyl fluoride, 60 μg/ml soybean trypsin inhibitor, 1 μg/ml leupeptin, 2.5 mM glutathione, and 250 mM sucrose) was added; after 15 min, cells were resuspended and sonicated on ice (3 × 20 s). The homoge-
enate was subjected to differential centrifugation at 10,000 g for 10 min and at 174,000 g for 1 h at 4°C. The pellet (microsomal fraction) was resuspended in 1 ml of homogenization buffer, and the protein concentration was determined by the Coomassie protein assay.

**Determination of PGE₂ Synthase Activity in Microsomes of A549 Cells.** Microsomal membranes of A549 cells were diluted in 0.1 M potassium phosphate buffer, pH 7.4, containing 2.5 mM glutathione (total volume, 100 μl), and test compounds or vehicle (DMSO) was added. After 15 min, PGE₂ formation was initiated by addition of PGH₂ (final concentration, 20 μM). After 1 min at 4°C, the reaction was terminated with 100 μl of stop solution (40 mM FeCl₃, 80 mM citric acid, and 10 μM 11β-PGE₂). PGE₂ was separated by solid-phase extraction on reversed phase (RP)-C18 material using acetonitrile/citric acid, and 10 μM acetonitrile for 10 min at room temperature, the samples were stimulated with 10 μg/ml lipopolysaccharide for 5 h at 37°C. PGE₂ formation was stopped on ice, the samples were centrifuged (2300 g; 10 min; 4°C) and the citric acid (30 μl; 2 M) was added to the supernatant. After another centrifugation step (2300 g; 10 min; 4°C), solid-phase extrac-
tion and HPLC analysis of PGE₂ were performed as described above. The PGE₂ peak (3 ml), identified by coelution with authentic standard, was collected, and acetonitrile was removed under a nitrogen stream. The pH was adjusted to 7.2 by addition of 10× PBS buffer, pH 7.2 (230 μl), before PGE₂ was quantified using a PGE₂ high-
sensitivity enzyme immunoassay kit (Assay Designs, Ann Arbor, MI) according to the manufacturer’s protocol.

**Activity Assays of Isolated COX-1 and -2.** Inhibition of the activities of isolated ovine COX-1 and human COX-2 was performed as described previously (Mitchell et al., 1999; Capdevila et al., 1995). Although the purified COX-1 is not of human origin, ovine COX-1 is generally used for inhibitor studies when examining the effective-
ness of compounds on the activity of isolated COX-1 enzyme (Mitch-
ell et al., 1993). In brief, purified COX-1 (ovine, 50 units) or COX-2 (human recombinant, 20 units) were diluted in 1-mL reaction mixture containing 100 mM Tris buffer, pH 8.0, 5 mM glutathione, 5 μM hemoglobin, and 100 μM EDTA at 4°C and preincubated with the indicated compounds for 5 min. Samples were prewarmed for 60 s at 37°C, and arachidonic acid (5 μM for COX-1; 2 μM for COX-2) was added to start the reaction. After 5 min at 37°C, the COX product 12-HHT was extracted and then analyzed by HPLC as described previously (Albert et al., 2002).

**Statistics.** Data are expressed as mean ± S.E. The program GraphPad Instat (GraphPad Software Inc., San Diego, CA) was used for statistical comparisons. Statistical evaluation of the data was performed by one-way ANOVAs for independent or correlated sam-
ple(s) followed by Tukey’s HSD post hoc tests.
Student’s t test for paired and correlated samples was applied. A p value of <0.05 (*) was considered significant.

Results

Effects of Licofelone on the Activity of Isolated COX-1/2. It was previously found that licofelone suppresses cellular COX-1 in bovine platelets (IC50 = 0.21 μM) (Laufer et al., 1994b) and thromboxane B2 synthesis in ionophore-stimulated whole blood (IC50 = 3.9 μM) (Tries et al., 2002b) or COX-2-mediated PGE2 synthesis in ionomycin-stimulated human subchondral osteoblasts (IC50 < 0.8 μM) (Paredes et al., 2002). To investigate the direct interference of licofelone with isolated COX enzymes, purified ovine COX-1 and purified human recombinant COX-2 were preincubated with licofelone (or vehicle, DMSO) for 5 min; arachidonic acid (5 μM for COX-1; 2 μM for COX-2) was added and after 5 min, the formation of 12-HHT as the major COX-1/2-derived product under these experimental conditions (Capdevila et al., 1995) was determined. Licofelone potently and concentration-dependently suppressed 12-HHT formation by COX-1, with an IC50 of 0.8 μM (Fig. 1B). In contrast, licofelone was less efficient in suppressing the activity of COX-2 under comparable assay conditions, with an IC50 value >30 μM (Fig. 1C), excluding a strong direct interaction of licofelone with COX-2.

In control experiments, the COX-2-selective celecoxib (5 μM) strongly suppressed the formation of 12-HHT by COX-2 as expected (Fig. 1C).

Effects of Licofelone on the Activity of mPGES-1. Induction of COX-2 by proinflammatory stimuli coincides with the expression of mPGES-1, resulting in substantial PGE2 formation (Murakami et al., 2000; Thoren and Jakobsson, 2000). Because licofelone potently reduced COX-2-coupled PGE2 formation in intact cells (Paredes et al., 2002), but it caused no significant inhibition of isolated COX-2, we investigated whether licofelone could interfere with mPGES-1 activity. Expression of COX-2 and mPGES-1 in A549 cells was induced by treatment with 1 ng/ml interleukin-1β for 72 h. Microsomes were isolated, preincubated with or without licofelone, and PGE2 formation was induced by addition of 20 μM PGH2. After 1-min incubation, PGE2 formation was terminated and PGE2 was determined by RP-HPLC. The mPGES-1 inhibitor MK-886 was used as reference drug, and in agreement with the literature (IC50 = 2.4 μM; Claveau et al., 2003; Riedeau et al., 2005), it concentration-dependently blocked PGE2 formation, with an IC50 = 2 μM (Fig. 2A). Licofelone potently and concentration-dependently suppressed PGE2 formation, with an IC50 = 6 μM, being almost equipotent to MK-886 (Fig. 2A). In contrast, neither the COX-1/2 inhibitor indomethacin nor the COX-2-selective celecoxib (up to 10 μM, each) significantly reduced the enzymatic conversion of PGH2 to PGE2 in this assay (data not shown).

To investigate whether licofelone blocks mPGES-1 in a reversible manner, microsomal preparations of A549 cells were preincubated with licofelone at 1 and 10 μM for 15 min, and for comparison with MK-886 at 0.3 and 3 μM each. The samples containing 3 μM MK-886 or 10 μM licofelone were divided: one part was diluted 10-fold with reaction buffer to obtain a final concentration of 0.3 and 1 μM inhibitor, respectively. Control samples without inhibitor received DMSO. Then, PGH2 was added to each sample to start the reaction. As shown in Fig. 2B, both MK-886 as well as licofelone slightly reduced PGE2 formation at 0.3 or 1 μM, respectively, whereas at 3 or 10 μM, PGE2 synthesis was potently inhibited. Dilution of these incubations before addition of substrate to a final concentration of 0.3 or 1 μM, respectively, reversed the inhibitory effect of both MK-886 and licofelone, implying that licofelone as MK-886 inhibits mPGES-1 in a reversible manner.

It seemed reasonable that the potency of licofelone to inhibit mPGES-1 may depend on the substrate concentration. However, decreasing the PGH2 concentrations from 20 to 1 μM did not significantly affect the potency of licofelone, rather excluding a competitive inhibitory mechanism (Fig. 2C). The potency of the reference inhibitor MK-886 (10 μM) varied at 1 μM PGH2 in individual microsomal preparations (data not shown); therefore, we normalized the activity data in Fig. 2C to vehicle control (100%) and 10 μM MK-886 (0%). Likewise, the effectiveness of MK-886 was essentially the same at 1 or 20 μM PGH2 (A. Koeberle, F. Pfallastro, H. Nordhoff, and O. Werz, submitted for publication).

Effects of Licofelone on Prostanoid Formation in Intact Cells. Interleukin-1β-stimulated A549 cells express mPGES-1 and COX-2, but not COX-1 (Asano et al., 1996; Thoren and Jakobsson, 2000). Because COX-2 preferably couples with mPGES-1 (Murakami et al., 2000; Thoren and Jakobsson, 2000), it is reasonable that PGE2 production in interleukin-1β-stimulated A549 cells is mainly dependent on COX-2-derived PGH2 and subsequent cleavage to PGE2 by mPGES-1. However, mPGES and mPGES-2 are still likely to contribute to PGE2 formation, although to a lesser extent (Park et al., 2006). A549 cells (pretreated with interleukin-1β for 72 h) were preincubated with licofelone, MK-886, or vehicle (DMSO) for 10 min before stimulation of the cells with 2.5 μM A23187 plus 1 μM arachidonic acid and [3H]arachidonic acid (18.4 kBq). Induction of PGE2 synthesis by A23187 and exogenous arachidonic acid is thought to exclude possible effects of licofelone on the level of receptor-coupled signal transduction and/or on cytosolic phospholipase A2-mediated arachidonic acid release. Thus, cell stimulation by A23187 circumvents receptor-coupled signal transduction by activating the cell via massive elevation of intracellular Ca2++, and coaddition of exogenous arachidonic acid circumvents the requirement for the supply of endogenous substrate for PGE2 formation provided by cytosolic phospholipase A2. Formed PGE2 was separated by RP-HPLC and quantified by liquid scintillation counting. Celecoxib (5 μM) almost abolished PGE2 production (Fig. 3A), and PGE2 was not detectable in the presence of 10 μM indomethacin (data not shown). MK-886 was hardly active at 10 μM, and at 33 μM it reduced PGE2 formation by 63%. The formation of PGE2 was potently and concentration-dependently reduced by licofelone, with an IC50 = 0.1 μM (Fig. 3A). Thus, licofelone is approximately 60-fold more potent in the cellular assay than in cell-free systems, and it is more than 100-fold as potent as MK-886. To exclude the possibility that the potent suppression of PGE2 is due to COX-2 inhibition by licofelone, we assessed the formation of the stable PGI2 degradation product 6-keto PGF1α in parallel as a parameter of cellular COX-2 activity in these A549 cells. However, neither licofelone nor MK-886 caused a significant reduction of 6-keto PGF1α formation in interleukin-1β-stimulated A549 cells, up to the highest con-

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centrations (33 μM for licofelone and 30 μM for MK-886) tested (Fig. 3B). Control experiments using 10 μM indomethacin or 5 μM celecoxib led to an efficient decrease of the 6-keto-PGF1α levels under these conditions. In contrast, for inhibition of cellular COX-1 activity, we could confirm the efficacy of licofelone in intact human platelets reported previously (Laufer et al., 1994b), in which an IC50 for 12-HHT formation of 0.24 μM was apparent in our assay (Fig. 3C).

Effects of Licofelone on the Formation of PGE2 in Human Whole Blood. Although the effects of licofelone on general PGE2 formation using a simple ELISA technique as a read-out system in human osteoarthritis subchondral osteoblasts were shown by others previously (Paredes et al., 2002), we established a novel assay that allowed us to specifically assess COX-2-mediated PGE2 synthesis in human whole blood (A. Koeberle, F. Pollastro, H. Northoff, and O. Werz, submitted for publication). Heparinized human whole blood was preincubated with licofelone for 10 min, before stimulation with 10 μg/ml lipopolysaccharide for 5 h. To minimize the contribution of COX-1 in PGE2 synthesis, and thus, to establish experimental settings where PGE2 is primarily produced via the COX-2/mPGES-1 pathway, COX-1 was inactivated by the use of 50 μM aspirin and thromboxane formation by platelets was blocked using the thromboxane synthase inhibitor CV4151 at 1 μM. For measuring PGE2, we first separated PGE2 from other arachidonic acid metabolites that may possibly interfere with commercially available PGE2 ELISA detection systems. Thus, PGE2 was isolated from plasma by solid-phase extraction, separated by RP-HPLC, and then quantified by ELISA. As shown in Fig. 4, licofelone concentration-dependently reduced PGE2 synthesis, with an IC50 of approximately 5 μM, but it failed to completely decrease the PGE2 level and approximately 30% PGE2 still remained. Likewise, MK-886 only partially reduced PGE2 formation at 30 μM (44 ± 8% versus vehicle control), and higher concentrations (up to 100 μM) caused no further decrease of PGE2 levels (data not shown). Celecoxib (20 μM) and indomethacin (50 μM) efficiently reduced PGE2 formation to 24 ± 13 and 16 ± 4%, respectively (Fig. 4), implying that COX inhibition (by indomethacin or celecoxib) might be a more efficient mean to reduce PGE2 formation in whole blood compared with mPGES-1 inhibition (by licofelone or MK-886).

![Fig. 2. Effects of licofelone and MK-886 on the activity of mPGES-1.](image-url)
Licofelone is an advanced dual inhibitor of the COX and 5-lipoxygenase pathway, exhibiting not only a remarkable efficacy as anti-inflammatory drug but also exerting significantly less adverse effects under chronic use that are usually associated with NSAIDs (i.e., gastrointestinal injury and renal irritations) or selective COX-2 inhibitors (cardiovascular risks) (Kulkarni and Singh, 2007). Our data show that licofelone is a direct and potent inhibitor of COX-1 in cell-free assays, confirming previous conclusions drawn from analysis of the compound in cell-based test systems (Lauffer et al., 1994b; Tries et al., 2002b). In contrast, licofelone hardly inhibits isolated COX-2, and it fails to reduce the formation of the COX-2 product 6-keto PGF1α in intact cells, despite potent suppression of COX-2-mediated PGE2 formation. Because licofelone blocked the conversion of PGH2 to PGE2 catalyzed by mPGES-1 in a cell-free assay, we conclude that suppression of PGE2 synthesis in the cell is due to interference with mPGES-1, rather than with COX-2. Taken together, the primary mechanism of action of licofelone is as a COX-1 inhibitor, but the additional suppression of mPGES-1 and of leukotriene biosynthesis may contribute to the overall efficacy and tolerability of this drug.

Initially, licofelone was proposed as a potent dual COX/5-lipoxygenase inhibitor based on its suppressive effect on COX and 5-lipoxygenase activity in intact bovine and human platelets and granulocytes (Lauffer et al., 1994a,b). Several subsequent investigations confirmed inhibition of the 5-lipoxygenase pathway and of PGE2 or thromboxane B2 formation by licofelone in cell-based models (Jovanovic et al., 2001; Paredes et al., 2002; Tries et al., 2002b; Fischer et al., 2007; Vidal et al., 2007). Moreover, reduction of prostanoids (i.e., PGE2 and thromboxane B2) and leukotriene B4 by licofelone in vivo was shown in animal models such as experimental dog osteoarthritis (Jovanovic et al., 2001; Lajeunesse et al., 2004) or N-formyl-L-methionyl-L-leucyl-L-phenylalanine-challenged rabbits (Rotondo et al., 2006). However, the experimental settings in all these studies did not allow the...
elucidation of the molecular mechanisms resulting in suppressed eicosanoid formation. In fact, an efficient suppression of leukotriene biosynthesis by licofelone was observed only in models with intact cells, where FLAP represents a pivotal cofactor for leukotriene B4 formation. In leukocyte homogenates or in test systems with purified recombinant 5-lipoxygenase, the inhibitory activity of licofelone was negligible (Fischer et al., 2007). FLAP inhibitors (e.g., MK-886 or BAY X1005) are well appreciated pharmacological tools for intervention with leukotriene synthesis (Gillard et al., 1989), and licofelone shares structural similarities with MK-886 and exhibits similar pharmacological properties (Fischer et al., 2007).

In analogy to cellular leukotriene biosynthesis, several different enzymes are required for the consecutive transformation of arachidonic acid to distinct PGs in the cell upon activation, including phospholipases A2, COX-1/2, and select PG synthases as well as the receptor for the external stimulus and the distal signaling molecules that eventually lead to release of arachidonic acid and its metabolism (Funk, 2001). Consequently, any test compound resulting in reduced biosynthesis of a certain PG in stimulated cells does not have to unequivocally act (solely) on COX but also may interfere with other components. Our data clearly show that licofelone is a potent inhibitor of isolated COX-1 (IC50 = 0.8 μM) but not of isolated human COX-2 (IC50 > 30 μM). These data are in line with the observation that in stimulated A549 cells, PGE2 formation was potently inhibited (IC50 = 0.1 μM), whereas the generation of 6-keto PGF1α (the stable metabolite of PGI2) remained unaffected by licofelone. Because the COX-1 route for supply of PGH2 can be ignored in A549 cells (Warner et al., 2006), the COX-1 inhibitory effect of licofelone might be negligible, and we conclude that interference with mPGES-1 is mainly responsible for suppression of PGE2 by licofelone. Note that due to supply of exogenous arachidonic acid in the A549 cellular assay, suppression of phospholipase A2 enzymes (required for arachidonic acid liberation) by licofelone as possible point of attack can be ruled out, supported also by the lack of interference to reduce the formation of the 12-lipoxygenase product 12-hydro(pero)xieicosatetraenoic acid from endogenously released arachidonic acid in thrombin-activated platelets (data not shown).

The effectiveness of licofelone as inhibitor of mPGES-1 was demonstrated in this study by 1) a cell-free assay measuring the direct conversion of PGH2 to PGE2 by mPGES-1 and 2) a cellular assay determining PGE2 from exogenously added arachidonic acid. It is interesting to note that licofelone was approximately 60-fold more potent in intact A549 cells compared with the cell-free assay, which is not readily understood. It is puzzling that MK-886 showed no such different potencies, and in the whole blood assay the efficacy of licofelone was comparable as in the cell-free test system. It is possible that additional A549-specific (intra)cellular components/mechanisms are operative, or licofelone interferes with other steps in the formation of PGE2 from endogenous arachidonic acid such as the transfer of PGH2 from COX-2 to mPGES-1. The loss of efficacy of licofelone in whole blood versus intact A549 cells could be due to binding to plasma albumin.

Pharmacological intervention with mPGES-1 in the therapy of PGE2-mediated disorders, including rheumatoid arthritis, fever, and pain, is proposed to have advantages over general suppression of all PGs by applying COX inhibitors (Jachak, 2007; Samuelsson et al., 2007). Drugs selective for COX-2 have originally been developed to reduce the gastrointestinal risk of unselective COX inhibitors, but the association with an increased incidence of major adverse cardiovascular events raises the need for alternative therapeutic strategies and targets. In particular, selective mPGES-1 inhibition is proposed to avoid the increased incidence of major adverse cardiovascular events of COX-2-selective drugs due to the lack of impaired PGI2 levels (Wang et al., 2006). Thus, deletion of mPGES-1 in mice impaired inflammatory reactions and pain responses (Trebin et al., 2003), but neither affected thrombogenesis nor blood pressure, and caused augmented PGI2 expression (Cheng et al., 2006), implying mPGES-1 as a target for therapeutic intervention in patients with cardiovascular risk. To date, MK-886 that was originally developed as inhibitor of leukotriene biosynthesis (Gillard et al., 1989), and structural derivatives of MK-886 have been reported as potent and specific inhibitors of mPGES-1 (Claveau et al., 2003; Riendeau et al., 2005). In a recent study, synthetic phenanthrene imidazoles were presented as selective, and orally active mPGES-1 inhibitors (IC50 = 0.42 to 1.3 μM in A549 cells and human whole blood, respectively) with significant analgesic effect in a guinea pig hyperalgesia model (Côte et al., 2007). Taken together, preferential inhibition of mPGES-1 over COX-2 by licofelone might be advantageous in clinical use.

There is accumulating evidence that inhibition of both the leukotriene and the PG biosynthetic pathway is superior over single interference, not only in terms of anti-inflammatory effectiveness but also due to a lower incidence of gastrointestinal toxicity, typically related to COX inhibition (Celotti and Lauffer, 2001; Kulkarni and Singh, 2007). In fact, licofelone markedly improved gastrointestinal tolerability in animal models and offered gastroprotection against NSAIDs-induced gastropathy (Wallace et al., 1994; Kulkarni and Singh, 2007). In healthy volunteers, licofelone showed significantly superior gastric tolerability and a lower incidence of ulcers compared with naproxen (Bias et al., 2004). Moreover, licofelone in contrast to COX-2-selective inhibitors may have a favorable cardiovascular profile because of the presumed lack of impaired PGI2 levels, and because of the ability to suppress the formation of leukotrienes, which are mediators of atherogenesis and cardiovascular disease (Peters-Golden and Henderson, 2007). In fact, licofelone reduced neointimal formation and inflammation in an atherosclerotic rabbit model (Vidal et al., 2007), protected rabbits from the cardiovascular derangement triggered by N-formyl-methionyl-l-leucyl-l-phenylalanine (Rotondo et al., 2006), and had a significant antithrombotic activity and a marked platelet aggregation-inhibiting effect in rats (Triets et al., 2002a). Although licofelone, based on its unique and favorable molecular pharmacological profile regarding intervention with eicosanoid biosynthesis, may represent a suitable drug for the therapy of chronic inflammatory diseases with low risks of adverse effects, long-term studies evaluating the cardiovascular toxicity of licofelone have to be conducted to judge its cardiovascular safety in chronic use.

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References


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