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 [≡ML3000; 2-([6-(4-chromophenyl)-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-buty1-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 isoenzymes imply that selective molecular interactions between COX and PGES isoenzymes 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

ABBREVIATIONS: 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-buty1-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, calciymc; ELISA, enzyme-linked immunosorbent assay; 12-HHT, 12-(5-hydroxy-5-cholesten-8E,14E)-dodecaenoic acid; ANOVA, analysis of variance; HSD, honestly significant difference; BAY X1005, 2-([4-(quinolin-2-yl-methoxy)phenyl]2-cyclopentylacetic acid; Indo, indomethacin; Cele, celecoxib; CV 4151, (E)-7-phenyl-1-[3-(3-pyriddyl)]-6-heptoenoic acid.
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 PGE₂ (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 [=ML3000; 2-(6-(4-chlorophenyl)-2,2-dimethyl-7-phenyl-2,3-dihydro-1H-pyrrolizin-5-yl) 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 Laufer, 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 PGE₂ 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 Laufer, 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 (IC₅₀ = 1.7 µM) is mainly related to its interference with 5-lipoxygenase-activating protein (FLAP) rather than 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 PGE₂ formation. Our data indicate that the potent inhibition of COX-2-mediated PGE₂ 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 PGF₁α 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); PGG₂ (Larodan, Malmö, Sweden); 11β-PGE₂, PGB₂, MK-886, 6-keto PGF₁α, 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 (4000g; 20 min; 20°C). Platelets were immediately isolated by density sedimentation and centrifugation on Nycoprep cushions (PAA Laboratories GmbH). The supernatants were mixed with PBS, pH 5.9, and 0.9% NaCl [1:1 (v/v)]. Washed platelets were finally resuspended in PBS, pH 5.9 and 0.9% NaCl [1:1 (v/v)]. Platelets were immediately isolated by density sedimentation and centrifugation on Nycoprep cushions (PAA Laboratories GmbH). The supernatants were mixed with 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 CaCl₂. For incubations with solubilized compounds, methanol or DMSO was used as vehicle, never exceeding 1% (v/v).

A549 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% CO₂. After 3 days, confluent cells were detached using 1× trypsin/EDTA solution and reseeded at 2 × 10⁶ cells in 20 ml of medium. Cell
viability was measured using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium dye reduction assay. A549 cells (4 × 10^4 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 licoferone or solvent (DMSO) was added, and the samples were incubated for another 5 h. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (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 (Victor4 plate reader; PerkinElmer, Rodgau-Juegesheim, Germany). Licoferone did not significantly reduce cell viability within 5 h (data not shown), excluding possible acute cytotoxic effects of the compound in the cellular assays.

**Induction of mPGES-1 and COX 459 Cells and Isolation of Microsomes.** Preparation of A549 cells was described as performed previously (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 interleukin-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 phenylmethylsulfonyl 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 homogenate 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 mPGES-1 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 gluthathione (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 FeCl₂, 80 mM citrate, and 10 μM 11β-PGE₂), and PGE₂ was separated by solid-phase extraction on reversed phase (RP-C18) material using acetonitrile (200 μl) as eluent and analyzed by RP-HPLC [30% aqueous acetonitrile + 0.007% trifluoroacetic acid (v/v); Nova-Pak C18 column, 5 × 100 mm, 4-μm particle size, flow rate 1 ml/min], with UV detection at 195 nm. 11-PGE₂ was used as internal standard to quantify PGE₂ product formation by integration of the area under the eluted peaks. For quantification of radiolabeled PGE₂, 0.5-ml fractions were collected and mixed with 2 ml of Ultima Gold for liquid scintillation counting in an LKB Wallac 1209 Rackbeta liquid scintillation counter (GE Healthcare, Chalfont St. Giles, UK).

For determination of 6-keto PGF₁α, 10⁶ cells in 1 ml of PBS containing 1 mM CaCl₂ were preincubated with the indicated compounds for 15 min at 37°C, and 6-keto PGF₁α formation was initiated by addition of 30 μM arachidonic acid. After 15 min at 37°C, the reaction was stopped by cooling on ice. Cells were centrifuged (300g; 5 min; 4°C), and the amount of released 6-keto PGF₁α was assessed by ELISA using a monoclonal antibody against 6-keto PGF₁α, according to the protocol described by Yamamoto et al. (1987). For the ELISA, the monoclonal antibody (0.2 μg/200 μl) was coated to microtiter plates via a goat anti-mouse immunoglobulin G antibody. 6-Keto PGF₁α (15 μg) was linked to bacterial β-galactosidase (0.5 mg), and the enzyme activity bound to the antibody was determined in an ELISA reader at 550 nm (reference wavelength, 630 nm) using chlorophenol-red-β-D-galactopyranoside (Roche Diagnostics, Mannheim, Germany) as substrate.

**Determination of PGE₂ in Whole Blood.** Peripheral blood from healthy adult volunteers, who had not received any medication for at least 2 weeks under informed consent, was obtained by venipuncture and collected in syringes containing 20 U/ml heparin. For determination of PGE₂, aliquots of whole blood (0.8 ml) were mixed with the thromboxane synthetase inhibitor CV4151 at 1 μM and aspirin at 50 μM. A total volume of 1 ml was adjusted with sample buffer (10 mM potassium phosphate buffer, pH 7.4, 3 mM KCl, 140 mM NaCl, and 6 mM D-glucose). After preincubation with the indicated compounds 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 (2300g; 10 min; 4°C) and citric acid (30 μl; 2 M) was added to the supernatant. After another centrifugation step (2300g; 10 min; 4°C), solid-phase extraction 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., 1993; 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 effectiveness of compounds on the activity of isolated COX-1 enzyme (Mitchell 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 mM hemoglobin, and 100 μM EDTA at 4°C and preincubated with the test 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 samples followed by Tukey’s HSD post hoc tests. Where appropriate,
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 (IC$_{50}$ = 0.21 μM) (Laufer et al., 1994b) and thromboxane B$_2$ synthesis in ionophore-stimulated whole blood (IC$_{50}$ = 3.9 μM) (Tries et al., 2002b) or COX-2-mediated PGE$_2$ synthesis in ionomycin-stimulated human subchondral osteoblasts (IC$_{50}$ < 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 IC$_{50}$ 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 IC$_{50}$ 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 PGE$_2$ formation (Murakami et al., 2000; Thoren and Jakobsson, 2000). Because licofelone potently reduced COX-2-coupled PGE$_2$ 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 PGE$_2$ formation was induced by addition of 20 μM PGH$_2$. After 1-min incubation, PGE$_2$ formation was terminated and PGF$_2$ was determined by RP-HPLC. The mPGES-1 inhibitor MK-886 was used as reference drug, and in agreement with the literature (IC$_{50}$ = 2.4 μM; Claveau et al., 2003; Rierendeau et al., 2005), it concentration-dependently blocked PGE$_2$ formation, with an IC$_{50}$ = 2 μM (Fig. 2A). Licofelone potently and concentration-dependently suppressed PGE$_2$ formation, with an IC$_{50}$ = 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 PGH$_2$ to PGE$_2$ 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, PGH$_2$ was added to each sample to start the reaction. As shown in Fig. 2B, both MK-886 as well as licofelone slightly reduced PGE$_2$ formation at 0.3 or 1 μM, respectively, whereas at 3 or 10 μM, PGE$_2$ 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 PGH$_2$ 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 PGH$_2$ 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 PGH$_2$ (A. Koeberle, F. Pollastro, H. Northoff, 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 PGE$_2$ production in interleukin-1β-stimulated A549 cells is mainly dependent on COX-2-derived PGH$_2$ and subsequent cleavage to PGE$_2$ by mPGES-1. However, cPGES and mPGES-2 are still likely to contribute to PGE$_2$ 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 PGE$_2$ 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 A$_2$-mediated arachidonic acid release. Thus, cell stimulation by A23187 circumvents receptor-coupled signal transduction by activating the cell via massive elevation of intracellular Ca$^{2+}$, and coaddition of exogenous arachidonic acid circumvents the requirement for the supply of endogenous substrate for PGE$_2$ formation provided by cytosolic phospholipase A$_2$. Formed PGE$_2$ was separated by RP-HPLC and quantified by liquid scintillation counting. Celecoxib (5 μM) almost abolished PGE$_2$ production (Fig. 3A), and PGE$_2$ 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 PGE$_2$ formation by 63%. The formation of PGE$_2$ was potently and concentration-dependently reduced by licofelone, with an IC$_{50}$ = 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 PGE$_2$ is due to COX-2 inhibition by licofelone, we assessed the formation of the stable PGL$_2$ degradation product 6-keto PGF$_1α$, 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 PGF$_1α$ formation in interleukin-1β-stimulated A549 cells, up to the highest con-
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 inhibition of cellular COX-1 activity, we could confirm the contribution of COX-1 in PGH₂ 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. 3. Effects of licofelone on prostanoid formation in intact cells. A, PGE\textsubscript{2} formation in A549 cells. Interleukin-1\textbeta-stimulated A549 cells (4 × 10\textsuperscript{5} cells/ml) were preincubated with the indicated compounds or vehicle (DMSO) for 5 min at 37°C and then 2.5 μM arachidonic acid (18.4 kbp) was added. After 5 min at 37°C, the amount of released 6-keto PGF\textsubscript{1α} was determined by RL-HPLC and liquid scintillation counting as described under Materials and Methods. B, 6-keto PGF\textsubscript{1α}, formation in A549 cells. Interleukin-1\textbeta-stimulated A549 cells (10\textsuperscript{6}/ml) were preincubated with the indicated compounds (or vehicle, DMSO) for 15 min, 30 μM arachidonic acid was added, and after 15 min at 37°C the amount of released 6-keto PGF\textsubscript{1α} was assessed by ELISA as described under Materials and Methods. MK-886 (30 μM), Indo (50 μM), Cele (20 μM), or vehicle (DMSO) was used as control. Data are given as mean ± S.E., n = 3. **, p < 0.05; ***, p < 0.01; or ****, p < 0.001 versus vehicle (0.1% DMSO) control, ANOVA + Tukey’s HSD post hoc tests.

Fig. 4. Effects of licofelone on PGE\textsubscript{2} biosynthesis in human whole blood. Heparinized human whole blood, treated with 1 μM thromboxane synthase inhibitor and 50 μM aspirin, was preincubated with the indicated concentrations of licofelone for 10 min at room temperature, and then PGE\textsubscript{2} formation was induced by addition of 10 μg/ml lipopolysaccharide. After 5 h at 37°C, PGE\textsubscript{2} was extracted from plasma by RP-18 solid-phase extraction, separated by RP-HPLC, and quantified by ELISA as described. MK-886 (30 μM), Indo (50 μM), Cele (20 μM), or vehicle (DMSO) was used as control. Data are given as mean ± S.E., n = 3. **, p < 0.05; ***, p < 0.01; or ****, p < 0.001 versus vehicle (0.1% DMSO) control, ANOVA + Tukey’s HSD post hoc tests.

Discussion

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 irrigations) 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 (Laufer 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 PGF\textsubscript{1α} in intact cells, despite potent suppression of COX-2-mediated PGE\textsubscript{2} formation. Because licofelone blocked the conversion of PGH\textsubscript{2} to PGE\textsubscript{2} catalyzed by mPGES-1 in a cell-free assay, we conclude that suppression of PGE\textsubscript{2} 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 (Laufer et al., 1994a,b). Several subsequent investigations confirmed inhibition of the 5-lipoxygenase pathway and of PGE\textsubscript{2} or thromboxane B\textsubscript{2} 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., PGE\textsubscript{2} and thromboxane B\textsubscript{2}) and leukotriene B\textsubscript{4} 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 bio-synthesis 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 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-l-methionyl-l-leucyl-l-phenylalanine (Rotondo et al., 2006), and had a significant antithrombotic activity and a marked platelet aggregation-inhibiting effect in rats (Tries 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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