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-chlorophenyl)-2,2-dimethyl-7-phept-2,3-dihydro-1H-pyrrrolizin-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 isozyme 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 select 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-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-trans-heptadecatrienoic 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-(7-(3-pyriddyl))-6-heptenoic 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 PGE
subscript {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 ([=ML3000; 2-6-(4-chlorophenyl)-2,2-dimethyl-7-phenyl-2,3-dihydro-1H-pyrrrolizin-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 PGE
subscript {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 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 mechanisms of the inhibition of the biosynthesis of 5-lipoxygenase products by licofelone, and we found that licofelone-mediated suppression of leukotriene synthesis (IC
subscript {50} = 1.7 \mu M) 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 PGE
subscript {2} formation. Our data indicate that the potent inhibition of COX-2-mediated PGE
subscript {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
subscript {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
subscript {2} (larodan, Malmo, Sweden); 11β-PGE
subscript {2}, PGB
subscript {1}, MK-886, 6-keto PGF
subscript {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 (4000g; 20 min; 20°C). Platelets were immediately isolated by dextran sedimentation and centrifugation on Nycoprep cushions (PAA Laboratories GmbH, Coelbe, Germany); 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 re-suspended in PBS, pH 7.4, and 1 mM CaCl
subscript {2}. 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 \mu g/ml streptomycin at 37°C and 5% CO
subscript {2}. After 3 days, confluent cells were detached using 1 \mu/ml trypsin/EDTA solution and reseeded at 2 \times 10^6 cells in 20 ml of medium.

Fig. 1. Effects of licofelone on the activity of isolated COX enzymes. A, chemical structure of licofelone. Effects of licofelone on the activity of isolated COX-1 (B) and COX-2 (C). Purified ovine COX-1 (50 units) or human recombinant COX-2 (20 units) were added to a COX reaction mix, containing 5 mM glutathione. The COX enzymes were preincubated with the test compounds for 5 min, and then the reaction was started with 5 \mu M (COX-1) or 2 \mu M (COX-2) arachidonic acid. After 5 min at 37°C, the formation of 12-HHT was determined by RP-HPLC as described in text. Indomethacin (Indo; 10 \mu M) and celecoxib (Cele; 5 \mu M) were used as controls. Data are given as mean ± S.E., n = 3 to 4. **, p < 0.01; ***, p < 0.001 versus vehicle (0.1% DMSO) control, ANOVA + Tukey’s HSD post hoc tests.
viability was measured using the colorimetric 3-(4,5-dimethylthia-
zol-2-yl)-2,5-diphenyldiazotrozo dye reduction assay. A549
cells (4 × 10⁴ 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-Dimethiazol-2-yl)-2,5-diphe-
yldiazotrozo (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-Juegesheim, 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.

Induction of mPGES-1 in A549 Cells and Isolation of Micro-
somes. Preparation of A549 cells was described as performed previ-
sely (Jakobsson et al., 1999). In brief, cells (2 × 10⁶ 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
with 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 homog-
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 citrat-
one (total volume, 100 μl), and test compounds or vehicle (DMSO)
were added. After 15 min, PGE₂ formation was initiated by addition of
PGH₂ (final concentration, 20 μM). At 1 min after 4°C, the reaction
was terminated with 100 μl of stop solution (40 mM FeCl₂, 80 mM
citric acid, and 10 mM NaCl; 2 M), and PGE₂ was separated by
solid-phase extraction on reversed phase (RP)-C18 material using
acetonitrile. Solid-phase extraction and HPLC analysis were per-
formed as described above. The amount of 11-HHT, identified by coelution with an authentic stan-
dard, was quantified 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 com-
ounds 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 mi-
nterpreter plates via a goat anti-mouse immunoglobulin G antibody.
6-Keto PGE₁α (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, Mann-
heim, Germany) as substrate.

Determination of COX-1 Product Formation in Washed
Platelets. Freshly isolated platelets (10⁶/ml PBS containing 1 mM
CaCl₂) were preincubated with the indicated agents for 5 min at
room temperature. After addition of 5 μM arachidonic acid and
further incubation for 5 min at 37°C, the COX-1 product 12(S)-
hydroxy-5-cis-8,10-trans-heptadecatrienoic acid (12-HHT) was ex-
tracted and then analyzed by HPLC as described previously (Albert et
al., 2002).

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 determi-
nation 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 β-glucose). After preincubation with the indicated compounds
for 10 min at room temperature, the samples were stimulated with
10 ng/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 extrac-
tion and HPLC analysis of PGE₂ were performed as described above.
The PGE₂ peak (3 ml), identified by coelution with authentic stan-
dard, 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 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 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 sam-
plest followed by Tukey’s HSD post hoc tests. Where appropriate,
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 Jakobs-son, 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-2 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. 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 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, cPGES 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 15 min, respectively, PGE₂ formation at 0.3 or 1 μM was 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-
Previously (Laufer et al., 1994b), in which an IC50 for 12-HHT efficacy of licofelone in intact human platelets reported pre-
inhibition of cellular COX-1 activity, we could confirm the contribution of COX-1 in PGH2 synthesis, and thus, to
formation of 0.24 µM PGH2 as substrate. The amount of PGE2 was quantified for 1 µM PGH2 by use of a PGE2 high-sensitivity enzyme immunoassay kit according to the manufacturer’s protocol. PGE2 production at 10 µM MK-886 was set 0% of vehicle control (100%) to compare both data sets. Data are given as mean ± S.E., n = 3 to 4. * p < 0.05 versus vehicle (0.1% DMSO) control, ANOVA + Tukey’s HSD post hoc tests. C, potency of licofelone for mPGES-1 inhibition was compared at 1 and 30 µM MK-886 (for licofelone) and 30 µM indomethacin (for MK-886).

Fig. 2. Effects of licofelone and MK-886 on the activity of mPGES-1. A, concentration-response curves for licofelone and MK-886. Microsomal preparations of interleukin-1β-stimulated A549 cells were preincubated with vehicle (DMSO) or the test compounds at the indicated concentrations for 15 min at 4°C, and the reaction was terminated with 20 µM PGH2. After 1 min at 4°C, the reaction was terminated using a stop solution, containing FeCl3 and 11β-PGE2 (1 nmol) as internal standard. B, reversibility of mPGES-1 inhibition by licofelone and MK-886. Microsomal preparations of interleukin-1β-stimulated A549 cells were preincubated with 3 µM MK-886 or 10 µM licofelone for 15 min at 4°C. An aliquot was diluted 10-fold to obtain an inhibitor concentration of 0.3 and 1 µM, respectively. For comparison, microsomal preparations were preincubated for 15 min with 0.3 µM MK-886 or 1 µM licofelone, or with vehicle (DMSO) and then 20 µM PGH2 was added (no dilution). Then, all samples were incubated for 1 min on ice, and PGE2 formation was analyzed as described by RP-HPLC under Materials and Methods. Data are given as mean ± S.E., n = 3 to 4. * p < 0.05 versus vehicle (0.1% DMSO) control, ANOVA + Tukey’s HSD post hoc tests. C, potency of licofelone for mPGES-1 inhibition was compared at 1 and 20 µM PGH2 as substrate. The amount of PGE2 was quantified for 1 µM PGH2 by use of a PGE2 high-sensitivity enzyme immunoassay kit according to the manufacturer’s protocol. PGE2 production at 10 µM MK-886 was set 0% of vehicle control (100%) to compare both data sets. Data are given as mean ± S.E., n = 3 to 4.

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 read-out system in human osteoarthritis subchondral osteo-
blasts 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 PGH2 synthesis, and thus, to establish experimental settings where PGE2 is primarily produced via the COX-2/mPGES-1 pathway, COX-1 was inacti-
vated by the use of 50 µM aspirin and thromboxane formation by platelets was blocked using the thromboxane syn-
Thase 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 licofe-
lone or MK-886).
**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 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 PGF1\(_\alpha\) 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
elicitation 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 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).

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