Pharmacological activities and pharmacokinetics of licofelone

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Abstract
Licofelone (ML3000) is a dual cyclooxygenase/lipoxygenase inhibitor being considered as a treatment for osteoarthritis. It has both an analgesic and an anti-inflammatory action. Inhibition of 5-lipoxygenase may reduce the gastrointestinal toxicity associated with other non steroidal anti-inflammatory drugs, which only inhibit cyclooxygenase. It has been tested in Phase III clinical trials. According to the published papers from 200-2009, authors introduced results of the pharmacological activities and drug metabolism and pharmacokinetics of licofelone.

Key words
drug metabolism; licofelone; pharmacokinetics; pharmacological activity

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Introduction
Licofelone (ML3000) is developed by the German pharmaceutical company, Merckle GmbH, together with EuroAlliance partners Alfa Wassermann and Lacer, licofelone (ML3000). Licofelone is a dual cyclooxygenase/lipoxygenase (COX/LOX) inhibitor being considered as a treatment for osteoarthritis.[1] Licofelone has both an analgesic and an anti-inflammatory action. Inhibition of 5-lipoxygenase (5-LOX) may reduce the gastrointestinal toxicity associated with other non steroidal anti-inflammatory drugs (NSAIDs), which only inhibit COX. It has been tested in Phase III clinical trials.[2] Pharmacological studies have demonstrated that the drug has analgesic, antipyretic, anti-inflammatory and significant anti-asthmatic activity without causing GI damage. It is currently in a phase III trial for the treatment of osteoarthritis.[3,4]
Licofelone, designed to aim at a dual inhibition of the LOX and COX pathways, has proved to be effective in reducing growth in cancer cell lines. Interestingly, a recently published report that uses a mathematical model to study the interactions of the AA metabolic network, has revealed that a dual inhibitor against
LOX/COX is more effective than a combination of single COX and LOX inhibitors.[5-7] Licofelone inhibits LOX-5, COX-1, and COX-2, decreases production of prostaglandins (PGs) and leukotrienes (LTs),[8,9] and presents lower GI toxicity compared with NSAIDs naproxen and rofecoxib.[10-12] Interestingly, it has been reported recently that Licofelone inhibits LOX/COX pathways and induces apoptosis in HCA-7 colon cancer cells.[13]

Licofelone, as a first member of a new class of COX and 5-LOX inhibitors, possesses analgesic and anti-inflammatory activities with a mechanism of action attributable to the inhibition of LTB₄ thereby IL-1β arrests the production of proteolytic enzymes responsible for damaging the structural changes of joints. Thus, licofelone, by arresting the pathophysiology, is going to be an ideal disease-modifying drug with better tolerability and acceptability.

Pharmacological activities of Licofelone

Biochemical pharmacological studies

Licofelone distinctly differs from NSAIDS since it inhibits not only COX but also 5-LOX, which is associated with the production of pro-inflammatory and gastrotoxic leukotrienes. Inhibition of COX alone by NSAIDS is expected to shunt arachidonic acid metabolism to 5-LOX pathway leading to increased production of gastrotoxic leukotrienes.[14] Thus, inhibition of 5-LOX in addition to COX is a new opening for having an agent possessing anti-inflammatory properties with reduced gastric toxicity. In fact, 5-LOX now stands implicated in the deterioration of joints in OA. Inhibition of 5-LOX, therefore, can be said to protect cartilage and connective tissue from damage and also slow the progression of the disease.

LTB₄ protects the 5-LOX pathway. It has been shown to regulate the synthesis of interleukin-1β (IL-1β) by synovium. Excess production of LTB₄ leading to up regulation of IL-1β synthesis in the synovium during osteoarthritis has been shown to be responsible for damage to the joints and progression of disease. NSAIDs by inhibiting only COX pathways are expected to make arachidonic acid more available for the formation of LTB₄ and thereby of IL-1β. This also explains how some NSAIDs accelerate the progression of osteoarthritis (OA). A recent study has reported that reduction of structural changes by licofelone in OA is associated with the reduction of synthesis of LTB₄ and IL-1β synthesis in the synovium.[15] The occurrence of structural changes during the course of OA are related, besides the above factors, a number of complex pathways and mechanisms including the excessive production of proteolytic enzymes that can degrade the cartilage matrix and soft tissue around the joint. Among the proteolytic enzymes, the matrix metalloproteinase’s (MMPs), especially MMP-13, Aggrecanase 1and 2, and a cathepsin are the ones most likely to be involved in the degradation of cartilage. In a study focused on the effects of licofelone on the gene expression and protein synthesis of the major collagenolytic enzymes (MMP-13, aggrecanase and cathepsin K) in OA cartilage in an experimental model, licofelone has been found to markedly reduce the mRNA expression/synthesis of the above noted enzymes and prevent the death of chondrocytes indicative of a promising disease-modifying effect of licofelone in OA.[16] The most promising compound, the tolylsulphonamide 11f, revealed an IC₅₀ of 2.1 µM and is equipotent to the literature reference molecule MK-886. Selected compounds also potently reduced 5-LOX product formation in intact cells. Inhibition of isolated COX was occasionally remarkably cut down.[17]

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, COX-1 and COX-2 as well as microsomal PG-E₂ synthase (mPGES)-1. Licofelone concentration-dependently inhibited isolated COX-1 (IC₅₀ = 0.8 µM), whereas isolated COX-2
was less affected (IC₅₀ > 30µmol). However, licofelone efficiently blocked the conversion of PG-H₂ to PG-E₂ mediated by mPGES-1 (IC₅₀ = 6 µmol) derived from microsomes of interleukin-1beta-treated A549 cells, being about equipotent to MK-886, a well recognized mPGES-1 inhibitor. In intact interleukin-1β-treated A549 cells, licofelone potently (IC₅₀ < 1 microM) 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.[18]

Gene expression of ML3000 treated and untreated rheumatoid arthritis synovial fibroblasts were measured with Affymetrix gene arrays. Downregulation of chemokine (C-X-C motif) ligands CXCL9, CXCL10 and CXCL11 was verified with Real-time polymerase chain reaction, CXCL10 protein levels were determined with ELISA to find previously unknown properties of ML3000, a competitive inhibitor of the cyclooxygenase and the LO pathway. Rheumatoid arthritis synovial fibroblasts were treated with the COX inhibitor naproxen, the 5-LO inhibitor BWA4C and the 5-lipoxygenase-activating protein (FLAP) inhibitor MK886, and consecutive changes in CXCL10 protein levels were determined with Western blot. Synovial fibroblasts and monocyte-derived macrophages ML3000 inhibited the tumour necrosis factor induced expression of CXCL9, CXCL10 and CXCL11, which are all ligands of the chemokine receptor CXCR3. No effect was observed in monocytes. Whereas inhibition of the cyclooxygenase pathway or the FLAP protein showed no effect, blockade of 5-LO significantly downregulated CXCL10 protein levels. 5-LO mRNA was detected in monocytes and in monocyte-derived macrophages. All tested cell types expressed 5-LO protein. ML3000 effectively downregulates CXCR3 ligands. This study confirms that a thorough analysis of the impact of a drug on its target cells cannot only reveal unexpected properties of a substance, but also helps to understand the underlying molecular mechanisms. Accordingly, our data provide the basis for further clinical studies testing the application of ML3000 in diseases such as rheumatoid arthritis or multiple sclerosis.[19]

**Antithrombotic activity**

The antithrombotic activity of ML3000 (10, 30 and 100 mg·kg⁻¹) and aspirin (30 and 100 mg·kg⁻¹) was measured in the mesenteric venules of rats using the laser-induced thrombus model. Both ML3000 and aspirin, at all doses tested, showed significant antithrombotic activity. The mean number of laser injuries necessary to induce a thrombus that blocked the vessel was 1.93± 0.28 in the control group, 3.3 ± 0.53, 3.6 ± 0.14 or 4.07 ± 0.37 in the groups treated with ML3000 at 10, 30 or 100 mg·kg⁻¹ p.o. and 3.4± 0.55 or 3.9 ±0.3 in the groups treated with Aspirin at 30 or 100 mg·kg⁻¹ p.o. The antithrombotic activity in this model was significant up to 12 h post-administration of 100mg·kg⁻¹ ML3000 or Aspirin. The aggregation inhibiting activity of ML3000 (1-100 µg·ml⁻¹) and indomethacin (1 µg·ml⁻¹) was studied using the following inducing agents: ADP (1 and 2 µM), epinephrine (25 and 50 µM), collagen (0.5 and 1 µg·ml⁻¹), and the thromboxane mimetic U46619 (0.8 and 1.6 µM). Aggregation inhibitory activity was observed with ML3000 in all assays except with the higher concentration of U46619 at 1.6 µM. Indomethacin (1 µg·ml⁻¹) inhibited aggregation in all assays. ML3000 has significant antithrombotic activity and a marked platelet aggregation inhibiting effect. Given its demonstrated antiinflammatory activity, platelet function inhibition, and antithrombotic effects along with a lack of effect on the GI mucosa, ML3000 may offer an alternative to the combination of a COX-2 inhibitor and aspirin in arthritis patients at risk for cardiovascular disease.[20]
Gay et al evaluated the dual acting antiinflammatory drug ML3000 to search for potential new therapies to inhibit the progression of joint destruction in patients with rheumatoid arthritis. 5-LOX as well as COX-1 and COX-2 in the rat model of adjuvant arthritis. On Day 0, female Lewis rats (5 per group) were injected intradermally with complete Freund's adjuvant at base of the tail. Treatment began on Day 2; the rats received ML3000 (20 or 80 mg·kg⁻¹ each day) twice daily 7 h apart for 28 days and were then sacrificed. To reduce pain, the positive control group and 2 treatment groups received paracetamol (3 mg·ml⁻¹ water). Joint histology was scored for synovial cell proliferation, fibroproliferative pannus, and cartilage and bone erosions, as well as diffuse leukocyte infiltrates.

Daily doses of 20 or 80 mg·kg⁻¹ ML3000 significantly reduced the arthritis associated deficiency of body growth, the edema/erythema score, and splenomegaly. In the ankle joint, ML3000 significantly reduced the overall histological score, synovial cell proliferation, and bone/cartilage erosions, and inhibited the appearance of fibroproliferative pannus. The addition of paracetamol in the drinking water had no influence. ML3000 is an antiarthritic drug with a high gastrointestinal tolerability, which can reduce synovial cell proliferation and joint erosion and is capable of markedly suppressing prostaglandin synthesis.

Using a human whole blood assay the effect of ML3000 on the synthesis of products of 5-LOX (LTB4, LTC4) and COX-1/2 (TXB-2, PGE2) in vitro and ex vivo in order to further elucidate the mechanism of action of ML3000. ML3000 (0.3, 1, 3, 10, 30 µg·ml⁻¹) and indomethacin (0.3, 1, 3, 10, 30 µg·ml⁻¹) concentration-dependently inhibited the synthesis of PGE-2 (IC₅₀ = 3.9 and 4.5 µM). In contrast to ML3000, indomethacin produced an increase in LTC4 of up to 155.5% of control. 5-LOX inhibition was further tested in a basophilic leukemia cell assay using RBL-1 cells. ML3000 (1-10 µM) inhibited the synthesis of LTB4 in a concentration related manner (IC₅₀: 3.6 µM). In carrageenan induced rat paw edema, ML3000 and indomethacin completely blocked the formation of PGE₂ in the inflamed tissue. The LTB₄ production in the inflamed paw was reduced to basal levels by ML3000 (10 ±1.4 pg/paw saline control and 7.5 ±1.3±5.9±3.2 pg/paw ML3000), whereas LTB₄ levels remained markedly elevated as compared to saline control by indomethacin (30.7 pg/paw).

5-LOX inhibition in the inflamed rat colon was investigated by measuring LTB₄ synthesis. MK-886 and ML3000 at 10 mg·kg⁻¹ p.o. reduced LTB₄ production to 29.8±4.9 and 30.1 ±2.8 pg·mg⁻¹ tissue as compared to control (54.2 ±7.4 mg·kg⁻¹ tissue). LTB₄ levels in the rat stomach were comparable to control (2.5 ±0.4 pg·g⁻¹ protein) after oral administration of ML3000 (10, 30, 100 mg·kg⁻¹), whereas oral treatment with indomethacin (0.3, 1, 3 mg·kg⁻¹) or diclofenac (1, 3 mg·kg⁻¹) increased LTB₄ up to 9.2± 2.3 or 8.9 ± 1.6 pg·mg⁻¹ protein. This effect was significant at 1 mg·kg⁻¹ diclofenac and 0.3 mg·kg⁻¹ indomethacin. These results provide further evidence, that ML3000 inhibits 5-LOX as well as COX-1 and COX-2 in vitro and in animal experiments. The favourable gastrointestinal (GI) tolerability of the compound is believed to be linked to the mechanism of combined 5-LOX and COX-1/2 inhibition of ML3000.

Licofelone inhibited LTC₄ formation by mixed polymorphonuclear leukocyte/platelet suspensions stimulated with A-23187 (IC₅₀ = 3.8 µM). Licofelone also inhibited the generation of reactive oxygen species, release of elastase by polymorphonuclear leukocytes, and homotypic polymorphonuclear leukocyte aggregation induced by N-formyl-methionyl-leucyl-phenylalanine (fMLP), complement fraction 5a (C5a) and platelet activating factor (PAF), respectively. These in vitro studies demonstrated that licofelone inhibits 5-LOX as well as COX-1 and COX-2 activity, and therefore, polymorphonuclear leukocyte responses relevant to the pathogenesis of inflammation. The pharmacodynamic profile of licofelone has been assessed and compared with widely used NSAIDs in various animal models. In a carrageenan-induced rat paw edema model, licofelone (10, 30 and 100 mg·kg⁻¹) demonstrated an ED50 value of 17 mg·kg⁻¹ po and completely inhibited both PGE2
and LTB4 secretion, compared with indomethacin (10 mg·kg⁻¹), which only inhibited secretion of PGE2.\[24\]

In another study using this model, licofelone (2.5 and 5 mg/kg/day for 8 weeks) markedly reduced the level of chondrocyte apoptosis, and significantly decreased the levels of caspase-3, COX-2 and iNOS in cartilage from both condyles and plateaus.\[25\] In vitro, licofelone (0.8 to 8 μM) inhibited the production of PGE2 and LTB4 by OA osteoblasts at the highest dose, dose-dependently stimulated 1,25-dihydroxy vitamin D-induced alkaline phosphatase activity, and inhibited osteocalcin release via its effect on LTB4 production.\[26\] These results suggest that licofelone could be used as a disease-modifying drug for the treatment of OA and rheumatoid arthritis (RA).

The gastric-sparing properties of licofelone have been investigated. The drug dose-dependently inhibited ATPase activity in pig gastric microsomes with an IC₅₀ value of 16.6 μM. When the drug was diluted by 100-fold, the inhibitory effect was abolished. Licofelone-treated human gastric adenocarcinoma cells secreted less baseline and IL-1β- induced IL-8 with IC₅₀ values of 0.82 and 1.2 μM, respectively.\[27\] The inhibition of COX and 5-LOX by licofelone was first determined in a bovine thrombocyte intact cell assay and intact bovine polymorphonuclear leukocytes, respectively (IC₅₀ values of 0.21 μM for COX and 0.18 μM for 5-LOX).\[28\]

**Effect on adjuvant arthritis**

In a rat adjuvant arthritis model, licofelone (20 or 80 mg·kg⁻¹/day bid for 28 days) significantly reduced the arthritis-associated deficiency of body growth, the edema/erythema score and splenomegaly. In the ankle joint, licofelone significantly reduced the overall histological score, synovial cell proliferation and bone/cartilage erosions, and also inhibited the appearance of fibroproliferative pannus.\[29\]

**Effect on yeast-induced hyperthermia and laser-induced thrombus**

In a brewer's yeast-induced hyperthermia model in rats, a significant antipyretic effect was noted with licofelone (10 mg·kg⁻¹ po), which lasted 3 h and was comparable to the effect of indomethacin (10 mg·kg⁻¹ po). Licofelone was highly effective and potent in a guinea pig model of AC-induced bronchoconstriction (ED₅₀ = 0.2 mg·kg⁻¹ with iv injection.\[30\] In addition, licofelone (100 mg), administered as an aerosol to allergic sheep before antigen challenge, significantly inhibited the early bronchial response and completely blocked late antigen-induced bronchoconstriction. It also attenuated airway hyper-responsiveness to aerosolized carbachol that occurred 24 h after antigen challenge. In a rat laser-induced thrombus model, licofelone (10, 30 and 100 mg·kg⁻¹ po) demonstrated significant antithrombotic activity comparable to aspirin (30 and 100 mg·kg⁻¹ po). An in vitro study demonstrated that licofelone (1 to 100 μg·ml⁻¹) had a marked platelet aggregation inhibiting effect.\[30\]

**Clinical pharmacological studies**

After the initial enthusiasm of the scientific community, a re-evaluation of some large, randomized double-blind clinical studies performed with two of these compounds, has disclosed that the late serious gastrointestinal complications are not significantly reduced in comparison with non-selective inhibitors and that cardiovascular concerns might arise particularly if theses drugs are utilized in patients with underlying heart diseases. A new promising class of drugs to control inflammatory diseases is in advanced clinical development. The balanced
inhibitors of 5-LOX and of COX-1 and COX-2 block the formation of all the enzymatically arachidonic acid-derived metabolites, both prostaglandins (like COX inhibitors) and LT; these drugs have been shown to possess a very good anti-inflammatory efficacy without serious side effects. Licofelone, previously known as ML3000, is the molecule in the most advanced phase of clinical development (phase III) among this class of compounds; it is a potent, competitive, and well balanced inhibitor of 5-LOX and COX pathways. The drug has been shown to possess analgesic, anti-inflammatory, antipyrretic antibranchectostrictory and antiplatelet properties at doses which are safe for the gastrointestinal tract. Moreover, the newly performed preclinical studies, here briefly reviewed, appear to indicate that the compound seems particularly suitable to protect the articular cartilage and the synovial space in degenerative joint disease and to exert a relevant antithrombotic activity. Preliminary results of clinical studies of licofelone in osteoarthritis indicate that the drug has a comparable or slightly better efficacy than that of naproxen but possesses a much better gastrointestinal safety. This latter important aspect has been also evaluated by an endoscopic study in normal volunteers randomly assigned to a 4-week treatment with licofelone, placebo or naproxen. The results indicate that no ulcers occurred in either licofelone group or the placebo group, while ulcers with unequivocal depth were present in 20% of the naproxen-treated subjects.\textsuperscript{[31]}

Pharmacokinetics of licofelone

Licofelone is a dual inhibitor of both cyclooxygenase isomers and 5-lipoxygenase and under development for treatment of osteoarthritis. In conventional in vitro assays using liver microsomes and NADPH as cosubstrate, a high metabolic stability of licofelone was observed. In the presence of UDP-glucuronic acid, licofelone is rapidly converted into the corresponding acyl glucuronide, M1. These results are in conflict with data from clinical studies. After administration of licofelone to humans, M1 plasma concentrations were negligibly low, whereas the exposure of the hydroxy-metabolite M2 achieved values of approximately 20% compared with that of the parent drug. Metabolism studies with human hepatocytes and dual-activity assays with microsomes, which allowed the simultaneous monitoring of

The ECL measurement was performed using the Perkin Elmer QPCR System 5000, while the BL methodology used a SeaLife Science AquaLite Aequorin-antibody conjugate, which was detected with a ML3000 luminometer. Both instruments were found to be extremely sensitive with accurate quantitation of label in the attomole range, allowing detection during the exponential phase of PCR amplification. In our hands, it was possible to detect $1.5 \times 10^{14}$ copies (18 cycles) of IL-2 PCR product using ECL and $1 \times 10^{13}$ copies (14 cycles) using BL technology. Overall, we found the BL assay to be a rapid, sensitive, and inexpensive way to quantitate PCR-generated products with a broad range of potential analytical applications.\textsuperscript{[32]} In a human whole blood assay, licofelone (0.3, 1.0, 3.0, 10 and 30 μg·ml$^{-1}$) and indomethacin (0.3, 1.0, 3.0, 10 and 30 μg·ml$^{-1}$) concentration-dependently inhibited the synthesis of PGE2 (IC$_{50}$ = 3.9 and 4.5 μM, respectively). In contrast to licofelone, indomethacin produced an increase in LTC4 of up to 155.5% of control. Furthermore, licofelone (1 to 10 μM) inhibited the synthesis of LTB4 in a concentrationrelated manner (IC$_{50}$ = 3.6 μM) in a basophilic leukemia cell assay using RBL-1 cells.\textsuperscript{[33]}
hydroxylation and glucuronidation reactions, were performed, and the metabolic pathway of licofelone was elucidated. After glucuronidation, predominantly catalyzed by UDP glucuronosyltransferase (UGT) isoforms UGT2B7, UGT1A9, and UGT1A3, M1 is converted into the hydroxy-glucuronide M3 in a CYP2C8-dependent reaction. The enzyme specificities were investigated using recombinant human cytochrome P450 and UGT isoforms as test systems. In vitro drug-interaction studies using the 6α-hydroxylation of paclitaxel as control reaction confirmed that neither licofelone nor M1 is a relevant inhibitor of CYP2C8. The formation of M3 was also observed with liver microsomes from cynomolgus monkeys, but in incubations with mouse and rat liver microsomes, M1 remained unchanged.[9]

**Biotransformation pathway**

![Chemical structure of licofelone and its proposed biotransformation pathway.](image)

The results from in vitro metabolism studies show that in humans hydroxylation of the glucuronide M1 represents the pivotal step in the biosynthesis of M2.[9] Although the cytochrome P450 (P450)-dependent hydroxylation of glucuronides has been described in the literature,[34,35] the formation of M2 represents a unique example as the systemic exposure of humans to this major metabolite is based on the glucuronidation of the parent drug followed by hydroxylation of the glucuronide.

In the presence of UDP-glucuronic acid, licofelone is rapidly converted into the corresponding acyl glucuronide, M1. These results are in conflict with data from clinical studies. After administration of licofelone to humans, M1 plasma concentrations were negligibly low, whereas the exposure of the hydroxy-metabolite M2 achieved
values of approximately 20% compared with that of the parent drug. Metabolism studies with human hepatocytes and dual-activity assays with microsomes, which allowed the simultaneous monitoring of hydroxylation and glucuronidation reactions, were performed, and the metabolic pathway of licofelone was elucidated. After glucuronidation, predominantly catalyzed by UDP glucuronosyltransferase (UGT) isoforms UGT2B7, UGT1A9, and UGT1A3, M1 is converted into the hydroxy-glucuronide M3 in a CYP2C8-dependent reaction. The enzyme specificities were investigated using recombinant human cytochrome P450 and UGT isoforms as test systems. In vitro drug-interaction studies using the 6- hydroxylation of paclitaxel as control reaction confirmed that neither licofelone nor M1 is a relevant inhibitor of CYP2C8. The formation of M3 was also observed with liver microsomes from cynomolgus monkeys, but in incubations with mouse and rat liver microsomes, M1 remained unchanged.[9]

**Phase I and Phase II metabolism in vitro**

Initial in vitro phase I biotransformations using liver microsomes from rat and human showed a high metabolic stability of licofelone. Two hydroxy-metabolites, M2 and M4, were detected, but concentrations, determined after 30 or 60 min incubation of 10 to 100 µmol licofelone, reflected that less than 2% of the substrate was converted. Assays with diclofenac were performed as control reactions. In incubations with HLM, only 0.06% of the initial substrate concentrations was converted into M2 compared with 1.8% M4. With RLM as test system, 1.1% M2 and 1.8% M4 were found. The formation of M4 was somewhat surprising because in plasma samples from laboratory animals, this metabolite was not detected, and in humans, quantifiable concentrations were determined only in plasma samples, which were collected during a clinical study after administration of supratherapeutic doses to determine the maximum tolerated dose. To identify the metabolizing enzymes involved in the formation of hydroxy-metabolites, experiments with different P450 isoforms were performed. Although the direct hydroxylation of licofelone has been considered as a quantitatively negligible pathway, a primary screening experiment, conducted with 10 µmol licofelone, gave evidence that CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2J2, and CYP3A4 are involved in the oxidation of licofelone. Thus, in a second experiment, 100 µmol licofelone was incubated in the presence of 200 pmol.ml⁻¹ of these isoenzymes. In all the incubations, both hydroxy-metabolites were detected, but M4 concentrations always exceeded those of M2 (Fig. 2).[9]

Fig 2. Biotransformation of licofelone in phase I assays with CYP isoforms (n = 2, rates given as means). Metabolite concentrations were determined by LC/MS/MS and expressed as percentage relative to the initial substrate concentration of 100 µM.

With more than 600 ng.ml⁻¹ M4, which corresponds to a substrate turnover of approximately 1.6%, CYP2C9*1 showed the highest activity toward formation of M4 followed by CYP2J2> CYP3A4> CYP2C8 > CYP2C19> CYP2D6*1. However, M4 concentrations formed with CYP2J2, CYP3A4, CYP2C8, CYP2C19, and CYP2D6*1 were less than the third compared with
the amount found in incubations with CYP2C9*1. With 67.8 ng·ml⁻¹, only CYP2J2 formed significant amounts of M2. CYP2C9*1 and CYP2C8 also formed M2 but to a much lower extent.[9]

**Biotransformation of Licofelone in Human Hepatocytes.**

The metabolism experiments using human hepatocytes showed that both biotransformation activities (i.e., hydroxylation and glucuronidation) were required to achieve an M2/M4 ratio that at least qualitatively reflects the human in vivo metabolism. However, the integrated phase I/II metabolic capacity of hepatocytes does not allow the elucidation of metabolic pathways. Furthermore, this test system is less suitable to identify the enzymes responsible for biotransformations of interest. Finally, hepatocytes from dogs, mice, or monkeys are not ubiquitously available, which limits their use for interspecies comparison of drug metabolism. Licofelone (30 and 100 µmol) was added to primary human hepatocytes, and aliquots of the supernatant were analyzed by LC/MS. The rate of metabolite formation was not linear throughout the observation period but significantly decreased after 4 h. Biotransformation rates, which were derived from metabolite concentrations determined at 4 and 24 h after incubation of 30 and 100 µmol licofelone, the glucuronide M1 accounted for 85% of the sum of metabolites and was clearly dominating after 24 h. Both hydroxy-metabolites M2 and M4 were present, but in contrast to phase I metabolism experiments using microsomes, M2 concentrations substantially exceeded those of M4, in particular if corresponding glucuronides were considered. The observation that M3 concentrations substantially exceeded those of M2 was interpreted as a result of the high glucuronidation activity of hepatocytes. M5 was detected, but throughout the observation period concentrations remained below the limit of quantification.[9]

![Fig 3. Biotransformation of licofelone in dual-activity assay with HLM (n= 3, rates given as mean ± S.D.). Bars illustrate the percentage biotransformation related to the initial substrate concentration, and the numbers above the bars correspond to absolute concentrations. Top, hydroxy-metabolites M2 and M4; bottom, glucuronide conjugates M1 and M3.][9]

**Dual-Activity Assays Using Liver Microsomes.**

The metabolism experiments using human hepatocytes showed that both biotransformation activities (i.e., hydroxylation and glucuronidation) were required to achieve an M2/M4 ratio that at least qualitatively reflects the human in vivo metabolism. However, the integrated phase I/II
metabolic capacity of hepatocytes does not allow the elucidation of metabolic pathways. Furthermore, this test system is less suitable to identify the enzymes responsible for biotransformations of interest. Finally, hepatocytes from dogs, mice, or monkeys are not ubiquitously available, which limits their use for interspecies comparison of drug metabolism.[9]

In Fig. 3, the biotransformation rates, relative to the initial substrate concentrations, and the concentrations are given. With 10 and 30 µmol licofelone, the sum of glucuronides (M1 < M3) was 3.41 and 9.92µmol, which correspond to biotransformation rates of 34 and 33%. With 100 µl of licofelone, the relative glucuronide content was 25%. The biotransformation rate of M2 and M3 decreased with ascending substrate concentrations. The highest M3 concentration (5.02 ± 0.11 µmol) was observed at 30 µmol, whereas only 2.41 ± 0.56µmol was determined after incubation of 100 µmol licofelone. These data indicate that the glucuronidation capacity of the test system was limited at concentrations greater than 30 µM. In contrast, hydroxylation of M1 was partly inhibited in incubations with 100 µmol. M4 concentrations increased with ascending substrate concentrations in an almost proportional manner. The biotransformation rate to M4 was essentially constant.[9]

Pharmacokinetics in animals
Plasma levels and distribution of radioactivity were examined using whole-body autoradiography after oral administration of 14C-labeled licofelone (13.7 to 28.6 mg·kg⁻¹) to female rats (Fig 4). Plasma levels of licofelone peaked at 3 to 4 h after administration, with a plasma t 1/2 of about 11 h. The highest tissue levels of licofelone were detected in the lung, liver, kidney, heart and intestine. Almost no penetration of the blood-brain barrier was noted; however, after 48 h there was a minor accumulation in fat. Of the total radioactivity, 58.3% was found in the feces and 7.9% in the urine(Fig 5).[36]

The whole-body autoradiograms (Fig 6) show that an increased radioactivity is left in the stomach, the highest tissue level is detected in the lung, liver, kidneys, heart, large intestine and small intestine(Fig 6); the tissue levels cumulation of radioactivity is also seen in the spleen, levels in the lung, liver and kidneys are lower relative to the intestine levels (Fig 6b); and at 48 h, the tissue distribution is similar to the result obtained after 24 h (Fig 6c).

Fig 4. Plasma level of radioactivity of 14C-ML3000 after oral administration of a single dose 28.6 mg.kg⁻¹ to rats.

Fig 5. Excretion of radioactivuy with feces(-) and urine (---) after oral administration of 28.6 mg.kg⁻¹ dose.

Pharmacokinetics in humans
In humans, after p.o. administration of immediate-release tablets, licofelone is rapidly absorbed from the gastrointestinal tract, and maximum plasma concentrations are achieved approximately 2 to 3 h after administration. Systemic elimination follows biphasic characteristics with a rapid initial decrease of plasma concentration [T½α= 1 h] and a slow
terminal elimination \([T_{1/2}β = 7–9 \text{ h}]\). In plasma, after single dosing, ML3000–1-\(O\)-acyl glucuronide (M1) and hydroxy-ML3000 (M2) were detected as metabolites. Relative to the parent drug, the systemic exposure remained less than 2%. After repeated administration, at steady state, the exposure of M2 increased to approximately 20% relative to that of the parent drug, and the rate of systemic elimination was below that of the parent drug (monophasic, \(T_{1/2}β = 10–12 \text{ h}\)).[9]

Fig 6. Whole-body autoradiograms after oral administration. a: 21.6 mg.kg\(^{-1}\at 24\text{h}, b:13.7 \text{ mg.kg}^{-1}\at 24\text{h}, c:28.6\text{ mg.kg}^{-1}\at 48\text{h}.

Licofelone (200 mg bid for 5 days and a single final dose of 200 mg on day 6) was administered in 18 healthy male and female young (mean age of 30.9 years) and elderly
(mean age of 72.1 years) individuals. Following the first dose, mean Cmax was similar for young (1665 ± 1151 ng·ml⁻¹) and elderly (1637 ± 903 ng·ml⁻¹) individuals. The maximum plasma concentrations were reached 0.74 to 4 h after administration, while the mean AUC(0 to 12) was 23% lower in the young individuals (5646 ± 2073 versus 4582 ± 1927 ng.h·ml⁻¹). Licofelone demonstrated similar Cmax values in the two groups at steady-state, with young individuals having a Cmax value of 1727 ± 829 ng·ml⁻¹ and elderly individuals having a Cmax value of 1744 ± 616 ng·ml⁻¹; the AUC was 20% higher in elderly individuals. t1/2(β) was greater in young individuals than elderly ones (11.1 ± 7.0 versus 8.7 ± 4.7 h), while the mean t1/2(α) value was 15% higher in the elderly study population. No pharmacokinetic interaction between licofelone and warfarin was observed, suggesting that the two drugs have different elimination pathways.

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References

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