Synthesis and Biological Evaluation of Licofelone Derivatives as Anticancer and Anti-inflammatory Agents

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Abstract: Two C5-substituted licofelone derivatives were developed and investigated for cytotoxicity against mammary (MCF-7 and MDA-MB 231) as well as colon carcinoma (HT-29) cancer cells. Both compounds were at least 2-fold more active than 5-fluorouracil (5-FU) and licofelone against mammary carcinoma cells. At HT-29 cells, they were less active, but nevertheless distinctly as active as 5-FU and still 2-fold more active than licofelone. However, variation of the C5-carboxylic group results in an occasionally remarkable decrease of anti-inflammatory potency in *in vitro* and *in vivo*.

Keywords: Anti-inflammatory activity, COX inhibition, Cytotoxicity, Licofelone, Synthesis.

INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are drugs with anti-inflammatory, anti-pyretic and analgesic effects. In recent years numerous experimental, epidemiologic, and clinical studies suggest that NSAIDs are promising anticancer agents. NSAIDs restore normal apoptosis in human adenomatous colorectal polyps and in various cancer cell lines that have lost adenomatous polyposis coli gene function. NSAIDs also inhibit angiogenesis in cell culture and rodent models of angiogenesis. Many epidemiologic studies have found that long-term use of NSAIDs is associated with a lower risk of cancers [1-5].

Recently, several companies were running drug-discovery programs to develop dual cyclooxygenase /lipoxygenase (COX/LOX) inhibitors as NSAIDs with an improved gastrointestinal safety. To date, various structural families of dual COX/LOX inhibitors have been designed and several compounds are currently undergoing clinical development as anti-inflammatory drugs [6-15]. One of these compounds, [2,2-dimethyl-6-(4-chlorophenyl)-7-phenyl-2,3-dihydro-1*H*-pyrrolizin-5-yl]acetic acid) (licofelone, Scheme **1**) showed in clinical trials anti-inflammatory and analgesic activity in osteoarthritis comparable to conventional NSAIDs with a safer gastrointestinal profile. It is a potent, competitive inhibitor of 5-LOX, COX-1 and COX-2 [6-14]. Researches also addressed that licofelone appears to suppress inflammatory prostaglandin E2 (PGE2) formation preferentially by inhibiting microsomal prostaglandin E2 synthase-1 (mPGES-1) at concentrations that do not affect COX-2, implying attractive and thus far unique molecular pharmacological dynamics as an inhibitor of COX-1, 5-LOX, and mPGES-1 [10, 11]. In addition, it enhanced apoptosis in prostate cancer cells as well as HCA-7 colon cancer cells through the mitochondrial pathway [12, 13].

Moreover, we recently reported a novel series of C5-substituted licofelone derivatives. The acetic acid of licofelone was replaced by an acetyl residue which was further substituted with lipophilic groups (alkyl or chlorine), formate, acetate, propionate, benzoates, and methylbenzoates. Dependent on the C5-substituent, the compounds showed high selectivity for MCF-7 cells. Especially acetyl benzoate derivatives were inactive at the MDA-MB 231 cell line and as active as 5-FU at MCF-7 cells. C5-acetyl, -acetyl formate, -acetyl acetate and –acetylpropionate derivatives showed growth inhibition at both cell lines, comparable with cisplatin [15].

Based on the encouraging antitumor activity of licofelone and its C5-substituted derivatives, we continue our efforts to vary the substituents at C5 of licofelone. In this paper, the acetic acid was replaced by an acetyl residue which was further substituted with methoxy group and 1,2,4-triazole. The novel compounds were tested for cytotoxicity against MCF-7, MDA-MB 231 breast cancer and HT-29 colon cancer cells as well as for anti-inflammatory potency in *in vitro* and *in vivo.*

MATERIALS AND METHODS

Materials

All reagents were purchased from Shanghai Chemical Reagent Company. Licofelone and 5-benzyl-3,3-dimethyl-3,4-dihydro-2*H*-pyrrole (**2**) were synthesized according to previous method [8, 9, 11, 16-18]. Column chromatography (CC): silica gel 60 (200–300 mesh). Thin-layer chromatography (TLC): silica gel 60 F254 plates (250 mm; Qingdao Ocean Chemical Company, China). Melting point: capillary

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tube; uncorrected. IR spectra: Shimadzu FTIR-8400S spectrophotometer. ^{1}H-NMR spectra: Bruker ACF-300 Q apparatus at 300 MHz (internal standard, TMS). Mass spectrometry (MS): Hewlett–Packard 1100 LC/MSD spectrometer; in m/z. Elemental analyses: CHN-O-Rapid instrument.

6-(4-chlorophenyl)-2,2-dimethyl-7-phenyl-2,3-dihydro-1H-pyrrolizine (3)

The 5-benzyl-3,3-dimethyl-3,4-dihydro-2*H*-pyrrole (**2**) (10 g) was dissolved in absolute ethanol (130 mL). The solution was cooled to 20 °C and 2-bromo-1-(4-chlorophenyl)ethanone (13 g) was added. After stirring for 30 min, $NaHCO_3$ (6 g) was added and the reaction was continued at 20-25 °C with protection from light. After about 24 h, the yellowish suspension was then filtered under suction and the solid was washed with a small amount of cold ethanol. The dried product was suspended in water at 40-45 °C. After stirring for 30-35 min, the product was then filtered under suction and dried in vacuo at 35-40 °C.

Yield 25%; MS (ESI, m/z): $[M+H]^+$ 322.2; IR (KBr, cm^{-1}): 2956, 2917, 2869, 1699, 1600, 832, 765, 699; ^{1}H-NMR ($CDCl_3$, 300MHz): δ 1.26 (s, 6H, 2-CH_3), 2.79 (s, 2H, -CH_2-), 3.73 (s, 2H, -CH_2-), 6.67 (s, 1H, =CH-); 7.03-7.26 (m, 9H, Ar-H); Anal. calcd. for $C_{21}H_{20}ClN$: C, 78.37; H, 6.26; N, 4.35%; Found C, 78.56; H, 6.15; N, 4.31%.

[2,2-dimethyl-6-(4-chlorophenyl)-7-phenyl-2,3-dihydro-1H-pyrrolizin-5-yl]acetic acid (licofelone)

3 (4 g, 12.7mmol) was dissolved by stirring in dry THF. The assembly was blanketed with N_2 atmosphere. The yellow colored solution was cooled to 10-15 °C and oxalyl chloride (2.8 ml, 19.6 mmol) was slowly added below 15 °C over a period of 30 min. The green colored solution was further stirred at 18-25 °C for 20-30 min after complete addition. The internal temperature did not exceed 20 °C, the reaction mixture was then quenched carefully with ice (50 g). The reaction mixture was then stirred at 20-30 °C for 5-10 min and was further diluted with diethylene glycol (32 ml) and hydrazine (6 ml, 120 mmol). The solvent THF was distilled off during the process until the gradual rise in internal temperature reaches 75-80 °C. The suspension was then cooled to 50-55 °C and KOH (9.04 g, 161 mmol) was added portion wise over a period of 30 min. The now yellowish liquid was then heated to 95-110 °C gradually. The temperature was then raised slowly to 140-145 °C and during the process aqueous distillate was collected. The batch was held at 130-145 °C for 2-3 h. The reaction temperature was then cooled to 35-40 °C and water and diethyl ether were added. The mixture was stirred vigorously for 15-20 min and until the stirring switched off the layers were allowed to settle. The aqueous phase was separated cooled below 5 °C and acidified to pH 1 using a solution of HCl maintaining temperature below 10 °C. The separated solid was taken up in diethyl ether and the ethereal extract was washed thoroughly with water. The ethereal extract was charcoalized before being evaporated under vacuo below 20 °C. The solid was slurried in heptane and filtered under suction and washed with heptane. The product was dried in vacuum at 45-55 °C.

Yield 55.6%; mp 157 °C; MS (ESI, m/z): $[M+H]^+$ 380, $[M+Na]^+$ 402; IR (KBr, cm^{-1}): 3402, 2958, 2917, 2900, 2849, 1725, 1711, 1450, 832, 699; ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 1.30 (s, 6H, 2-CH_3), 2.85 (s, 2H, -CH_2-), 3.58 (s, 2H, -CH_2-), 3.75 (s, 2H, -CH_2-), 7.03-7.26 (m, 9H, Ar-H); Anal. calcd. for $C_{23}H_{22}ClNO_2$: C, 72.72; H, 5.84; N, 3.69%; Found C, 72.59; H, 5.98; N, 3.82%.

2-Chloro-1-(2-(4-chlorophenyl)-6,6-dimethyl-1-phenyl-6,7-dihydro-5H-pyrrolizin-3-yl)ethanone (5)

The reactions were performed in a 100 mL three neck round bottom flask fitted with a gas inlet port. The flask was charged with 50 mL of absolute diethyl ether, 0.4 g (1.27 mmol) of **3**, 0.25 mL of corresponding chloroacetyl chloride and 3.72 mmol of Lewis acid ($AlCl_3$ or BF_3-Et_2O). The resulting solution was magnetically stirred at room temperature and purged by nitrogen gas for 3 h. Subsequently, the reaction mixture was hydrolyzed with 60 mL of water and the product was extracted with diethyl ether. The organic layer was dried over Na_2SO_4 and the solvent was distilled off to give the crude product, which was then purified by flash column chromatography with mixture eluent of petroleum ether and ethyl acetate (40:1) to give a white solid.

Yield (40.1% ($AlCl_3$) or 59.3 % (BF_3-Et_2O)); mp 157-159 °C; MS (ESI, m/z): $[M+H]^+$ 398.2, $[M+Na]^+$ 420.2; IR (KBr, cm^{-1}): 2919, 2839, 1645, 1595, 1450, 1012, 748, 704; ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 1.31 (s, 6H, 2-CH_3), 2.84 (s, 2H, -CH_2-), 3.85 (s, 2H, -CH_2-), 4.24 (s, 2H, -CH_2-), 6.95-7.38 (m, 9H, Ar-H). Anal. calcd. for $C_{23}H_{21}Cl_2NO$: C, 69.35; H, 5.31; N, 3.52%; Found C, 69.07; H, 5.41; N, 3.71%.

1-(6-(4-chlorophenyl)-2,2-dimethyl-7-phenyl-2,3-dihydro-1H-pyrrolizine-5-yl)-2-(1,2,4-triazole-1-yl)-ethanone (6)

The flask was charged with 10 mL of absolute acetone, 0.14 g (2 mmol) of 1*H*-1,2,4-triazole and 0.21 g (2 mmol) of sodium carbonate, and 0.4 g (1.00 mmol) of **5**. The resulting solution was stirred at room temperature for over 24 h. Then the solvent was distilled off to give the crude product, which was then purified by flash column chromatography with mixture eluent of petroleum ether and ethyl acetate (1:1) and followed by recrystallization from acetone to give a white solid.

Yield: 75.0%; mp: 121-122 °C; MS (ESI, m/z): $[M+H]^+$ 431, $[M+Na]^+$ 453; IR (KBr, cm^{-1}): 3113, 2969, 2854, 1640, 1490, 972, 883, 669; ^{1}H-NMR ($CDCl_3$, 300MHz): δ 1.30 (s, 6H, 2-CH_3), 2.85 (s, 2H, -CH_2-), 4.19 (s, 2H, -CH_2-), 4.69 (s, 2H, -CH_2-), 6.97-7.42 (m, 9H, Ar-H), 7.92 (s, 1H, =CH-), 8.02 (s, 1H, =CH-); Anal. Calcd. for $C_{25}H_{23}ClN_4O$: C, 69.68; H, 5.38; N, 13.00%; Found C, 69.72; H, 5.31; N, 12.87%.

1-(6-(4-chlorophenyl)-2,2-dimethyl-7-phenyl-2,3-dihydro-1H-pyrrolizine-5-yl)-2-methoxy-ethanone (7)

The flask was charged with 10 mL of absolute methanol, 0.1 g (2 mmol) of sodium methoxide and 0.4 g (1.00 mmol) of **5**. The resulting solution was stirred at room temperature for over 24 h. Then the solvent was distilled off to give the crude product, which was then purified by flash column chromatography with mixture eluent of petroleum ether and ethyl acetate (20:1) to give a white solid.

Yield: 72.5%; mp 128-130 °C; MS (ESI, m/z): $[M+H]^+$ 394.2; IR (KBr, cm^{-1}): 3437, 2959, 1650, 1515, 967, 923,

773, 699, 644. ^{1}H-NMR ($CDCl_3$, 300MHz): δ 1.31 (s, 6H, 2-CH_3), 2.82 (s, 2H, -CH_2-), 3.23 (s, 3H, -CH_3), 3.76 (s, 2H, -CH_2-), 4.23 (s, 2H, -CH_2-), 6.95-7.37 (m, 9H, Ar-H); Anal. Calcd. for $C_{24}H_{24}ClNO_2$: C, 73.18; H, 6.14; N, 3.56%; Found C, 73.07; H, 5.98; N, 3.84%.

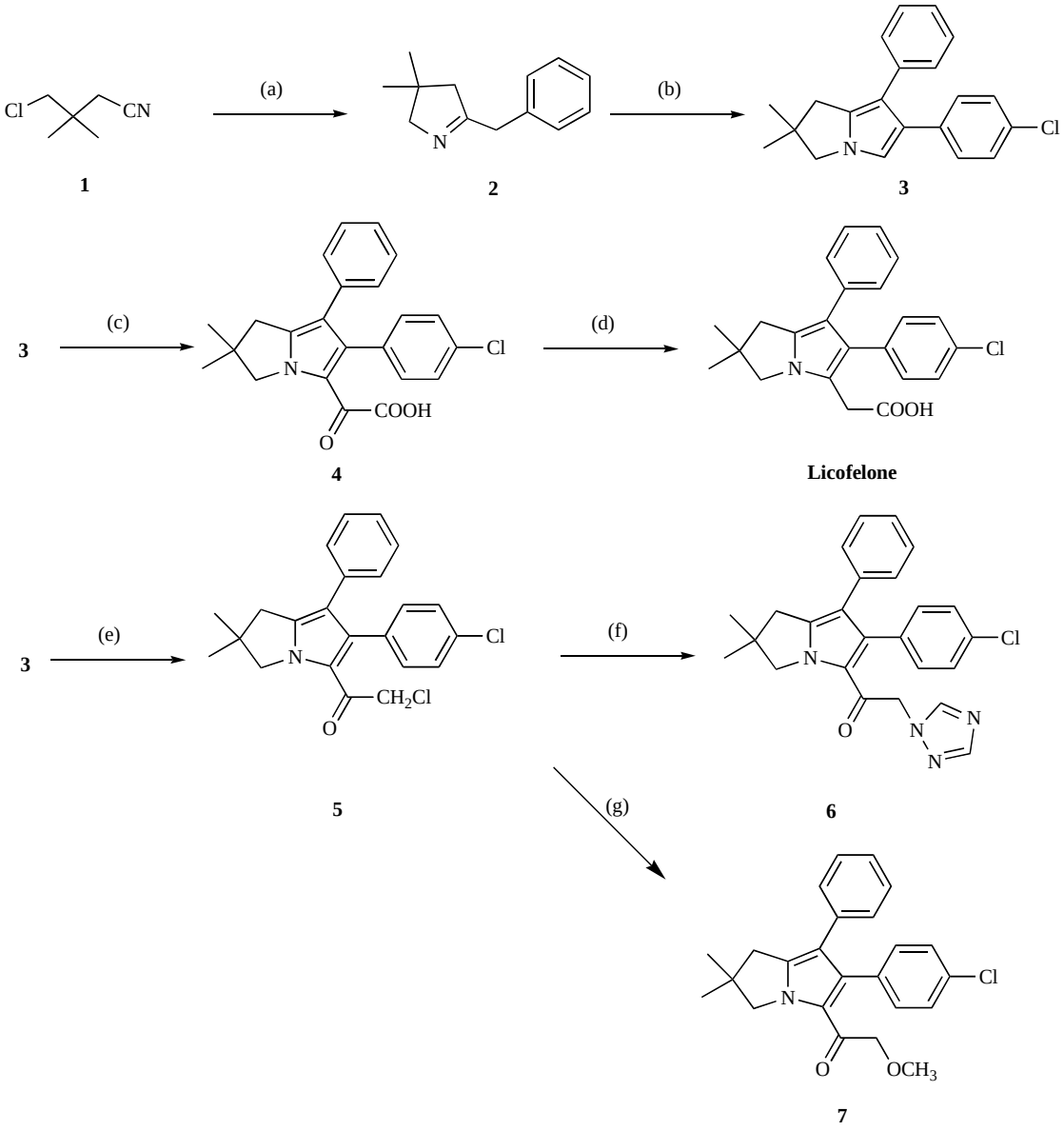
PHARMACOLOGY

Cell Culture

The human MCF-7, MDA-MB 231 breast cancer cell lines and HT-29 colon cancer cell line were obtained from the American Type Culture Collection. All cell lines were maintained as a monolayer culture in L-glutamine containing Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose (PAA Laboratories, Austria), supplemented with 5% fetal bovine serum (FBS; Biochrom, Germany) in a humidified atmosphere (5% CO_2) at 37 °C.

Cytotoxicity

The experiments were performed according to established procedures with some modifications [15]. In 96 well plates 100 μL of a cell suspension in culture medium at 7500 cells/mL (MCF-7 and MDA-MB 231) or 3000 cells/mL (HT-29) was plated into each well and was incubated for three days under culture conditions. After the



Scheme 1. Synthetic routes of licofelone, **6** and **7**.[a]

[a] Reagents and conditions: (a) benzylmagnesium chloride (Grignard species provided in situ from benzylchloride and Mg 1:1), initially absolute Et_2O, 2 h, reflux, then toluene, 3 h, reflux, 70%; (b) 2-bromo-1-(4-chlorophenyl) ethanone, absolute ethanol, $NaHCO_3$, 36 h, rt, 25%; (c) oxalyl chloride, THF, 10-15 °C, then add H_2O; 25-30 °C, 20 mins; (d) $N_2H_4{\cdot}H_2O$, KOH, ethylene diglycol, 85 °C, 5 h, then to 140-145 °C, 2 h, 55%.; (e) $ClCH_2COCl$, Lewis acid, absolute Et_2O, rt, 3 h, (40% ($AlCl_3$) and 59% (BF_3-Et_2O)). (f) 1-*H*-1,2,4-triazole, Na_2CO_3, absolute acetone, rt, over overnight, 75%. (g) $NaOCH_3$, absolute CH_3OH, rt, overnight, 72.5%.

addition of various concentrations of the test compounds, cells were incubated for up to 144 hours. Then the medium was removed, the cells were fixed with glutardialdehyde solution (1%) and stored under phosphate buffered saline (PBS) at 4 °C. Cell biomass was determined by a crystal violet staining, followed by extraction of the bound dye with ethanol and a photometric measurement at 590 nm. Mean values were calculated and the effects of the compounds were expressed as % Treated/Control$_{corr}$ values according to the following equations:

$$T/C_{corr}\,[\%] = \frac{T - C_0}{C - C_0} \cdot 100$$

(C_0 control cells at the time of compound addition; C control cells at the time of test end; T probes/samples at the time of test end)

The IC_{50} value was determined as the concentration causing 50% inhibition of cell proliferation and calculated as mean of at least three independent experiments (OriginPro 8).

Inhibition of COX Enzymes

The inhibition of isolated ovine COX-1 and human recombinant COX-2 was determined with 10 μM of the respective compounds by ELISA ("COX inhibitor screening assay", Cayman Chemicals). Experiments were performed according to the manufacturer's instructions. Absorption was measured at 415 nm (Victor2, Perkin Elmer). Results were calculated as the means of duplicate determinations.

Xylene-Induced Ear Edema

Animals

The experiments with animals were approved by Research Ethic Committee of Jiang-Shu province, China. Kunming male mice of approximately 20 g were obtained from experimental animal center of China Pharmaceutical University, and fed with rat food and water ad libitum. All animals fasted for 12 h before the experiments. The temperature (25 °C) and humidity (60%) in the animal room were well controlled.

Method

Mice were allotted to groups of 6 animals each. Thirty minutes after i.p. injection of **6, 7**, licofelone, ibuprofen or celecoxibe, 0.015 ml of xylene was applied to the anterior and posterior surfaces of the right ear. The left ear was considered as control. Two hours after xylene application, mice were killed and both ears were removed. Circular sections were taken, using a cork borer with a diameter of 7 mm, weighed and measured. The degree of ear swelling was calculated based on the weight and thickness of left ear without xylene.

Statistical Analysis

The measurement data were expressed as the mean ± SD. Data were subjected to one-way analysis of variance (ANOVA), followed by multiple comparison with least significant differences (LSD) test or Dunnett's test as appropriate. Statistical significance was considered with $P < 0.05$. The analysis of data was performed by software SPSS 13.0.

RESULTS AND DISCUSSION

Chemistry

6,7-Diaryl-2,3-dihydro-1*H*-pyrrolizine (**3**) and licofelone were synthesized according to previously published methods [8, 9, 11, 16-18] and outlined in Scheme **1**.

We condensed **1** with a commercially available benzyl-Grignard, and accomplished ring closure to rather unstable 5-benzyl-3,3-dimethyl-3,4-dihydro-2*H*-pyrrole (**2**). 6,7-Diaryl-2,3-dihydro-1*H*-pyrrolizine (**3**) was cyclized with the 2-bromo-1-(4-chlorophenyl)ethanone and **2** in ethanol/ aqueous $NaHCO_3$ solution at room temperature (rt) for 36 h with moderate yields. Friedel-Craft acylation of **3** with oxalyl chloride and subsequent Wolff–Kishner reduction with hydrazine hydrate yielded licofelone (Scheme **1**).

Analogously, **5** was obtained by reaction of **3** with chloroacetyl chloride by means of BF_3-Et_2O or $AlCl_3$ as catalysts (Scheme **1**). The reaction yield depended on the catalyst, while in the case of **5** both catalysts work as well (40% ($AlCl_3$) and 59% (BF_3-Et_2O)). Compound **6** was obtained by reaction of **5** with 1*H*-1,2,4-triazole and sodium carbonate, while compound **7** was obtained by reaction of **5** with sodium methoxide in methanol solution.

Under these synthetic conditions IR, ESI-MS, ^{1}H NMR of **3**, licofelone, **5-7** were recorded. The IR spectra of compounds **5-7** are very similar. In accordance with the structure, the IR spectra showed ν(CO) bands at about 1650 cm^{-1}. The ^{1}H NMR of **6** showed two triazole protons at δ 7.92 ppm and 8.02 ppm compared with **5**.While the ^{1}H NMR of **7** showed three methyl protons with single peaks at δ 3.23 ppm compared with **5**. Both compounds were further supported by positive mode ESI mass spectra, which documented a base peak corresponding to the $[M+H]^+$ fragment for **6** and **7**.

BIOLOGICAL ACTIVITY

In vitro cytotoxicity assays were performed to obtain an insight into the antitumor activity of licofelone and its derivatives. In addition to licofelone and its derivatives, the established antitumor drug 5-FU was screened against hormone dependent MCF-7, hormone independent MDA-MB 231 breast cancer and HT-29 colon cancer cell lines. MCF-7 cells have a basal level of COX-1 and a barely detectable and transient COX-2- inducible expression, whereas MDA-MB 231 cells show a low expression of COX-1 but a constitutive level of COX-2 [19]. HT-29 human colon cancer cells constitutively express COX-2 [20]. Therefore, their growth is sensitive to NSAID treatment [1-3].

The experiments were performed according to established procedures [15]. A number of known cells were exposed to increasing concentrations of compounds on a 96-well tissue culture plate and incubated for a given period of time. Due to the poor solubility of the substances, DMSO stock solutions had to be used. Because of the cytotoxicity of

Table 1. Antiproliferative Effects Against MCF-7, MDA-MB 231 and HT-29 Cells

	Cytotoxicity IC_{50}, [μM][a]		
Compound	**MDA-MB 231**	**MCF-7**	**HT-29**
6	4.8 ± 0.1	2.2 ± 0.2	6.0 ± 0.2
7	5.3 ± 0.1	2.0 ± 0.2	11.8 ± 0.3
Licofelone	36.7 ± 3.2	5.5 ± 0.6	22.6 ± 1.2
5-FU	9.6 ± 0.3	4.7 ± 0.4	7.3 ± 1.0

[a]The IC_{50} values represent the concentration which results in a 50% decrease in cell growth after 72 h incubation.

DMSO in higher concentrations, final DMSO concentrations were limited to 0.1% in all samples. IC_{50} values for these compounds were calculated (OriginPro 8) and are presented in Table **1**. Doses dependent antiproliferative effects of compounds in three cell lines are showed in Fig. (**1**).

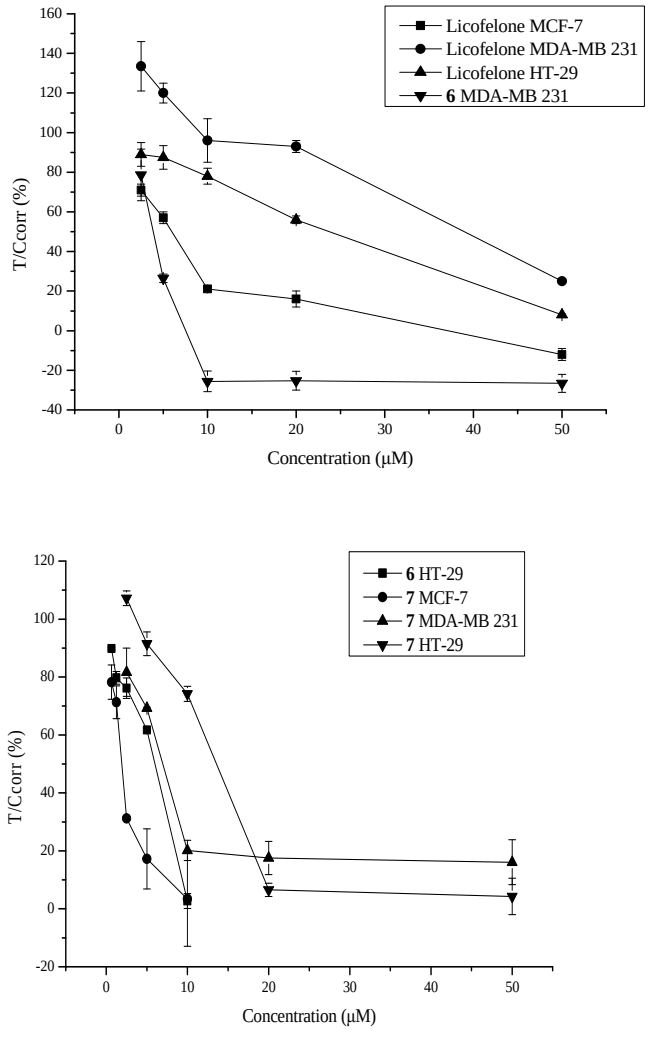


Fig. (1). Dose dependent antiproliferative effects of the novel compounds **6** and **7** as well as licofelone at MCF-7, MDA-MB 231 and HT-29 cells. In some cases the error bars are hidden behind the symbols.

Licofelone showed at the MCF-7 cell line an IC_{50} = 5.5 μM very similar to 5-FU (IC_{50} = 4.7 μM). Against MDA-MB 231 (IC_{50} = 36.7 μM) and HT-29 cells (IC_{50} = 22.6 μM), it was only marginally active indicating at least 4-fold selectivity for MCF-7 cells.

Both novel compounds **6** and **7** showed promising antiproliferative effects at MCF-7 cells (IC_{50} = 2.2 μM and 2.0 μM, respectively) and MDA-MB 231 cells (IC_{50} = 4.8 μM and 5.3 μM, respectively), approximately twice as active as 5-FU (IC_{50} = 4.7 μM, MCF-7; IC_{50} = 9.6 μM, MDA-MB 231). At HT-29 cells, compounds **6** and **7** were less active (IC_{50} = 6.0 μM and 11.8 μM, respectively) but nevertheless distinctly as active as 5-FU (IC_{50} = 7.3 μM)

In comparison with the results of licofelone, exchange of the C5-acetic acid moiety enormously increased the growth inhibitory effects. Both novel compounds were at least 2-fold as active as licofelone in three cell lines. However, the high selectivity for MCF-7 cells was lost. Moreover, the cytotoxicity of both compounds was comparable to C5-acetyl, -acetyl formate, -acetyl acetate and –acetylpropionate derivatives at MCF-7 and MDA-MB 231 cells [15].

The time-activity curves of **6** presented in Fig. (**2**) show a marginal recuperation of the tumor cells after a prolonged exposition. Because exponential cell growth is guaranteed for at least 140 h of incubation, the rise of the growth curve can be explained by the development of drug resistance. The onset of antiproliferative effects was observed early. **6** showed its maximum activity after 48 h, therefore we evaluated the IC_{50} value of the compounds after an incubation time of 72 h.

In order to estimate a possible participation of the COX inhibition on the cytotoxic properties of licofelone and its derivatives, their influences against the isolated isoenzymes were determined (Fig. **2**). During the recent years, COXs, especially COX-2 became very interesting targets in cancer chemotherapy. In addition to the well-established pathophysiological role that COX-2 plays in inflammation, recent evidence implies that this isoform may also be involved in multiple biological events throughout the tumorigentic process. Further, COX-2 is chronically overexpressed in many premalignant, malignant, and metastatic human cancers, and levels of overexpression have been shown to significantly correlate to invasiveness, prognosis, and survival in some cancers. Pharmacological studies consistently demonstrate that COX-2 inhibitors show dose dependent inhibition tumor growth and metastasis in various relevant animal models of cancer. Importantly, several investigators have also shown that COX-2 inhibitors may act additively or synergistically with currently used cytotoxics and molecularly targeted agents [1-4, 19-22].

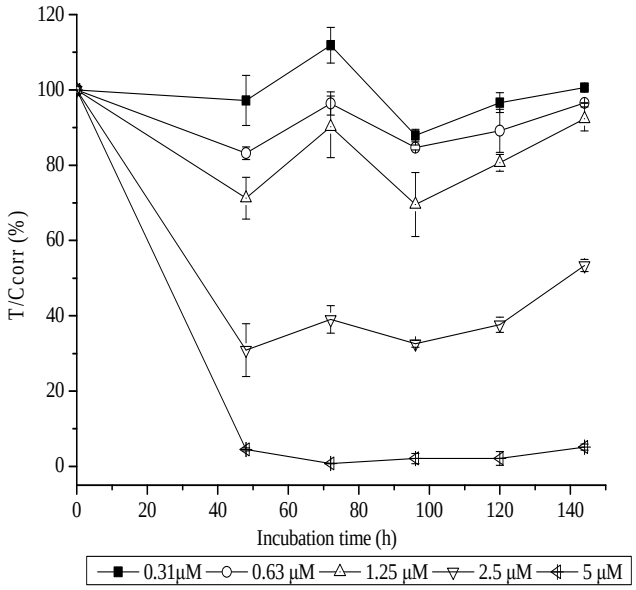


Fig. (2). Time dependent antiproliferative effects of compound **6** at MCF-7 cells.

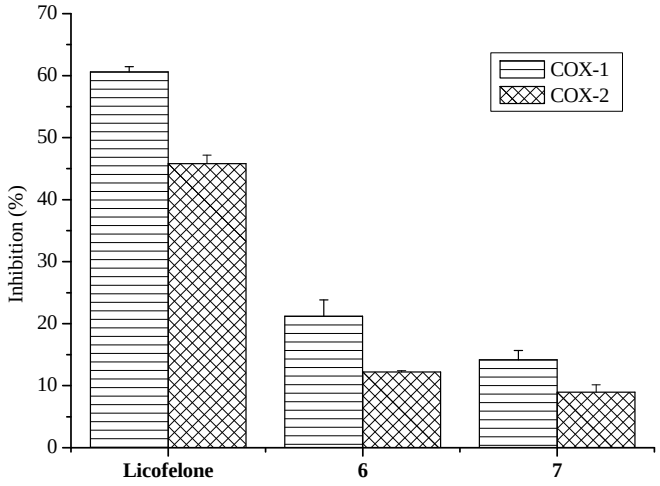


Fig. (3). Inhibition of COX-1 (ovine) and COX-2 (human recombinant) activity after treatment with compounds in the concentration of 10 µM (negative control (DMSO) was set as 0%).

A drug concentration of 10 µM was used for the experiments since licofelone inhibited the COX at this concentration by about 50% (COX-1 (60.6%) and COX-2 (45.8%)). Therefore, we used this concentration and performed the ELISA without calculation of the IC_{50} values. Under the same test conditions, compounds **6** and **7** lowered the activity of COX-1 (14% and 24%, respectively) and COX-2 (8% and 12%, respectively) (Fig. **3**). In each case, the COX-1 isoenzyme was more affected by the inhibitor than COX-2 indicating a comparable selectivity as demonstrated for licofelone. This finding is in accordance with previous investigations on the derivatization of licofelone [11, 15, 18]. Variation of the C5-carboxylic group results in an occasionally remarkable decrease of COX-activity. However, a correlation of COX inhibition and cytotoxicity against cancer cells was not visible.

Nevertheless, the anti-inflammatory effects of the licofelone derivatives were studied in *in vivo* using the xylene-induced ear swelling in mice. As shown in Table **2**, licofelone influenced the ear swelling in a dose-dependent manner, with an about 50% inhibition at a dose of 100 mg/kg as well as NSAIDs ibuprofen and celecoxib. Similar to the results of COX inhibition, **6** and **7** lowered the *in vivo* activity with anti-inflammatory effects of about 7-33%.

CONCLUSION

Two licofelone C5-substituted derivatives were developed and investigated for their biological activity. Both compounds exhibited high antiproliferative potencies and reduced anti-inflammatory effects. The anti-inflammatory properties and the COX-1/2 inhibition did not correlate with the growth inhibition of cancer cells. However, these results clearly documented that modification at position 5 of the 2,3-dihydro-1*H*-pyrrolizine core allows an optimization of licofelone for an effective tumor therapy. Additional investigations to get an insight into the mechanism of action (such as LOX inhibition [22] or other molecular pathways [15]) as well as into structure–activity relationships are in progress and will be part of a forthcoming paper.

Table 2. Effect of the Compounds on Xylene-Induced Ear Swelling in Mice (n = 6, $\bar{X}$ ± S)

	Dose [mg/kg]	Swollen extent; weight [mg]	Inhibition (%)	Swollen extent; thickness [mm]	Inhibition (%)
Control		5.7 ± 1.4		0.138 ± 0.017	
Licofelone	25	3.8 ± 2.2*	33.3%	0.070 ± 0.032**	49.2%
	100	3.2 ± 0.8**	43.9%	0.066 ± 0.013**	52.2%
	200	1.3 ± 1.9**	77.2%	0.030 ± 0.029**	74.6%
6	100	5.3 ± 0.4	7.0%	0.092 ± 0.019**	33.3%
7	100	4.2 ± 0.5*	26.3%	0.096 ± 0.034*	30.4%
Ibuprofen	100	2.78 ± 0.37**	51.2%	0.063 ± 0.006**	54.3%
Celecoxib	100	3.06 ± 0.49**	46.3%	0.072 ± 0.013*	47.9%

*P < 0.05, **P < 0.01. Data were subjected to one-way ANOVA.

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