Full Paper

Licofelone–Nitric Oxide Donors as Anticancer Agents

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Five licofelone ([2,2-dimethyl-6-(4-chlorophenyl)-7-phenyl-2,3-dihydro-1*H*-pyrrolizin-5-yl]acetic acid) nitric oxide donor conjugates were developed by a parallel synthesis approach. The biological screening revealed that compounds with a propyl (**6b**), butyl (**6c**), or octyl (**6d**) chain between licofelone and the nitric oxide donor exhibited high antiproliferative potency at MCF-7 and MDA-MB−231 breast cancer as well as at HT-29 colon cancer cells. Moreover, **6b–d** possessed at least 2-fold higher cytotoxicity at MDA-MB-231 cells than the parent compound licofelone although they showed less inhibitory activity at COX-1 and COX-2. A correlation between COX inhibition and growth inhibitory properties is not visible. However, the high levels of nitric oxide production of the compounds may result in their high cytotoxic activity.

Keywords: COX inhibition / Cytotoxicity / Licofelone / Nitric oxide

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Introduction

During the past years, nitric oxide (NO) releasing drugs have come into the focus in the treatment of cancer. Besides their positive effects against inflammation and vascular diseases, NO plays various physiological roles in tumor tissues [1–5]. Studies demonstrated for NO potent growth-regulatory potency in different cell lines. High concentration of NO could induce the apoptosis of tumor cells, prevent tumors from metastasizing and inhibit the epidermal growth factor-induced DNA synthesis to kill tumor cells [1, 3–5].

The so-called ''NO-releasing drugs'' have their pioneers in nitric oxide-donating non-steroidal anti-inflammatory drugs (NO-NSAIDs) [6]. NSAIDs have been used for the suppression of pain and inflammation in the clinic for many years. Their main mode of action is the inhibition of the cyclooxygenase enzymes (i.e., COX-1 and COX-2) leading to a reduction in the synthesis of prostaglandins, the messenger molecules in the process of inflammation. In recent years, several epidemiological, clinical and experimental studies have shown that NSAIDs also exhibit anticancer properties [7]. Moreover, it was demonstrated that long term use of NSAIDs significantly reduces the recurrence risk in various malignancies such as breast and colon cancer [8–10].

A large number of well-known NSAIDs have been conjugated with a NO-donor group to confer an improved pharmacological profile [6, 11, 12]. Among these compounds, NO-donating aspirin (NO-ASA; Fig. 1) and NO-donating indomethacin (NO-indomethacin; Fig. 1) with the NO-releasing $-ONO_2$ group are representative examples for a successful drug optimization for the treatment of cancer. In *in-vitro* studies, NO-ASA inhibited the growth of colon, prostate, tongue, pancreatic, lung, and breast cancer cells 10–6000 fold relative to its parent compound ASA, while NO-indo-

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Abbreviations: cyclooxygenase (COX); enzyme-linked immunosorbent assay (ELISA); 5-fluorouracil (5-FU); 5-lipoxygenase (5-LOX); microsomal prostaglandin E2 synthase-1 (mPGES-1); nitroglycerinum (NG); nitric oxide (NO); nitric oxide-donating non-steroidal anti-inflammatory drugs (NO-NSAIDs); NO-donating aspirin (NO-ASA); NO-donating indomethacin (NO-indomethacin); prostaglandin E2 (PGE2); phosphate buffer solution (PBS); room temperature (rt).

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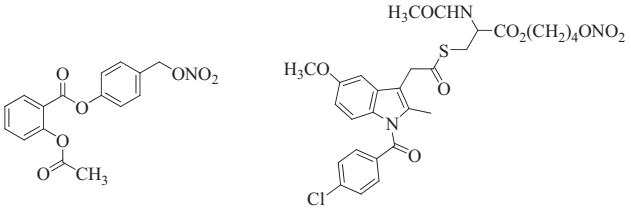


Figure 1. The structures of NO-ASA and NO-indomethacin with the same NO-releasing moiety ($-ONO_2$).

methacin inhibited the growth of pancreatic and colon cancer cell lines 4–18 fold relative to indomethacin [6, 11, 12].

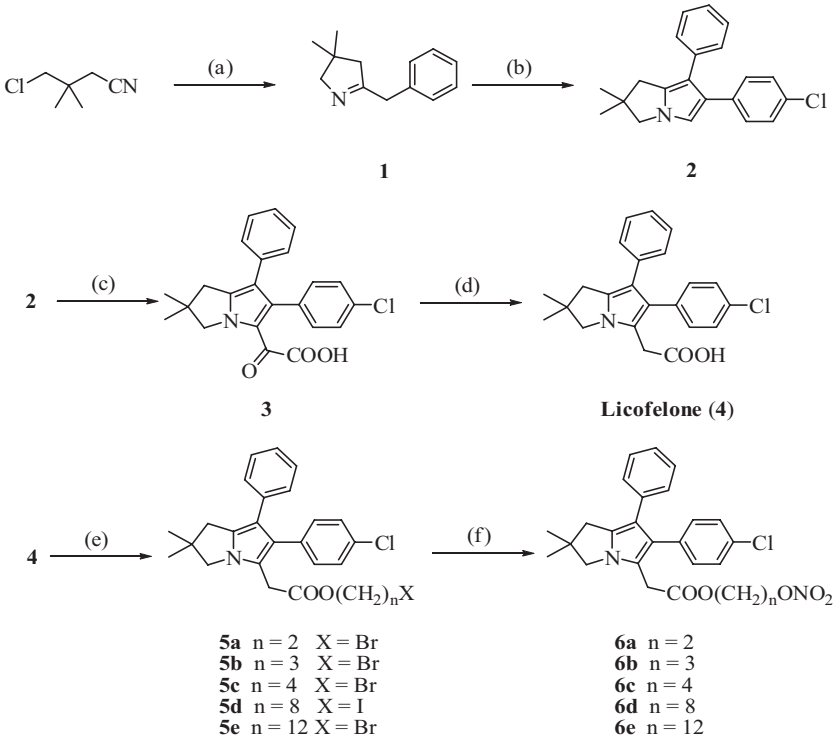
Further very interesting lead structures can be selected from the class of 2,3-dihydro-1*H*-pyrrolizines which are widely investigated inhibitors of the arachidonic acid pathways [13–24]. A compound of this series, [2,2-dimethyl-6-(4-chlorophenyl)-7-phenyl-2,3-dihydro-1*H*-pyrrolizin-5-yl]acetic acid) (licofelone, Scheme 1) showed in clinical trials anti-inflammatory and analgesic activity in osteoarthritis comparable to conventional NSAIDs with a better gastrointestinal profile. It is a potent, competitive inhibitor of 5-lipoxygenase (5-LOX) and cyclooxygenase isoenzymes COX-1 and COX-2 [13, 14]. Recently 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 [15, 16]. Furthermore, it enhanced apoptosis in prostate cancer cells as well as in HCA-7 colon cancer cells through the mitochondrial pathway. All these results show that licofelone has also a good perspective as antitumor drug [17, 18].

The above mentioned results induced us to equip licofelone with a NO-releasing group to optimize the tumor cell growth inhibiting properties. We modified licofelone at the carboxylic acid group because we [19] and others already showed that derivatization at C5 improved the activity profile and reduced undesirable side effects [16, 20, 21]. In this article, we describe the synthesis and the *in-vitro* cytotoxicity. Additionally we evaluated the influence of this structural modification on the COX inhibitory properties.

Result and discussion

Chemistry

6,7-Diaryl-2,3-dihydro-1*H*-pyrrolizine (**2**) and licofelone were synthesized according to previously published methods (Scheme 1) [13, 16, 19, 22]. 4-Chloro-3,3-dimethyl-butyronitrile was condensed with the commercially available benzyl-Grignard, followed by ring closure to the rather unstable 5-benzyl-3,3-dimethyl-3,4-dihydro-2*H*-pyrrole (**1**). 6,7-Diaryl-2,3-dihydro-1*H*-pyrrolizine (**2**) was obtained in moderate yields by



5a n = 2 X = Br
5b n = 3 X = Br
5c n = 4 X = Br
5d n = 8 X = I
5e n = 12 X = Br

6a n = 2
6b n = 3
6c n = 4
6d n = 8
6e n = 12

Scheme 1. Synthetic routes of compounds **6a–e** [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 min; (d) $N_2H_4 \cdot H_2O$, KOH, ethylene diglycol, 85°C, 5 h, then to 140–145°C, 2 h, 55%.; (e) dihalogenalkanes, K_2CO_3, CH_3COCH_3, 56°C, 43–70%; (f) CH_3CN, $AgNO_3$, refluxed, 61–82%.

cyclization of 2-bromo-1-(4-chlorophenyl)ethanone and **1** in ethanol/aqueous $NaHCO_3$ solution at room temperature (rt). Friedel-Craft acylation of **2** with oxalyl chloride and subsequent Wolff–Kishner reduction with hydrazine hydrate yielded licofelone (Scheme 1).

Licofelone was further treated with dihaloalkanes and K_2CO_3 in acetone at 56°C to generate the haloalkyl esters **5a–e** in 43–70% yields. Finally, reaction of **5a–e** with $AgNO_3$ in CH_3CN afforded the corresponding nitrates **6a–e** in satisfying yields (61–82%) after purification by column chromatography. All compounds were characterized by ^{1}H-NMR, MS, and elemental analysis.

Biological activity

The NO-licofelone derivatives, licofelone as well as the established antitumor drug 5-fluorouracil (5-FU) were screened for growth inhibitory effects 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 [25]. Therefore, their growth is sensitive to NSAID treatment [9, 10].

The experiments were performed according to established procedures [26]. DMSO was used to prepare a stock solution (10^{-2} M) of each compound. The final drug concentrations (between 2.5 to 50 μM) were achieved by dilution with cell culture medium. Because of the cytotoxicity of DMSO at higher concentrations, final DMSO concentrations were limited to 0.1% in all samples. IC_{50} values were calculated (OriginPro 8) and presented in Table 1. Concentration-dependent antiproliferative effects of **6a–e** in three cell lines are shown in Fig. 2.

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 ($IC_{50} = 22.0$ μM) it was only marginally active indicating at least 4-fold selectivity for MCF-7 cells. All NO-licofelone compounds showed promising antiproliferative activities at MCF-7 cells with IC_{50} between 4.4 μM and 17.9 μM. The 3-nitrooxypropyl derivative **6b** was even as active as licofelone and 5-FU.

At the MDA-MB-231 line, the compounds **6b–d** were less active ($IC_{50} = 10.7$–15.2 μM) but nevertheless distinctly more active than licofelone ($IC_{50} = 36.7$ μM) and as active as 5-FU ($IC_{50} = 9.6$ μM). With the 2-nitrooxyethyl (**6a**) and 2-nitrooxyododecyl (**6e**) derivatives, it was impossible to reach a 50% inhibition (Fig. 2).

HT-29 cells were less sensitive to the licofelone and its NO-derivatives. Licofelone, **6b**, and **6c** showed IC_{50} values of 22.0, 19.1, and 19.7 μM, respectively. Besides compounds **6a** ($IC_{50} > 50$ μM) and **6e** ($IC_{50} > 50$ μM), **6d** ($IC_{50} = 33.6$ μM) was nearly inactive. It should be mentioned that none of the new compounds reached the growth inhibitory effects of 5-FU.

These results clearly demonstrate a dependence of the growth inhibition on the length of the linker between the licofelone moiety and NO-donor group. The maximal effects are achieved with C3 to C8 chains. While the results of licofelone and the NO-donor derivatives at the MCF-7 and HT-29 cell lines are comparable, increased activity was determined at MDA-MB-231 cells (**6b–d** possessed at least 2-fold higher cytotoxicity compared to licofelone).

The drug design presented in this paper allows a mode of action which might include the inhibition of COX enzymes and the release of NO. Both effects can be involved in the reduction of tumor cell growth as already mentioned above. Therefore, we firstly studied the COX-interaction of the most active compounds **6b–d** *in vitro* in an enzyme-linked immunosorbent assay (ELISA) using the isolated iso-enzymes (Fig. 3). A drug concentration of 10 μM was used for the experiments, because licofelone inhibited the COX at this concentration by about 50% (COX-1 (60.6%) and COX-2 (45.8%)).

The NO-donor derivatives did not reduce the COX activity to the same extent as licofelone. At COX-1 an inhibition of only 1.6–8% were measured for **6b–d**, while at COX-2 **6b** and **6c** showed inhibitory effects of 14.8% and 25%, respectively. Compound **6d** was completely inactive at the tested concentration. In contrast to licofelone, **6c** especially demonstrated (about 4-fold) COX-2 selectivity.

Nevertheless, the NO-donor derivatives were less active than licofelone. This finding is in accordance with previous investigations on the derivatization of licofelone [16, 19]. Variation of the C5-carboxylic group results in an occasionally remarkably decrease of COX activity.

Furthermore, these results indicated sufficient stability of **6b–d** under the test conditions. Enzymatic ester cleavage would lead to a release of licofelone resulting in higher

Table 1. Growth inhibitory effects against MDA-MB–231, MCF-7, and HT-29 cells.

	Cytotoxicity IC_{50} [μM] [a]		
Compound	**MDA-MB-231**	**MCF-7**	**HT-29**
6a	>50 [b]	17.9 ± 2.0	>50 [b]
6b	10.7 ± 0.1	4.6 ± 0.1	19.1 ± 0.7
6c	12.8 ± 1.0	8.0 ± 0.2	19.7 ± 0.3
6d	15.2 ± 3.7	9.9 ± 0.4	33.6 ± 1.9
6e	>50 [b]	16.2 ± 1.5	>50 [b]
Licofelone (**4**)	36.7 ± 3.2	5.5 ± 0.6	22.0 ± 0.5
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. [b] IC_{50} value above 50 μM.

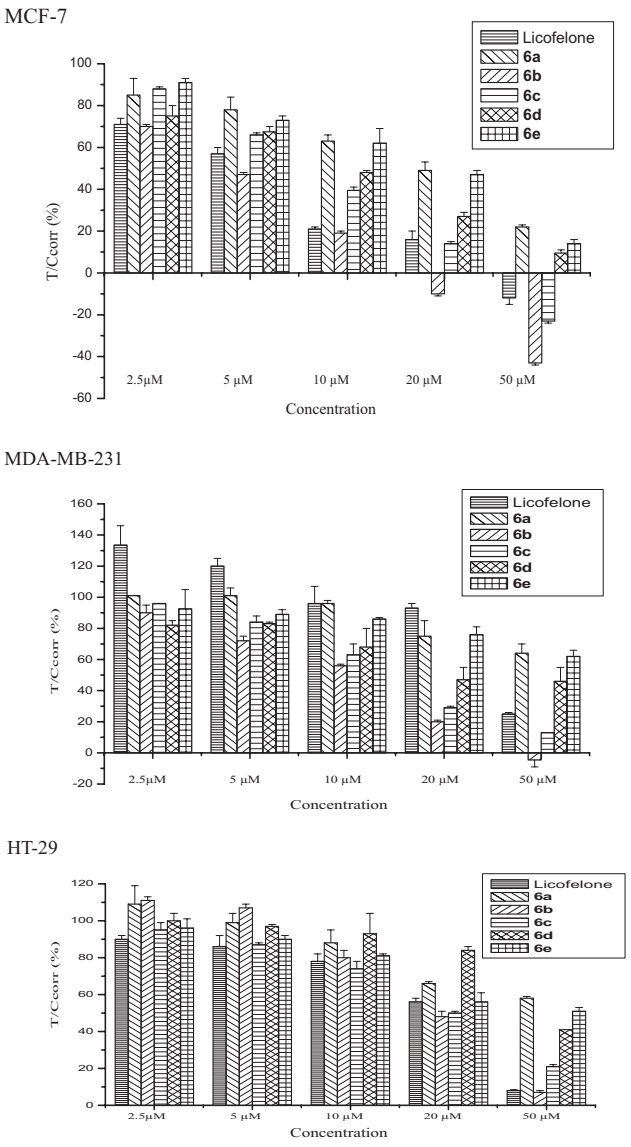


Figure 2. Concentration dependent antiproliferative effects of NO-licofelone compounds and licofelone at MCF-7, MDA-MB-231, and HT-29 cells. In some cases the error bars are hidden behind the symbols.

COX-inhibition. The chemical stability was already proven under cell culture condition (aqueous solution, pH 7.4, 37°C) and indicated no break down (data not shown).

In the next step the NO release was quantified using the Griess method in a given time scale to find a possible correlation with cell growth inhibitory effects. This assay is an indirect NO measurement by quantifying its stable derivatives NO_2^- or NO_3^- using an UV/VIS spectrophotometer. It has been reported that a reduced thiol group e.g. of L-cysteine, L-cysteamine, or glutathione has to be present to achieve NO

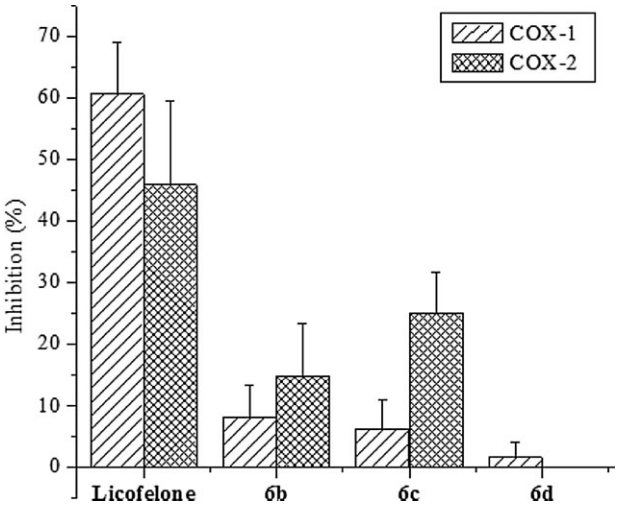


Figure 3. Inhibition of COX-1 (ovine) and COX-2 (human recombinant) activity after treatment with the compounds in the concentration of 10 μM (negative control (DMSO) was set as 0%).

release from certain donor agents [27, 28]. Therefore, **6b–d** as well as the reference drug nitroglycerinum (NG) were incubated at a concentration of 1 mM with L-cysteine (18 mM) at 37°C and the NO release was measured over 11 h (Fig. 4).

The significance of L-cysteine upon NO-release was demonstrated on the example of **6b** (see Fig. 4). In phosphate buffer solution (PBS) at pH 7.4 the degradation of **6b** was low (<12%). While in the presence of L-cysteine the breakdown increased to 72%.

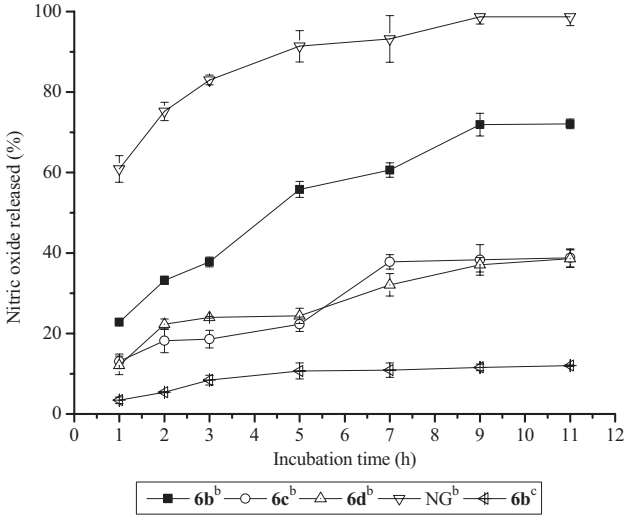


Figure 4. Time dependent NO release from compounds **6b–d** and NG. [a)]

[a)] In some cases the error bars are hidden behind the symbols. [b)] Incubated in the presence of 18 mM L-cysteine in PBS (pH 7.4) at 37°C. [c)] Incubated in PBS (pH 7.4) at 37°C.

As depicted in Fig. 4, all compounds released NO very fast in the beginning and reached their maximum after incubation for about 9 h. The efficacy of **6b** was about 2-fold higher compared to **6c** (38%) or **6d** (37%). These results correlate with the growth inhibitory effects, which demonstrated for **6b** the best results (see Table 2). Therefore, we propose the participation of NO release on the mode of action because high dose of NO induced potent cytotoxicity against tumor cells [1–6, 11, 12].

It might be possible, that reduced cytotoxicity as a consequence of the reduced COX-inhibitory effects can be overcome by NO toxicity. Nevertheless, an enzymatic ester cleavage after accumulation into the tumor cells cannot be excluded. In this case licofelone would participate on the biological properties, too. Thus, we will focus in a forthcoming study on the biological effects of **6b–d** to understand the pharmacokinetic and pharmacodynamic of these NO–licofelone compounds.

Conclusion

A series of novel NO-licofelone derivatives were synthesized and their primary biological activities were evaluated. Among these novel compounds, **6b-d** exhibited high antiproliferative potency in three cell lines. Especially at the MDA-MB-231 cells, they were at least 2-fold more cytotoxic than their parent compound licofelone. The high NO release indicated a possible participation of NO on the mode of action. It might be possible that a decreased cytotoxicity resulting from reduced COX inhibition can be overcome. The presented results, in accordance with previous reports, demonstrated that NO donating compounds often have enhanced pharmacological activity compared to their parent compounds [6, 11, 12]. Moreover, our data suggest that this structural modification of licofelone can enhance its cancer growth inhibitory properties. Additional investigations to get deeper insight into the mode of action as well as into a structure activity relationship are in progress.

Experimental

Chemistry

General: All reagents were purchased from Shanghai Chemical Reagent Company. 6-(4-Chlorophenyl)-2,2-dimethyl-7-phenyl-2,3-dihydro-1*H*-pyrrolizine (**2**) and licofelone (**4**) were synthesized according to previous methods [13, 16, 19, 22]. 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). ^{1}H-NMR spectra: Varian NOVA-400 spectrometer at 400 MHz (internal standard, TMS). Mass spectrometry (MS): Varian CH-7A (70 eV) spectrometer for electron impact (EI) MS; in *m/z*. Elemental analyses: CHN-O-Rapid instrument.

*Typical procedure of synthesis of **5a–e***

Licofelone (760 mg, 2.00 mmol), dihalogenalkanes (3.00 mmol) and K_2CO_3 (1382 mg 10 mmol) in 20 mL acetone were stirred at refluxed temperature for 2–8 h and cooled to r.t. Then the mixture was filtered and concentrated. The product was purified with column chromatography (silica gel, petroleum ether/ethyl acetate 20:1) to give pale yellow oily **5a–e**.

*2-Bromoethyl-2-(6-(4-chlorophenyl)-2,2-dimethyl-7-phenyl-2,3-dihydro-1H-pyrrolizin-5-yl)acetate **5a***

Yield 60.3%; MS (*m/z*): 485 $[M]^+$; ^{1}H-NMR ($CDCl_3$): δ 1.30 (s, 6H, 2 $-CH_3$), 2.85 (s, 2H, $-CH_2-$), 3.53 (t, 2H, J = 6.0 Hz, $-CH_2Br$), 3.57 (s, 2H, $-CH_2COO$), 3.77 (s, 2H, $-CH_2N-$), 4.45 (t, 2H, J = 6.0 Hz, $-CH_2O-$), 7.03–7.24 (m, 9H, Ar-H).

*3-Bromopropyl-2-(6-(4-chlorophenyl)-2,2-dimethyl-7-phenyl-2,3-dihydro-1H-pyrrolizin-5-yl)acetate **5b***

Yield 68.7%; MS (*m/z*): 499 $[M]^+$; ^{1}H-NMR ($CDCl_3$): δ 1.30 (s, 6H, 2 $-CH_3$), 2.13–2.19 (m, 2H, J = 6.0 Hz, $-CH_2-$), 2.85 (s, 2H, $-CH_2-$), 3.40 (t, 2H, J = 6.4 Hz, $-CH_2Br$), 3.54 (s, 2H, $-CH_2COO-$), 3.74 (s, 2H, $-CH_2N-$), 4.26 (t, 2H, J = 6.4 Hz, $-CH_2O-$), 7.03–7.24 (m, 9H, Ar-H).

*4-Bromobutyl-2-(6-(4-chlorophenyl)-2,2-dimethyl-7-phenyl-2,3-dihydro-1H-pyrrolizin-5-yl)acetate **5c***

Yield 56.9%; MS (*m/z*): 513 $[M]^+$; ^{1}H-NMR ($CDCl_3$): δ 1.30 (s, 6H, 2 $-CH_3$), 1.76–1.97 (m, 4H, 2 $-CH_2-$), 2.85 (s, 2H, $-CH_2-$), 3.38 (t, 2H, J = 6.0 Hz, $-CH_2Br$), 3.52 (s, 2H, $-CH_2COO-$), 3.74 (s, 2H, $-CH_2N-$), 4.14 (t, 2H, J = 6.0 Hz, $-CH_2O-$), 7.02–7.26 (m, 9H, Ar-H).

*8-Iodooctyl-2-(6-(4-chlorophenyl)-2,2-dimethyl-7-phenyl-2,3-dihydro-1H-pyrrolizin-5-yl)acetate **5d***

Yield 70.1%; MS (*m/z*): 617 $[M]^+$; ^{1}H-NMR ($CDCl_3$): δ 1.29 (s, 6H, 2 $-CH_3$), 1.25–1.39 (m, 8H, 4 $-CH_2-$), 1.61–1.73 (m, 4H, 2 $-CH_2-$), 2.84 (s, 2H, $-CH_2-$), 3.35 (t, 2H, J = 6.8 Hz, $-CH_2I$), 3.51 (s, 2H, $-CH_2COO-$), 3.75 (s, 2H, $-CH_2N-$), 4.11 (t, 2H, J = 6.4 Hz, $-CH_2O-$), 7.03–7.24 (m, 9H, Ar-H).

*12-Bromododecyl-2-(6-(4-chlorophenyl)-2,2-dimethyl-7-phenyl-2,3-dihydro-1H-pyrrolizin-5-yl)acetate **5e***

Yield 43.1%; MS (*m/z*): 625 $[M]^+$; ^{1}H-NMR ($CDCl_3$): δ 1.29 (s, 6H, 2 $-CH_3$), 1.28–1.45 (m, 16H, 8-CH_2-), 1.60–1.70 (m, 4H, 2 $-CH_2-$), 2.84 (s, 2H, $-CH_2-$), 3.18 (t, 2H, J = 7.2 Hz, $-CH_2Br$), 3.51 (s, 2H, $-CH_2COO-$), 3.75 (s, 2H, $-CH_2N-$), 4.11 (t, 2H, J = 6.4 Hz, $-CH_2O-$), 7.03–7.26 (m, 9H, Ar-H).

*Typical procedure of synthesis of NO-licofelone compounds **6a–e***

A mixture of **5a–e** (1 mmol), silver nitrate (340 mg, 2 mmol), and acetonitrile (10 ml) was stirred at refluxed temperature for 2–8 h. The precipitate was filtered off, and the solvent was carefully evaporated. The residue was taken up in ethylacetate, washed with H_2O and brine, dried (Na_2SO_4), and evaporated. The crude residue was purified by column chromatography (silica gel, petroleum ether/ethyl acetate 20:1) to afford colorless oily **6a–e**.

2-(Nitrooxy)ethyl- 2-(6-(4-chlorophenyl)-2,2-dimethyl-7-phenyl-2,3-dihydro-1H-pyrrolizin-5-yl)acetate ***6a***

Yield 61.3%; MS (*m/z*): 468 $[M]^+$; ^{1}H-NMR ($CDCl_3$): δ 1.30 (s, 6H, 2 $-CH_3$), 2.85 (s, 2H, $-CH_2-$), 3.58 (s, 2H, $-CH_2COO-$), 3.73 (s, 2H, $-CH_2N-$), 4.41 (t, 2H, $J = 4.4$ Hz, $-CH_2O-$), 4.68 (t, 2H, $J = 6.0$ Hz, $-CH_2O-$), 7.04–7.24 (m, 9H, Ar-H); Anal. calcd. for $C_{25}H_{25}ClN_2O_5 \cdot H_2O$: C, 61.66; H, 5.59; N, 5.75%; found: C, 61.34; H, 5.78; N, 6.05%.

3-(Nitrooxy)propyl-2-(6-(4-chlorophenyl)-2,2-dimethyl-7-phenyl-2,3-dihydro-1H-pyrrolizin-5-yl)acetate ***6b***

Yield 73.9%; MS (*m/z*): 482 $[M]^+$; ^{1}H-NMR ($CDCl_3$): δ 1.29 (s, 6H, 2 $-CH_3$), 2.03–2.09 (m, 2H, $-CH_2-$), 2.85 (s, 2H, $-CH_2-$), 3.55 (s, 2H, $-CH_2COO-$), 3.73 (s, 2H, $-CH_2N-$), 4.21 (t, 2H, $J = 6.0$ Hz, $-CH_2O-$), 4.48 (t, 2H, $J = 6.0$ Hz, $-CH_2O-$), 7.03–7.24 (m, 9H, Ar-H); Anal. calcd. for $C_{26}H_{27}ClN_2O_5$: C, 64.66; H, 5.63; N, 5.80%; found: C, 64.89; H, 5.25; N, 5.79%.

4-(Nitrooxy)butyl-2-(6-(4-chlorophenyl)-2,2-dimethyl-7-phenyl-2,3-dihydro-1H-pyrrolizin-5-yl)acetate ***6c***

Yield 82.1%; MS (*m/z*): 496 $[M]^+$; ^{1}H-NMR ($CDCl_3$): δ 1.29 (s, 6H, 2 $-CH_3$), 1.74–1.77 (m, 4H, 2 $-CH_2-$), 2.85 (s, 2H, $-CH_2-$), 3.54 (s, 2H, $-CH_2COO-$), 3.73 (s, 2H, $-CH_2N-$), 4.14 (t, 2H, $J = 6.0$ Hz, $-CH_2O-$), 4.45 (t, 2H, $J = 4.2$ Hz, $-CH_2O-$), 7.03–7.24 (m, 9H, Ar-H); Anal. calcd. for $C_{27}H_{29}ClN_2O_5$: C, 65.25; H, 5.88; N, 5.64%; found: C, 65.23; H, 5.79; N, 5.94%.

8-(Nitrooxy)octyl-2-(6-(4-chlorophenyl)-2,2-dimethyl-7-phenyl-2,3-dihydro-1H-pyrrolizin-5-yl)acetate ***6d***

Yield 71.8%; MS (*m/z*): 552 $[M]^+$; ^{1}H-NMR ($CDCl_3$): δ 1.29 (s, 6H, 2 $-CH_3$), 1.30–1.45 (m, 8H, 4 $-CH_2-$), 1.61–1.71 (m, 4H, 2 $-CH_2-$), 2.84 (s, 2H, $-CH_2-$), 3.51 (s, 2H, $-CH_2COO-$), 3.75 (s, 2H, $-CH_2N-$), 4.11 (t, 2H, $J = 6.4$ Hz, $-CH_2O-$), 4.41 (t, 2H, $J = 6.4$ Hz, $-CH_2O-$), 7.03–7.26 (m, 9H, Ar-H); Anal. calcd. for $C_{31}H_{37}ClN_2O_5$: C, 67.32; H, 6.74; N, 5.06%; found: C, 67.01; H, 7.02; N, 5.03%.

12-(Nitrooxy)dodecyl-2-(6-(4-chlorophenyl)-2,2-dimethyl-7-phenyl-2,3-dihydro-1H-pyrrolizin-5-yl)acetate ***6e***

Yield 69.9%; MS (*m/z*): 608 $[M]^+$; ^{1}H-NMR ($CDCl_3$): δ 1.26–1.40 (m, 16H, 8 $-CH_2-$), 1.29 (s, 6H, 2 $-CH_3$), 1.61–1.74 (m, 4H, 2 $-CH_2-$), 2.84 (s, 2H, $-CH_2-$), 3.51 (s, 2H, $-CH_2COO-$), 3.75 (s, 2H, $-CH_2N-$), 4.11 (t, 2H, $J = 6.4$ Hz, $-CH_2O-$), 4.43 (t, 2H, $J = 6.4$ Hz, -CH_2O-), 7.03–7.26 (m, 9H, Ar-H); Anal. calcd. for $C_{35}H_{45}ClN_2O_5$: C, 69.00; H, 7.45; N, 4.60%; found: C, 68.74; H, 7.75; N, 4.80%.

Biological Activity

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 [26]. 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) were plated into each well and were incubated for three days under culture conditions. After the addition of various concentrations of the test compounds, cells were incubated for up to 144 h. 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 assay, followed by extracting 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 equation:

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

where 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 two or 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.

In-vitro NO Releasing Assays

In-vitro NO release was assayed according to established procedures with some modifications [29].

Incubation with 18 mM L-Cysteine in PBS (pH 7.4)

A solution of the test compound (1 mL of 2 mM solution in 0.2 M PBS, pH 7.4) was mixed thoroughly with a freshly prepared solution of L-cysteine (1 mL of a 36 mM solution in 0.1 M PBS, pH 7.4), and the mixture was incubated at 37°C for up to appropriate incubation time in the absence of air. After exposure to air for 10 min at 25°C, an aliquot of the Griess reagent (1 mL) [freshly prepared by mixing equal volumes of 1.0% sulfanilamide and 0.1% *N*-naphthylethylenediamine dihydrochloride in water] was added to an equal volume (1 mL) of each test compound's incubation solution with mixing. After 10 min had elapsed, absorbance was measured at 540 nm using a Shimadzu UV 2100 UV-VIS scanning spectrophotometer. Solutions of 0–60 μM sodium nitrite were used to prepare a nitrite absorbance versus concentration curve under the same experimental conditions. The percent NO release (quantified as nitrite ion) was calculated (± SEM, $n = 3$) from the standard nitrite versus concentration curve.

Incubation with PBS (pH 7.4)

This assay was performed as described above except that a solution of the test compound (2 mL of a 1 mM solution in 0.1 M PBS pH 7.4) was used and no L-cysteine was added.

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References

[1] P. G. Wang, M. Xian, X. Tang, X. Wu, Z. Wen, T. Cai, A. J. Janczuk, *Chem. Rev.* **2002**, *102*, 1091–1134.

[2] J. O. Lundberg, E. Weitzberg, M. T. Gladwin, *Nat. Rev. Drug Discovery* **2008**, *7*, 156–167.

[3] S. Ekmekcioglu, C. H. Tang, E. A. Grimm, *Curr. Cancer Drug Targets* **2005**, *5*, 103–115.

[4] D. Hirst, T. Robson, *J. Pharm. Pharmacol.* **2007**, *59*, 3–13.

[5] R. Sullivan, C. H. Graham, *Curr. Pharm. Des.* **2008**, *14*, 1113–1123.

[6] B. Rigas, J. L. Williams, *Nitric Oxide* **2008**, *19*, 199–204.

[7] J. Marx, *Science* **2004**, *306*, 966–968.

[8] A. Mantovani, P. Allavena, A. Sica, F. Balkwill, *Nature* **2008**, *454*, 436–444.

[9] D. Pereg, M. Lishner, *J. Internal Med.* **2005**, *258*, 115–123.

[10] A. Agrawal, I. S. Fentiman, *Int. J. Clin. Pract.* **2008**, *62*, 444–449.

[11] R. K. Yeh, J. Chen, J. L. Williams, M. Baluch, T. R. Hundley, R. E. Rosenbaum, S. Kalala, F. Traganos, F. Benardini, P. del Soldato, K. Kashfi, B. Rigas, *Biochem. Pharmacol.* **2004**, *67*, 2197–2205.

[12] K. Kashfi, Y. Ryan, L. L. Qiao, J. L. Williams, J. Chen, P. Del Soldato, F. Traganos, B. Rigas, *J. Pharmacol. Exp. Ther.* **2002**, *303*, 1273–1282.

[13] S. Laufer, J. Augustin, G. Dannhardt, W. Kiefer, *J. Med. Chem.* **1994**, *37*, 1894–1897.

[14] H. Ulbrich, B. Fiebich, G. Dannhardt, *Eur. J. Med. Chem.* **2002**, *37*, 953–959.

[15] A. Koeberle, U. Siemoneit, U. Buhring, H. Northoff, S. Laufer, W. Albrecht, O. Werz, *J. Pharmacol. Exp. Ther.* **2008**, *326*, 975–982.

[16] A. J. Liedtke, P. R. Keck, F. Lehmann, A. Koeberle, O. Werz, S. Laufer, *J. Med. Chem.* **2009**, *52*, 4968–4972.

[17] N. K. Narayanan, D. Nargi, M. Attur, S. B. Abramson, B. A. Narayanan, *Anticancer Res.* **2007**, *27*, 2393–2402.

[18] S. Tavolari, M. Bonafe, M. Marini, C. Ferreri, G. Bartolini, E. Brighenti, S. Manara, V. Tomasi, S. Laufer, T. Guarnieri, *Carcinogenesis* **2008**, *29*, 371–380.

[19] W. Liu, J. Zhou, K. Bensdorf, H. Zhang, H. Liu, Y. Wang, H. Qian, Y. Zhang, A. Wellner, G. Rubner, W. Huang, C. Guo, R. Gust, *Eur. J. Med. Chem.* **2011**, *46 (3)*, 907–913.

[20] S. Laufer, H. G. Striegel, G. Dannhardt, Patent 94-4419247 [4419247], 22. DE. 1-6- 1994, **1995** (Merckle GmbH, Germany).

[21] A. Kirschning, M. Ries, S. Domann, W. Martin, W. Albrecht, P. Arnold, S. Laufer, *Bioorg. Med. Chem. Lett.* **1997**, *7*, 903–906.

[22] G. Dannhardt, T. Kammermaier, P. Merckle, H. G. Striegel, S. Laufer, WO Patent 2003/018583, *Chem. Abstr.* **2003**, *138*, 204938.

[23] S. Laufer, H. G. Striegel, K. Neher, P. Zechmeister, C. Donat, K. Stolingwa, S. Baur, S. Tries, T. Kammermeier, G. Dannhardt, W. Kiefer, *Arch. Pharm.* **1997**, *330*, 307–312.

[24] G. Dannhardt, W. Kiefer, U. Nowe, *Arch. Pharm.* **1995**, *328*, 681–686.

[25] X. H. Liu, D. P. Rose, *Cancer Res.* **1996**, *56*, 5125–5127.

[26] A. Hille, R. Gust, *Arch. Pharm.* **2009**, *342*, 625–631.

[27] M. Civelli, P. Caruso, M. Giossi, M. Bergamaschi, R. Razzetti, S. Bongrani, A. Gasco, *Br. J. Pharmacol.* **1996**, *118*, 923–928.

[28] W. Liu, C. Liu, C. Gong, W. Lin, C. Guo, *Bioorg. Med. Chem. Lett.* **2009**, *19*, 1647–1649.

[29] E. Naimi, A. Zhou, P. Khalili, L. I. Wiebe, J. Balzarini, E. De Clercq, E. E. Knaus, *J. Med. Chem.* **2003**, *46*, 995–1004.

