Licofelone, a dual COX/5-LOX inhibitor, induces apoptosis in HCA-7 colon cancer cells through the mitochondrial pathway independently from its ability to affect the arachidonic acid cascade

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Nowadays, no data are available concerning the potential use of dual cyclooxygenase (COX)/5-lipoxygenase (LOX) inhibitors as anticancer agents in colon cancer treatment. Here, we report, for the first time, that the dual COX/5-LOX inhibitor licofelone triggers apoptosis in a dose- and time-dependent manner in HCA-7 colon cancer cells. Induction of apoptosis was related to the recruitment of the intrinsic mitochondrial apoptotic pathway, as shown by loss in mitochondrial membrane potential, cytochrome c release, caspase-9 and 3 activation and poly-(ADP-ribose) polymerase-1 cleavage. Moreover, licofelone induced the cleavage of the full-length p21^WAF1 into p16^Bax, a more potent inducer of the apoptotic process than the uncleaved form. Pre-treatment of HCA-7 cells with the pan-caspase inhibitor z-VAD-fmk significantly blocked licofelone-induced apoptosis, confirming that this process occurred primarily in a caspase-dependent pathway. We also present evidences that licofelone was able to affect the arachidonic acid (AA) cascade, as it blocked the activity of 5-LOX and COX enzymes, and it induced, through the phosphorylation of cytoplasmic phospholipase A2 (cPLA2), the release of unesterified AA from HCA-7 membrane phospholipids. However, apoptosis induction was not related to the ability of licofelone to affect the AA cascade, since neither exogenous prostaglandin E2 and leukotriene B4 addition, nor pharmacological inhibition of cPLA2, was able to rescue HCA-7 cells from apoptosis. Even if further studies are needed to clarify the mechanism of licofelone-induced apoptosis, this study suggests that this drug, as well as similar dual COX/5-LOX inhibitors, may represent a novel and promising approach in colon cancer treatment.

Introduction

Colon cancer is the second leading cause of cancer-related mortality in Western population and its pathogenesis, together with its chemoprevention, are currently area of intense investigation. Recent studies regarding the relationship between arachidonic acid (AA) cascade and carcinogenesis are revealing novel molecular targets for cancer treatment. AA is an essential polyunsaturated fatty acid, typically esterified at the sn-2 position of cellular phospholipids and hydrolyzed by phospholipase A2 enzymes (1,2). Once released, unesterified AA is mainly metabolized by two important classes of enzymes: cyclooxygenases (COXs) and lipoxygenases (LOXs). The involvement of COX-2 and its metabolite prostaglandin E2 (PGE2) in colon carcinogenesis is well documented. A large body of studies indicate that PGE2 can increase the proliferation, the motility and the metastatic potential of tumour cells, can promote tumour angiogenesis, induce local immunosuppression and inhibit apoptosis (3). On the other hand, COX-2 can contribute to colon carcinogenesis through mechanisms that are independent from PGE2 action. COX-2 peroxidase activity can in fact transform many procarcinogens into ultimate carcinogens that, in turn, are able to activate many genes involved in cell proliferation (4). Moreover, through the reduction of the levels of unesterified AA, which has a proapoptotic action (5), COX-2 can promote tumour cell survival.

More recently, an involvement of 5-LOX enzyme and its products, in particular leukotriene B4 (LTB4), in the development and progression of colon cancer, has emerged (6,7). Similarly to COX-2, the expression and activity of 5-LOX have been found to be up-regulated during colon carcinogenesis (8) and closely related to tumour size, depth and vessel invasion (6). Even if the exact mechanism connecting 5-LOX to cancer aetiology remains to be fully elucidated, it seems likely that COX-2 and 5-LOX may represent an integrated system that regulates the proliferative, metastatic and proangiogenic potential of cancer cells. Both enzymes, in fact, induce cell cycle progression and block apoptosis, enhance cancer cells chemoresistance and stimulate angiogenesis, with a convergent targeting on vascular endothelial growth factor expression and release (9). The frequent co-expression of these two enzymes and the striking analogy of their biological functions suggest that molecules which are able to block equally well both COX-2 and 5-LOX pathways, may represent a novel and promising alternative in colon cancer treatment.

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 (10), but no data are available concerning their potential use as anticancer agents in colon cancer treatment. On this basis, we decided to evaluate the possible anti-proliferative effect of the dual COX/5-LOX inhibitor licofelone [6-(4-chlorophenyl)-2,2-dimethyl-7-phenyl-2,3-dihydro-1H-pyrazin-5-yl] acetic acid, Figure 1A], in a human colon cancer line expressing both COX-2 and 5-LOX enzymes. Among dual COX/LOX inhibitors, licofelone is the molecule in the most advanced phase of clinical trials (phase III) as anti-inflammatory drug (11), and its safety and efficacy, in comparison with the non-steroidal anti-inflammatory drugs (NSAIDs) naproxen and rofecoxib, are reviewed (12,13). This drug is a potent, competitive and well-balanced inhibitor of the 5-LOX and COX pathways; because of its conformational similarity to AA, licofelone is able, in fact, to bind the active sites of both classes of enzymes, thus blocking their catalytic activity (14).

In this paper we show, for the first time, that licofelone is able to trigger apoptosis in HCA-7 colon cancer cells, through the recruitment of the intrinsic mitochondrial apoptotic pathway and independently from its ability to affect the AA cascade. Taken together, these data suggest that this drug, as well as similar dual COX/5-LOX inhibitors, may represent a really interesting therapeutic approach for colon cancer treatment.

Materials and methods

Reagents and antisera
Licofelone (Merckle–ratiopharm, GmbH, Ulm, Germany) and celecoxib were a generous gift of Prof. Stefan Lauffer (Department of Pharmaceutical-Medicinal...
Chemistry, Eberhard Karls Universität Tübingen, Germany); naproxen was purchased from Sigma Chemical Co. (Darmstadt, Germany). The pancaspase inhibitor z-VAD-fmk was from R&D Systems (Minneapolis, MN); methyl arachidonyl fluorophosphate (MAFP), UO126, SB203587 and SP600125 were from Biomol (Plymouth Meeting, PA), whereas bromoenol lactone and p-bromophenacyl bromide were purchased from Sigma Chemical Co. Primary antibody anti-β-actin (1:1000) and anti-caspase-9 (1:300) were from Sigma Chemical Co.; anti-COX-1 (1:250), anti-COX-2 (1:250) and anti-caspase-3 (1:1000) were from Cayman Chemical Company (Ann Arbor, MI); anti-Bcl-2 (1:500) was from Trevigen (Gaithersburg, MD); anti-Bax (Ab-1, 150–165, 1:2000) was from Calbiochem (Darmstadt, Germany); anti-5-LOX (1:250) was from BD Transduction Laboratories (Lexington, KY); anti-poly-(ADP-ribose)polymerase-1 (PARP-1) (1:500) was from Santa Cruz Biotechnology (Santa Cruz, CA); anti-cytoplasmic phospholipase A2 (cPLA2) (1:500) was from Chemicon International (Temecula, CA) and anti-phospho-cPLA2 Ser505 (1:1000) was from Cell Signaling Technology (MA, USA). Monoclonal and polyclonal horseradish peroxidase-conjugated secondary antibodies (1:1000) were from Sigma Chemical Co.

Cells and cell culture

The human colon cancer cell lines LS174T and CACO-2 were a generous gift of Prof. F.Dall’Olio (Department of Experimental Pathology, University of Bologna, Italy), whereas HCA-7 cells were obtained from European Collection of Cell Cultures. All the cell lines were cultured in Dulbecco’s modified Eagle’s Medium with 4.5 g/l glucose (Cambrex Bioscience, Milan, Italy), supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (Cambrex Bioscience), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma Chemical Co.). Cells were grown at 37°C in a humidified atmosphere of 95% air and 5% CO2 and routinely passaged using trypsin–EDTA 0.025% (Sigma Chemical Co.).

Treatment with drugs

For treatment with licofelone, celecoxib and naproxen, exponentially growing cells were trypsinized and seeded in complete medium for 24 h. At the end of incubation, fresh medium, alone or supplemented with the drugs, was added and completely replaced every 48 h. Naproxen was directly dissolved in culture medium, whereas licofelone and celecoxib were dissolved in dimethyl sulfoxide (Sigma Chemical Co.) and diluted into the medium to obtain the required final concentration before each experiment. To control for a possible effect of dimethyl sulfoxide, control samples were treated in parallel with an equivalent concentration of this solvent.

Cell viability assay

The effect of licofelone, celecoxib and naproxen on HCA-7 cell viability was determined by trypan blue exclusion assay. Briefly, HCA-7 were plated on
a six-well plate at the density of \(6 \times 10^6\) and treated with the drugs for 24 and 48 h, respectively. At the end of incubation, cells were harvested by trypsinization, stained with trypan blue solution (0.04% wt/vol) and counted in a Neubauer haemocytometer chamber (Sigma Chemical Co.). Three plates were assayed for each condition and each experiment was repeated in triplicate.

**Nuclear morphological changes**

Morphological changes in the nuclear chromatin of cells undergoing apoptosis were detected by the DNA-binding fluorochrome bis-benzimide stain (Hoechst 33342, Sigma Chemical Co.). Briefly, HCA-7 were seeded on a six-well plate at the density of \(6 \times 10^6\) and cultured in the presence or absence of licofelone for the indicated times. At the end of incubation, 1 \(\mu\)g Hoechst 33342 was added to each well for 30 min at 37°C in the dark. Cells were then washed with phosphate-buffered saline (PBS) and promptly observed under a fluorescence photomicroscope (Nikon Eclipse 90i, Nikon Instruments Italia, Firenze, Italy).

**Flow cytometric analysis**

For the analysis of DNA content, HCA-7 were seeded on a six-well plate at the density of \(6 \times 10^6\) and then incubated with licofelone for 24 and 48 h, respectively. At the end of incubation, cells were harvested by trypsinization, washed in PBS and incubated at 4°C for 16 h in the DNA-staining solution, containing 0.1% Triton X-100, 0.1% sodium citrate and 50 \(\mu\)g/ml propidium iodide. DNA content analysis was performed by FACSAsiaTM cell sorter (Becton Dickinson, Franklin Lakes, NJ).

**Mitochondrial membrane potential (\(\Delta \psi_m\)) measurement**

Tetramethyl rhodamine methyl ester (Invitrogen–Molecular Probes, Milan, Italy) is a fluorescent probe suitable for cytometric measurements of changes in \(\Delta \psi_m\) (15). Briefly, HCA-7 were seeded on a six-well plate at the density of \(6 \times 10^6\) and treated with licofelone for 12 h. Staining was performed by incubating cells in a 10 \(\mu\)M tetramethyl rhodamine methyl ester solution in culture medium at 37°C for 30 min. Cells were washed and immediately assessed by FACSAsiaTM cell sorter (Becton Dickinson). Morphological evaluation of changes in \(\Delta \psi_m\) was carried out by the cationic fluorochrome JC-1 (Invitrogen–Molecular Probes), as described previously (16). In cells with normal mitochondrial function, JC-1 forms aggregates that fluoresce red; when mitochondrial membrane potential decreases, as occurs when mitochondrial pores are formed during the apoptotic process, JC-1 becomes monomeric and fluoresces green. Briefly, HCA-7 cells were seeded onto ethanol-sterilized glass coverslips in a six-well plate at the density of \(6 \times 10^6\) and then incubated with licofelone for 12 h. At the end of incubation, cells were stained with 2.5 \(\mu\)g/ml JC-1 for 30 min at 37°C in the dark, washed and promptly viewed under a fluorescence photomicroscope (Nikon Eclipse 90i, Nikon Instruments Italia).

**Cytochrome c release from mitochondria**

The concentration of cytochrome c inside or outside mitochondria was measured by ELISA (Assay Designs, Ann Arbor, MI). Briefly, HCA-7 were seeded on a six-well plate at the density of \(7 \times 10^6\) in a 75 cm² flask and treated with licofelone for 12 h. At the end of incubation, cytoplasmatic and mitochondrial fractions were obtained according to the manufacturer’s instructions. Fractions were then run in the assay and values are expressed as nanogram per milligram of total protein from each fraction.

**Western blotting analysis**

Western blotting analysis was carried out as described previously (17). Briefly, cells were seeded in a 25 cm² flask at the density of \(1.5 \times 10^6\) and grown in complete medium for 24 h. Cells were then treated with licofelone for the indicated times and, at the end of incubation, scraped from flasks and lysed in a buffer containing 10 mM Tris, 1% sodium dodecyl sulphate, 1 mM Na-orthovanadate and a protease inhibitor cocktail (Roche, Penzberg, Germany). After addition of sodium dodecyl sulphate–polyacrylamide gel electrophoresis buffer and boiling, 50 \(\mu\)g of denatured proteins were separated in pre-cast gels 4–12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA), transferred to nitrocellulose paper and probed with anti-bodies. Detection was performed by using the enhanced chemiluminescence procedure developed by Amersham Biosciences (Little Chalfont, UK) and antibody intensities were determined using a densitometric image analysis software (Image Master, Pharmacia Biotech, Uppsala, Sweden). Afterwards, membranes were re-blotted with anti-\(\beta\)-actin antibody for normalization and equal protein loading.

**Enzyme immunohistoassays**

Determination of PGE1 and LTB4 levels in HCA-7 culture medium was evaluated by ELISA (Cayman Chemical Company). Briefly, cells were seeded in a 24-well plate at the density of \(1.2 \times 10^5\) cells per well and exposed to licofelone in serum-free medium for 24 h. The harvested medium was centrifuged at 500g for 5 min (4°C) to remove floating cells and the supernatant was collected and assayed following the customer’s instructions. Results are expressed as picogram per milliliter of medium. Determinations were carried out on three similarly treated wells and the medium from each experiment was assayed at three different dilutions.

**Release of unesterified AA from HCA-7 membrane phospholipids**

For the determination of unesterified AA release from HCA-7 membrane phospholipids, cells were seeded in a 25 cm² flask at the density of \(1.5 \times 10^6\), grown in complete medium for 24 h and then treated with licofelone for 24 h. At the end of incubation, membrane pellets were obtained by centrifugation of HCA-7 cells washed twice with PBS. Each sample contained a final concentration of \(3 \times 10^6\) cells/ml PBS and the experiment was repeated for four times. Phospholipids extraction and transesterification to fatty acid residues as the corresponding methyl esters were performed, and the analysis of the fatty acid composition was carried out according to the published procedures (18). The evaluation of AA concentration in control and treated samples was carried out by using a commercially available methyl arachidonate standard as the external reference in the gas chromatography (GC) analysis.

**Statistical evaluation**

Results are expressed as mean or percentage mean ± SD and reproducibility was confirmed at least in three separate experiments. Data were analysed by Student’s t-test in Excel program and a P value < 0.05 was considered to be statistically significant.

**Results**

**Licofelone decreases HCA-7 viability through the induction of apoptosis**

To test the putative anti-proliferative effect of the dual COX-5-LOX inhibitor licofelone, preliminary experiments were carried out in order to select a human colon cancer cell line expressing both COX and 5-LOX enzymes. HCA-7, CACO-2 and LS174T cell lines, which were available in our laboratory, were analysed for this purpose. As shown in Figure 1B, the level of the constitutively expressed COX-1 protein was similar in all the three cell lines tested and the highest amounts of COX-2 and 5-LOX enzymes were observed in HCA-7 cells, which were consequently chosen for further studies. Since this was the first study to assess the possible effect of licofelone on human colon cancer cell viability, increasing concentrations of this drug, ranging from 1 \(\mu\)M to 1 mM, were tested for 24 and 48 h, respectively. As shown in Figure 1C, we observed that licofelone, starting from the dose of 100 \(\mu\)M, significantly decreased HCA-7 cell viability in a time- and dose-dependent manner half maximal inhibitory concentration was much more potent than naproxen: IC\(_{50}\) (4.25 ± 0.07 mM) and similar to that observed for celecoxib (IC\(_{50}\) 48 h: 78 ± 3.9 \(\mu\)M). It is worth underlining that, as we found a relevant cytotoxic effect at doses of licofelone > 150 \(\mu\)M (data not shown), we decided not to go beyond this dose in our subsequent experiments.

We also observed that licofelone, after 24 h incubation, deeply affected HCA-7 morphology, as treated cells became rounded, detached from the culture flask and showed membrane blebbing (Figure 1D), suggesting that an apoptotic pathway did occur. To test this hypothesis, we performed flow cytometric analysis that revealed, after 24 and 48 h of incubation, a substantial time- and dose-dependent increase in the hypodiploid (subG1) peak, which represents the fraction of fragmented apoptotic cells (Figure 2A). An increase in typical apoptotic figures, such as fragmented or irregularly fragmented apoptotic nuclei, was observed in treated cells also upon Hoechst 33342 staining (Figure 2B). To study the possible involvement of caspase activation in licofelone-induced apoptosis, we firstly examined, by western blotting, the cleavage of caspase-3. This enzyme is, in fact, one of the most important executor of the apoptotic process and its activation, which depends on the proteolytic cleavage of procaspase-3 to a smaller and enzymatically active form, is responsible for the subsequent PARP-1 cleavage (19). As shown in Figure 2C, we found that licofelone 150 \(\mu\)M induced, starting from 16 h treatment onward, a strong and time-dependent cleavage of the 34 kDa pro-enzyme caspase-3 into its active 17 kDa form. Caspase-3 activation...
was rapidly followed by PARP-1 cleavage, seen as a typical 89 kDa band, which was almost complete after 24 h of treatment (Figure 2D). These results were in agreement with Hoechst 33342 staining, which displayed typical apoptotic figures not earlier of 16 h of treatment (Figure 2E). To better understand the role of caspase activation in licofelone-induced apoptosis, HCA-7 cells were pre-treated for 2 h with the pan-caspase inhibitor z-VAD-fmk (25 μM) and then co-treated with z-VAD-fmk (25 μM) and licofelone 150 μM for further 24 h. As shown in Figure 2F, treatment of HCA-7 cells with licofelone alone resulted in ~53% of apoptosis, whereas co-treatment with z-VAD-fmk resulted only in 10% of apoptotic cells, suggesting that, in our in vitro model, licofelone-induced apoptosis occurred primarily in a caspases-dependent manner.

Licofelone triggers apoptosis through the recruitment of the intrinsic mitochondrial pathway

Next, we were interested in determining the possible mechanism of licofelone-induced apoptosis. Many 5-LOX and COX inhibitors, alone or in association, have been reported to induce apoptosis through the intrinsic mitochondrial pathway (20,21), commonly characterized by early events, such as loss of $\Delta \psi_m$ and cytochrome c release from mitochondria to cytoplasm. On this basis, we decided to analyse the status of HCA-7 mitochondria after treatment with licofelone. As shown in Figure 3A (panels a–d), cytofluorimetric analysis of tetramethyl rhodamine methyl ester-stained cells revealed, after 12 h of treatment with licofelone, a dose-dependent loss in $\Delta \psi_m$ (~90% at the dose 150 μM). Fluorescence microscopy analysis of

Fig. 2. Licofelone induces apoptosis in HCA-7 colon cancer cells through caspases activation. (A) Flow cyt fluorometric analysis of DNA content in HCA-7 cells, after 24 and 48 h incubation with licofelone 100–150 μM, as previously described in the Materials and Methods. (B) Fluorescence microscopy analysis of HCA-7 cells incubated for 24 and 48 h with licofelone 100–150 μM and stained with 1 μg/ml Hoechst 33342, as previously described in the Materials and Methods (bar scale, 20 μm). (C and D) Time course of caspase-3 activation and PARP-1 cleavage, evaluated by western blotting, in HCA-7 cells treated with licofelone 150 μM. Representation of three separate experiments with similar findings. Histograms show full-length and cleaved caspase-3 and PARP-1 levels normalized to the corresponding β-actin level, as previously described in Materials and Methods. Asterisks indicate significance, with $^*P < 0.001$ (very significant).

Fig. 3. Licofelone causes the loss in $\Delta \psi_m$ and induces cytochrome c release from mitochondria to cytoplasm in HCA-7 cells. (A) Flow cytofluorimetric (panels a–d) and fluorescence microscopy (panels e and f) analysis of loss in $\Delta \psi_m$ in HCA-7 cells, after 12 h of treatment with licofelone 100–150 μM, as previously described in the Materials and Methods (bar scale, 5 μm). (B) Evaluation of cytochrome c level in the cytoplasmic and mitochondrial fraction, in HCA-7 cells treated for 12 h with licofelone 100–150 μM. Results are expressed as mean ± SD of three independent experiments. Asterisks indicate significance, with $^*P < 0.001$ (very significant).
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Fig. 4. Licofelone induces caspase-9 activation and the cleavage of p21Bax into p18Bax. (A) Time course of caspase-9 activation, evaluated by western blotting, in HCA-7 cells treated with licofelone 150 μM. Representation of three separate experiments with similar findings. Histograms show full-length and cleaved caspase-9 levels normalized to the corresponding β-actin level, as previously described in Materials and Methods. Asterisks indicate significance, with *P < 0.05 (significant) and **P < 0.001 (very significant). (B) Western blotting analysis of bcl-2 and Bax expression in HCA-7 cells treated for 12 h with licofelone 100–150 μM. Note that anti-Bax detected both the full-length p21Bax and the cleaved form p18Bax. Histograms show bcl-2, full-length and cleaved Bax levels, normalized to the corresponding β-actin level, as previously described in Materials and Methods. Representation of three separate experiments with similar findings. Asterisks indicate significance, with *P < 0.05 (significant) and **P < 0.001 (very significant).

for GC analysis by the transesterification procedure under mild conditions (30). As shown in Figure 6A, we found that, after 24 h of treatment with licofelone, the level of membrane-bound AA was reduced in a dose-dependent manner, indicating that a progressive

LC-1-stained cells confirmed the loss in ρm, as shown by the red/green fluorescence shift in treated cells (Figure 3A, panels e–h). It is worth underlining that, at this time, we did not observe any nuclear hallmark of apoptosis, thus suggesting that mitochondrial dysfunction was an event that preceded DNA fragmentation. Loss in ρm was associated to a dose-dependent release of cytochrome c from mitochondria to cytoplasm, as shown by its decrease in the mitochondrial fraction and its accumulation in the soluble cytoplasmic fraction after 12 h of treatment with licofelone (Figure 3B). As shown in Figure 4A, caspase-9, which is known to bind to cytochrome c–Apaf-1 complex, was cleaved and activated starting from 12 h of treatment onward, concurrent with the release of cytochrome c from HCA-7 mitochondria. The release of cytochrome c into the cytosol prompted us to analyse the involvement of upstream regulators of mitochondrial membrane perturbations, such as the proapoptotic and anti-apoptotic members of the Bcl-2 family, p21Bax and bcl-2. Accordingly, we evaluated their levels in HCA-7 cells treated for 12 h with increasing doses of licofelone. As shown in Figure 4B, while we did not detect any significant change in bcl-2 expression at all the doses tested, we observed, by using an antibody directed against the 150–165 amino acid sequence of human Bax, a dose-dependent cleavage of the full-length p21Bax into p18Bax fragments in the whole HCA-7 cells lysate. Such cleavage, due to the removal of the 33 N-terminal amino acids during apoptosis, has been shown to be a more potent inducer of the apoptotic process than p21Bax in several cell lines (22).

Licofelone-induced apoptosis is not related to its ability to affect the AA cascade

As both PGE2 and LTB4 have been shown to promote colon cancer cell proliferation and to act as anti-apoptotic agents (23–25), we first decided to verify whether licofelone-induced apoptosis was related to a reduction of these two AA metabolites in HCA-7 culture medium. As shown in Figure 5A, after 24 h treatment, licofelone reduced in a dose-dependent manner the level of LTB4 (the 5-LOX metabolite) and strongly inhibited PGE2 (the COX metabolite) production at all the doses tested. This effect was not due to a down-regulation of COX and 5-LOX protein levels (data not shown) and confirmed the ability of licofelone to bind the active sites of both classes of enzymes, thus blocking their catalytic activity (14). We next treated HCA-7 cells for 48 h with licofelone 150 μM in association with exogenous PGE2 and LTB4, in a concentration range mimicking their endogenous production. We found that, neither the addition of PGE2 nor LTB4 alone or in association (data not shown), were able to reverse licofelone-induced apoptosis, suggesting that this effect was not related to the ability of this drug to decrease PGE2 and LTB4 levels in HCA-7 culture medium (Figure 5B).

Next, since it has been reported that accumulation of intracellular unesterified AA is a common mechanism that links inhibitors of AA metabolism to induction of apoptosis (5,26,27), we were interested in evaluating whether licofelone could trigger apoptosis by this pathway. In resting cells, the levels of unesterified AA are maintained very low because it is rapidly esterified, typically at the sn-2 position of membrane phospholipids (1). However, following the activation of PLA2 enzymes, phospholipids are hydrolyzed at the sn-2 position and unesterified AA is released from membranes, thus becoming available for the biosynthesis of eicosanoids (28). To evaluate the effect of licofelone on AA release, a different approach from the detection of free fatty acids (FFAs) released in the cellular aqueous medium was followed. FFAs can in fact be identified by GC analysis only after esterification using diazomethane, a toxic and explosive gas not easily obtainable in biochemistry laboratories (29). Moreover, it is well known that FFAs cannot be detected and quantified directly by the GC methodology alone, since large errors in the manipulation steps cannot be avoided. In addition, the concentration of FFAs under normal conditions is very low, sometimes below detection limits, thus making untreated cells not very useful as controls. On this basis, we evaluated the cell membrane compartment and the fatty acid residues present in membrane phospholipids, which can be easily transformed
release of unesterified AA from HCA-7 membrane phospholipids has occurred. Among the several types of PLA2 enzymes, cPLA2 is the most well-characterized isoform and it is widely expressed in colon carcinomas (2,31). It is now recognized that this enzyme, which exhibits a significant selectivity towards phospholipids bearing AA at the sn-2 position, is a central regulator of stimulus-coupled cellular AA mobilization (2). Accordingly, we investigated whether licofelone-induced AA release may be related to cPLA2 activation. As shown in Figure 6B, we found that this drug induced, in a time-dependent manner and without changing total protein level, the phosphorylation of cPLA2 at Ser505, reaching the maximum after 24 h of treatment with licofelone 100–150 μM. Results are expressed as mean ± SD of three independent experiments. Asterisks indicate significance, with *P < 0.05 (significant) and **P < 0.001 (very significant).

Fig. 5. Licofelone-induced apoptosis is independent from the decrease of LTB4 and PGE2 levels in HCA-7 culture medium. (A) Evaluation of LTB4 and PGE2 levels in HCA-7 culture medium after 24 h treatment with licofelone 100–150 μM. Results are expressed as mean ± SD of three independent experiments. Asterisks indicate significance, with *P < 0.05 (significant) and **P < 0.001 (very significant). (B) Evaluation of the percentage of apoptotic cells in HCA-7 treated for 48 h with licofelone 150 μM, in association with increasing concentrations of exogenous PGE2 or LTB4. Results are expressed as percentage mean ± SD of three independent experiments. Asterisks indicate significance, with **P < 0.001 (very significant).

Discussion

The loss of cell ability to self-destroy by an apoptotic process is considered a pivotal factor for colon carcinogenesis (33). Thereby, molecules which are able to restore the equilibrium between proliferation and apoptosis in colonic epithelial cells and/or to promote apoptosis in cancer cells could be of relevant therapeutic interest. Epidemiological and clinical studies, as well as animal models of colon carcinogenesis, have clearly demonstrated that NSAIDs may be effective against the development and progression of colorectal cancer (34). However, the frequent and often severe gastrointestinal side effects limit their use as anticancer agents (35). Although the introduction of selective COX-2 inhibitors seemed promising, it is now well established that these compounds are not devoid of serious side effects, above all on the cardiovascular system (36). Therefore, novel molecules that maintain the same effectiveness of NSAIDs or selective COX-2 inhibitors, associated to a more favourable toxicological profile, may open a promising approach in colon cancer treatment.

To date, no data are available concerning the potential use of dual COX/5-LOX inhibitors as anticancer drugs in colon cancer treatment. In this regard, the new class of anti-inflammatory drugs named dual COX/5-LOX inhibitors, and in particular, dual COX/5-LOX inhibitors, may represent a good alternative. Both COX and 5-LOX inhibitors, alone or in association, have been reported in fact to induce apoptosis in many cancer cell types (7,26,37,38); moreover, the concurrent inhibition of 5-LOX and COX enzymes has the potential to offer clinically relevant advantages in terms of overall safety (39).
viability in a time- and dose-dependent manner, with an effectiveness much higher than naproxen and similar to that observed for the COX-2 selective inhibitor celecoxib. The decrease in cell viability was associated to the induction of apoptosis, as shown by the fact that, when exposed to licofelone, HCA-7 cells displayed the typical hallmarks of apoptotic death, such as membrane blebbing, unfragmented or irregularly fragmented pycnotic nuclei, caspase-3 activation and PARP-1 cleavage. Treatment with licofelone was also associated to mitochondrial dysfunction, an effect that is related to the recruitment of the intrinsic apoptotic pathway (40). This pathway is in fact characterized by early mitochondrial events, such as the loss in \( \Delta \psi_m \) and cytochrome \( c \) release from mitochondria to cytosol. As the level of cytochrome \( c \) in cytosol increases, caspase-9, which binds to cytochrome \( c \)-Apaf-1 complex, is cleaved and activated, thus leading to the activation of the executioner caspase-3 and subsequent PARP-1 cleavage. Consistent with these reports, we observed that licofelone induced, in a dose-dependent manner, the loss in \( \Delta \psi_m \) and cytochrome \( c \) release from mitochondria, which was associated to caspase-9 activation, an event that preceded caspase-3 activation and PARP-1 cleavage. The involvement of caspase activation in licofelone-induced apoptosis was further investigated using the pan-caspase inhibitor z-VAD-fmk. Co-treatment of HCA-7 cells with licofelone and z-VAD-fmk resulted in a significant decrease in the percentage of apoptotic cells, thus indicating that licofelone-induced apoptosis occurred primarily in a caspase-dependent manner.

The Bcl-2 protein family plays an important role in regulating cytochrome \( c \) release during apoptosis (41). In particular, proteolytic cleavage of full-length p21\(^{\text{ras}} \) into p18\(^{\text{ras}} \) has been reported to occur
in tumour cells treated with various chemotherapeutic agents that are able to activate the intrinsic apoptotic pathway (42). In agreement with these data we found, after treatment with licofelone, a dose-dependent decrease of full-length p21\(^{\text{Bax}}\), that was associated to the concurrent appearance of the cleaved form p18\(^{\text{Bax}}\), which has been reported to be more potent in disrupting mitochondrial integrity and inducing apoptotic cell death with respect to p21\(^{\text{Bax}}\) (43,44). It has been hypothesized, in fact, that the removal of the 33 N-terminal amino acids from p21\(^{\text{Bax}}\) may expose the hydrophobic BH3 domain (amino acids 59–73), involved in dimer formation and required for the proapoptotic function (45). Such a conformational change may facilitate Bax oligomerization and potential formation of death pores in the outer mitochondrial membrane that, in agreement with what we observed after treatment with licofelone, leak cytochrome c into the cytosol to initiate caspase activation. Taken together, these data suggest that disruption of \(\nabla_{\text{\text{mito}}}\) could be a possible, but not necessarily the only, mechanism of licofelone-induced apoptosis in HCA-7 cells.

An aberrant AA metabolism has been related to carcinogenic processes and its modulation has been suggested to be an important strategy for cancer prevention and treatment. In this regard, we investigated whether licofelone could be able to affect the AA cascade and also evaluated its possible involvement in HCA-7 apoptosis induction. We found that licofelone not only was able to block the activity of the AA-metabolizing enzymes 5-LOX and COX (as shown by the strong inhibition of LTB\(_4\) and PGE\(_2\) production), but also induced, through cPLA\(_2\) phosphorylation, the release of unesterified AA from HCA-7 membrane phospholipids. However, we ascertained that apoptosis induction was not related to the ability of licofelone to affect the AA cascade, as neither the exogenous addition in culture medium of the two AA metabolites, LTB\(_4\) and PGE\(_2\), nor the pharmacological inhibition of cPLA\(_2\), rescued HCA-7 cells from apoptosis. The observation that cPLA\(_2\) phosphorylation was significantly blocked following treatment with the inhibitors of p38 MAPK and JNK, also showed stress-activated kinases, led us to hypothesize a possible involvement of oxidative stress in licofelone-induced apoptosis. Even if this hypothesis will be closely examined in a next paper, preliminary data obtained in our laboratory have shown, following treatment with licofelone, an early increase in reactive oxygen species production, parallel to p38 MAPK and JNK activation (data not shown). The presence of oxidative stress could explain, at least in part, the reason why, although we observed cPLA\(_2\) activation and unesterified AA release following treatment with licofelone, pharmacological inhibition with MAFP failed to reverse HCA-7 cell apoptosis. Oxidative stress is in fact a phenomenon acting on a broad spectrum of molecular targets, including lipids, proteins and nucleic acids, thus making, as we hypothesize in our case, PLA\(_2\) inhibition not a sufficient countermeasure to block oxidative stress-induced cell damages.

The concentrations of licofelone required to induce apoptosis in our in vitro model are higher than those reached in plasma samples (46). In this regard, it is worth reminding that cell culture systems are extremely simplified experimental models, where it is not possible to reproduce the complexity of the human organism as a whole. In vivo tumour growth is in fact determined by the interaction between factors intrinsic to tumour cells, extracellular matrix, stromal cells and other host factors. All these elements, not reproducible in culture systems, may be responsible of the discrepancy between the doses of an agent and at doses significantly lower than those employed in vivo, ptosis induction in HCA-7 cells could be due, at least in part, to the fact that, in vitro, the concurrent anti-angiogenic effect (which probably occurs in vivo) cannot be elicited. In summary, results obtained in our study have shown, for the first time, that licofelone is able to trigger apoptosis in colon cancer cells, suggesting that this drug, as well as similar dual COX/5-LOX inhibitors, may represent a novel and promising approach in colon cancer chemoprevention and therapy.

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References

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