The decrease of cell membrane fluidity by the non-steroidal anti-inflammatory drug Licofelone inhibits epidermal growth factor receptor signalling and triggers apoptosis in HCA-7 colon cancer cells

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A B S T R A C T

The ability to induce changes in cell membrane properties is nowadays considered an additional mechanism to explain the pharmacological effects of non-steroidal anti-inflammatory drugs (NSAIDs). We previously demonstrated that the NSAID Licofelone, a dual cyclooxygenase/5-lipoxygenase inhibitor, triggers apoptosis in HCA-7 colon cancer cells independently from the inhibition of these enzymes. Here, we provide evidence that, in HCA-7 cells, the pro-apoptotic effect of this drug relies, at least in part, on its ability to inhibit epidermal growth factor receptor (EGFR) signalling by a decrease of cell membrane fluidity. Indeed, Licofelone induced a relevant change in the relative proportions of some saturated, monounsaturated and polyunsaturated fatty acids constituting HCA-7 phospholipid fraction and significantly increased the levels of cholesterol in HCA-7 cell membrane. All of these changes resulted in a remarkable decrease of membrane fluidity. Such phenomenon was associated with the block of EGFR kinase activity and of its downstream targets, the p44-42 mitogen-activated protein kinase (MAPK) and AKT cascades, whose inhibitions were found to induce apoptosis in HCA-7 cells. Overall, these findings provide a new additional mechanism by which NSAIDs are effective toward colon cancer cells.

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1. Introduction

Several epidemiological and clinical studies have provided compelling evidences on the antitumoural efficacy of non-steroidal anti-inflammatory drugs (NSAIDs) toward colon cancer [1], an effect exclusively ascribed for a long time to the inhibition of cyclooxygenase-2 (COX-2) enzyme. During the last decade, however, additional mechanisms have emerged [2]. Among these, the ability to induce changes in the biophysical properties of cell membrane, in particular fluidity, is nowadays considered of relevant importance to explain the pharmacological effects of these drugs [3–6]. Indeed, perturbations of membrane fluidity are known to affect many crucial cellular events, such as proliferation, differentiation and apoptosis, due to changes in the localization and activity of proteins embedded in the lipid bilayer and functioning as ion channels, receptors, enzymes and signal transducers [7].

We previously demonstrated that the NSAID Licofelone, a dual COX/5-lipoxygenase (5-LOX) inhibitor, triggers apoptosis in HCA-7 colon cancer cells independently from the inhibition of COX and 5-LOX enzymes [8]. Here, we investigated whether the pro-apoptotic effect of this drug could be related to modifications of HCA-7 cell membrane structure and properties. In Licofelone-treated cells, a significant variation in the relative proportions of some saturated, monounsaturated and polyunsaturated fatty acids (SFAs, MUFAs and PUFAs) constituting the HCA-7 membrane phospholipid fraction was observed. Such changes were parallel to a relevant increase in cholesterol content and resulted in a remarkable reduction of membrane fluidity. This last phenomenon was associated with the inhibition of epidermal growth factor receptor (EGFR) phosphorylation and of its downstream targets, the p44-42 mitogen-activated protein kinase (MAPK) and AKT cascades, whose aberrant activations are involved in cancer onset and progression [9,10]. The inhibition of these two cascades was found to result in the induction of apoptosis in HCA-7 cells. Notably, all of these effects were significantly reversed when Licofelone was co-administrated with oleic acid, a MUFA known to be a membrane fluidizer [11].
Overall, the data obtained in this study suggest that Licofelone-induced apoptosis in HCA-7 colon cancer cells may be related to the ability of this drug to decrease cell membrane fluidity and, consequently, to inhibit EGRF signalling.

2. Materials and methods

2.1. Cells and cell culture

The human colon cancer cell line HCA-7 (colony 29) was obtained from European Collection of Cell Cultures (ECACC) and cultured in Dulbecco’s modified Eagle’s Medium with 4.5 g/L glucose (Cambrex Bioscience, Italy), supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Cambrex Bioscience), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma Chem. Co., Dunnstadt, Germany). Cells were confirmed to be free of Mycoplasma contamination, grown at 37 °C in a humidified atmosphere of 95% air and 5% CO2 and routinely passaged using trypsin–EDTA 0.025% (Sigma) to maintain them in a logarithmic phase of growth.

2.2. Drugs

Licofelone was kindly supplied from Merckle-ratiopharm (GmbH, Ulm, Germany). U0126, epidermal growth factor (EGF) and oleic acid were from Sigma, Geofflin (S1025) was from Selleck Chemicals (Houston, Texas, USA) and LY294002 was a kind gift of Prof. A.M. Martelli (Cellular Signalling Laboratory, Department of Anatomical Sciences, University of Bologna, Italy).

2.3. Cytofluorimetric analysis

For the analysis of DNA content, HCA-7 were seeded on a 6-well plate at the density of 5 × 10^5 and then incubated for 48 h with U0126, LY294002 and gefitinib, or with Licofelone in presence/absence of oleic acid or EGF. At the end of incubation, cells were harvested, washed in phosphate buffer saline (PBS) and incubated at 4 °C for 16 h in the DNA staining solution, containing 0.1% Triton X-100, 0.1% sodium citrate, 50 µg/ml propidium iodide. DNA content analysis was performed by FACSAria™ cell sorter (Becton Dickinson, Franklin Lakes, NJ, USA).

2.4. Lipid extraction and fatty acids analysis

HCA-7 cells were seeded in a 25 cm² flask at the density of 1.5 × 10^6, grown in complete medium for 24 h and then treated with Licofelone, in presence/absence of oleic acid, for the indicated times. 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 1 × 10^6 cells/500 µL of PBS; of these, 80 µl were employed for cholesterol measurement (see below). Lipids were extracted in chloroform: methanol 2:1 (vol/vol), in the presence of 0.01% butylated hydroxytoluene. The chloroform phase was separated, evaporated under nitrogen and frozen at -20 °C until use. Lipids were re-suspended with 2 ml of KOH 0.5 M, heated at 80 °C for 10 min and transesterified with 2.5 ml of 14% BF3 in methanol (80 °C for 10 min). Methyl esters were extracted two times by adding 3 ml of n-hexane; combined n-hexane were evaporated under nitrogen and fatty acid methyl esters (FAMEs) were dissolved in 100 µl of cyclohexane. 2–4 µl were injected in gas-chromatography–mass spectrometry (GC/MS) analyses. GC separation was performed according to a previously published method [12], slightly modified for PTV injection and MS analysis. Briefly, the separation was performed in an Agilent HP6890 GC, equipped with PTV injector; GC/MS conditions were the following: column was a SUPELCO 30m × 0.25 mm (0.2 µm film thickness); carrier gas: Helium, initial 0.5 ml/min, constant pressure mode, Temperature Programmed Injector (PTV): solvent vent mode; temperature from 60°C to 220°C during injection cycle. Initial column temperature was 100°C for 1.25 min; then ramps to 185°C at 30°C/min and to 205°C at 5°C/min. All cycle covered 32 min. Mass Spectrometer, an HP5973, operated in electron impact mode at 70 eV. Ion source, GC interface were respectively 230°C, 280°C; MS Quad temp, was 150°C. Total ion Monitoring was performed, with Full data scan from m/z 40 to m/z 550. Peak identification was based on comparison of both retention times and mass spectra of unknown peak to those of standards and GC–MS Wiley® N database library. Results were reported in percentage and were calculated from the sum of the peak area of all analytes of interest using the following equation:

\[
\text{Analyte percentage} = \left(\frac{\text{Peak area}}{\text{summed areas of all peak of interest}}\right) \times 100
\]

Membrane saturation index was calculated as previously reported [13].

2.5. Membrane cholesterol estimation

The levels of cholesterol in HCA-7 cell membrane was evaluated according to Zlatkis et al. [14], operating a consistently reduction in the volumes of incubation. Briefly, 600 µl of glacial acetic acid were added to 80 µl of cell suspension (at the concentration of 1 × 10^6 cells/500 µL PBS). Then, 400 µl of ferric chloride solution (10% w/v in glacial acetic acid) was added to each sample and absorbance was measured at 560 nm, when tubes reached room temperature. Cholesterol was then quantified comparing the absorbance values of each sample with a calibration curve ranging from 15 to 50 µg, and normalizing values for the protein content of each samples.

2.6. Membrane fluidity assay

Membrane fluidity was evaluated employing the Membrane Fluidity kit (Marker Gene Technologies, Inc., Oregon, USA). Briefly, HCA-7 cells were seeded in a 6 well plate at the density of 5 × 10^5 and then treated with Licofelone, in presence/absence of oleic acid for 180 min. At the end of incubation, cells were processed following the manufacturer’s instructions. The imaging was performed under a fluorescence Olympus CKX41 inverted microscope (Olympus Italia), equipped with an Olympus C5060-ADU camera (Olympus, Italy).

2.7. Western blotting analysis

HCA-7 cells were lysed and blotted as previously described [9]. Primary antibodies employed were: rabbit anti-Phospho-EGFr (Tyr1173), rabbit anti-EGFr, rabbit anti-phospho-c-Raf (Ser338), rabbit anti-r-c-Raf, rabbit anti-phospho-MEK1/2 (Ser217/221), rabbit anti-MEK1/2, mouse anti-phospho-p44/p42 (Thr202/Tyr204), rabbit anti-p44/p42, rabbit anti-phospho-akt (Ser473), mouse anti-AKT (all from Cell Signalling Technology, Danvers, MA, USA) and rabbit anti-β-actin (Sigma). Goat anti-rabbit and anti-mouse horseradish peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology (CA, USA). Detection was performed using the ECL procedure developed by Amersham Biosciences (Little Chalfont, UK). Bands were acquired and densitometrically analyzed with Fluor-S MultiImager (Bio-Rad, Hercules, CA) using Quantity One software (Bio-Rad). After probing for phosphorylated proteins, membranes were stripped of bound antibodies by Re-Blot Plus Western Blot Recycling Kit (Chemicon International, Temecula, CA, USA) and re-probed with an antibody against the corresponding total protein.

2.8. EGRF competition binding assay

Competition binding assay was performed as previously reported [15]. Briefly, HCA-7 cells were seeded at the density of 4 × 10^5 in a 96-well plate for 24 h. Cells were then washed twice with PBS and fixed in 0.5% formaldehyde for 15 min at room temperature. Cells were then washed three times with PBS and incubated 30 min at 37°C in 3% PBS. At the end of incubation, PBS was removed and a solution containing 50 ng/ml biotinylated EGF (Invitrogen, Milan, Italy), in presence/absence of Licofelone 150 µM in DMEM supplemented with 0.1% BSA and 20 mM Hepes (pH 7.4) was added, and plate was incubated for 1 h at 37°C. Cells were then washed three times with PBS containing 0.3 M NaCl and incubated with streptavidin-conjugate horseradish peroxidase (HRP) at 37°C for 1 h. Cells were then re-washed in PBS containing 0.3 M NaCl and exposed to ABTS (Invitrogen) and hydrogen peroxide for 25 min. Absorbance values were finally read at 405 nm in a spectrophotometric microplates reader (Bio-Rad, Hercules, CA).

2.9. Immunofluorescence

HCA-7 were seeded on glass coverslips and treated with Licofelone, in presence/absence of oleic for the indicated times. At the end of incubation, cells were fixed for 5 min at room temperature in 2% paraformaldehyde, washed three times with PBS, permeablized for 4 min with PBS-Trition X-100 0.2% and incubated for 30 min in PBS–BSA 3% to minimize possible unspecific binding. Fixed cells were then incubated with mouse anti-Phospho-p44/p42 (Thr202/Tyr204) for 1 h, washed three times in PBS–BSA 3% and incubated for 45 min with anti-mouse FITC-conjugated secondary antibody (Santa Cruz Biotechnology). At the end of incubation, cells were washed three times in PBS and coverslips were mounted in glycerol–PBS medium containing 50 mg/ml DABCO. The imaging was performed under a fluorescence Olympus C5030X1 inverted microscope, equipped with an Olympus C5060-ADU camera (Olympus).

2.10. Statistical evaluation

Results are expressed as mean ± standard deviation. Statistical analysis was performed using one-way ANOVA followed by Bonferroni’s post hoc test, using GraphPad Prism software version 5.01 for Windows (GraphPad Software, San Diego, CA, USA). A p value <0.05 was considered statistically significant.

3. Results

3.1. Licofelone decreases HCA-7 membrane fluidity

In a first set of experiments, we investigated whether Licofelone administration could affect the structure and fluidity of HCA-7 cell
Time course up to 180 min with Licofelone 150 μM showed no changes on the level of mirystic, linoleic and α-linolenic fatty acids (data not shown). Conversely, we found a significant decrease of the MUFA oleic and of the PUFAs arachidonic and docosahexaenoic, concomitant to a relevant increase of the SFAs palmitic and stearic (Fig. 1A). These changes resulted in a remarkable increase of HCA-7 membrane saturation index, from 1.8 to 4.1 after 120 and 180 min of treatment, respectively (Fig. 1B).

Table 1. Membrane fatty acids analyzed for each sample.

<table>
<thead>
<tr>
<th>FAME carbon number</th>
<th>Systematic name</th>
<th>Common name</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>Tetradecanoic acid</td>
<td>Palmitic acid</td>
</tr>
<tr>
<td>18:0</td>
<td>Octadecanoic acid</td>
<td>Stearic acid</td>
</tr>
<tr>
<td>18:1n-9</td>
<td>cis-9-Octadecenoic acid</td>
<td>Oleic acid</td>
</tr>
<tr>
<td>18:2n-6</td>
<td>cis(9,12)-Octadecadienoic acid</td>
<td>Linoleic acid</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>cis(9,12,15)-Octadecatrienoic acid</td>
<td>α-Linolenic acid</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>cis(5,8,11,14)-Eicosatetraenoic acid</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>cis(4,7,10,13,16,19)-Docosahexaenoic acid</td>
<td>DHA</td>
</tr>
</tbody>
</table>

Fig. 1. Licofelone decreases HCA-7 cell membrane fluidity. (A) Fatty acid methyl esters (FAMEs) analysis of membrane phospholipids fraction in HCA-7 cells treated for the indicated times with Licofelone 150 μM. (B) Evaluation of membrane saturation index in HCA-7 cells treated for the indicated times with Licofelone 150 μM. (C) Membrane cholesterol estimation in HCA-7 cells treated for the indicated times with Licofelone 150 μM. (D) Pyrene labeling of HCA-7 membrane phospholipids after 0–180 min of treatment with Licofelone 150 μM. Scale bar: 10 μm. A representative picture of cells with high (white arrow) and low (black arrow) excimer fluorescence is also reported. *p < 0.05 and **p < 0.001.

Then, we evaluated the level of cholesterol in HCA-7 following Licofelone treatment. As shown in Fig. 1C, cholesterol levels grew remarkably in treated cells, reaching about a 50% increase compared to control ones. This result, together with the above observed increase of membrane saturation index, strongly suggested a progressive loss of HCA-7 membrane fluidity following Licofelone administration. To confirm this data, HCA-7 cells were labelled with the lipophylic probe pyrene that, when inserted into membranes as a substituent of one or both phospholipid acyl chains, is able to form excimers upon spatial interaction. When excimers form, the emission spectrum of pyrene shifts to the red, fluorescence intensity is proportional to their concentration and in turn, is proportional to membrane fluidity [18]. As shown in Fig. 1D, after 180 min of treatment with Licofelone, the majority of treated cells displayed a lower intensity of pyrene fluorescence in respect to control cells, indicating that this drug decreases membrane fluidity in HCA-7 colon cancer cells.

3.2. Licofelone inhibits the EGFR downstream p44-42 MAPK and AKT cascades in HCA-7 cells

Changes in membrane fluidity have been reported to affect several cell signalling events, due to variations in the localization and activity of many membrane-associated proteins [7]. Among these, EGFR, an important member of tyrosine kinase receptors, is frequently overexpressed and/or mutationally activated in several human cancers, including colorectal one [19]. Previous literature data have reported that EGFR tyrosine kinase activity is modulated by membrane fluidity variations [20]. In particular, the functional reconstitution of EGFR into liposomes with different lipid compositions has demonstrated that such receptor has a higher kinase activity in a fluid membrane [20]. We therefore investigated whether the decrease of membrane fluidity induced by Licofelone was associated with changes in EGFR activation and signalling. We observed that EGFR phosphorylation was completely blocked after 2 h of treatment with Licofelone (Fig. 2A). A more detailed time course showed that inhibition of EGFR phosphorylation occurred from 90 min onwards (Supplementary Fig. 1), a timing that, intriguingly, was consistent with the decrease of HCA-7 membrane fluidity. Notably, we observed that EGFR inhibition by Licofelone...
was not due to a direct binding of this drug to such receptor, as revealed by competition binding assay (Fig. 2B).

The block of EGFR activation was followed by a sequential inhibition of the phosphorylated protein levels of the EGFR downstream targets AKT, c-Raf, MEK1-2 and p44-42 MAPK (Fig. 2C). Moreover, immunofluorescence analysis revealed that, in treated cells, the decrease of phospho-p44-42 MAPK protein expression was accompanied by a progressive accumulation of this protein from the nucleus to the cytoplasm, starting at 8 h of incubation with Licofelone (Fig. 2D).

As the inhibition of AKT and p44-42 MAPK signalling has been related to the induction of apoptosis in some cancer cell lines [9,10,21], we investigated whether their inhibition could trigger apoptosis in our experimental model. We found that treatment for 48 h with the MEK inhibitor UO126 and the PI3K inhibitor LY294002 (in a concentration that almost completely inhibited both p44-42 MAPK and AKT phosphorylation, Fig. 3A) significantly induced apoptosis in HCA-7 cells (Fig. 3B). Similar results were obtained with the EGFR tyrosine kinase inhibitor Gefitinib (Fig. 3A and B). This last finding, in particular, is consistent with previous results obtained by Cunningham et al. [22] in DiFi colon cancer cells that, as HCA-7, express a high basal level of phosphorylated EGFR.

Overall, these data indicate that the inhibition of the EGFR downstream targets p44-42 MAPK and AKT triggers apoptosis in HCA-7 cells and that Licofelone-induced apoptosis occurs, at least in part, by the inhibition of such cascades.

### 3.3. Oleic acid addition associates with the recovery of membrane fluidity in HCA-7 cells treated with Licofelone

Oleic acid is known to be a membrane fluidizer [11,23]. We therefore analyzed whether the exogenous addition of this MUFA could reverse the loss of fluidity in Licofelone-treated cells, when administered in combination with this drug. As shown in Fig. 4A, in respect to Licofelone alone (see Fig. 1A), oleic acid addition was associated with a significant increase in the phospholipid saturation index (Fig. 4B), when compared to Licofelone used as single agent (Fig. 1B). Moreover, in respect to Fig. 1C, a remarkable diminution of membrane cholesterol was found (Fig. 4C). All of these data suggested that oleic acid administration was associated with a significant recovery of HCA-7 membrane fluidity. These results were confirmed by pyrene labelling that displayed, after 180 min of treatment, an increase of cells with a high-fluorescence intensity (Fig. 4D), when compared to those ones treated with Licofelone alone (Fig. 1D).

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**Fig. 2.** Licofelone inhibits EGFR signalling in HCA-7 cells. (A) Western blotting analysis of phosphorylated and total EGFR protein expression in HCA-7 cells treated for the indicated times with Licofelone 150 μM. Numbers indicate densitometric analysis of phosphorylated EGFR level normalized to corresponding EGFR total level. β-Actin was assessed as quantitative control for equal loading; (B) EGFR competition binding assay in HCA-7 cells incubated with Licofelone 150 μM and EGF 50 ng/ml, alone and in combination, as previously described in Section 2. (C) Western blotting analysis of phosphorylated and total AKT, c-Raf, MEK1-2 and p44-42 MAPK protein expression in HCA-7 cells treated for the indicated times with Licofelone 150 μM. Numbers indicate densitometric analysis of phosphorylated AKT, c-Raf, MEK1-2 and p44-42 MAPK total level normalized to corresponding AKT, c-Raf, MEK1-2 and p44-42 MAPK total level. β-Actin was assessed as quantitative control for equal protein loading. (D) Immunofluorescence analysis of phospho-p44-42 MAPK localization in HCA-7 cells treated for the indicated times with Licofelone 150 μM. Scale bar: 10 μm. *p < 0.001.
3.4. Oleic acid addition re-activates EGFR signalling and decreases HCA-7 apoptotic cells

We then assessed whether the recovery of membrane fluidity induced by oleic acid was associated with the re-activation of EGFR signalling and rescue from apoptosis in HCA-7 cells. As shown in Fig. 5A, in respect to the matched controls reported in Fig. 2A and C, phosphorylated EGFR, AKT, c-raf, MEK1/2 and p44-42 MAPK were detectable until 24 h treatment. Moreover, phospho-p44-42 MAPK was found to be localized exclusively in the nucleus (Fig. 5B). Activated p44-42 MAPK and AKT pathways are known to be involved in cancer cell resistance to apoptosis [9,24]. In agreement with these findings, combined addition of oleic acid and Licofelone to HCA-7 cells caused, after 48 h, a significant reduction in the percentage of apoptotic cells (about 65% vs. Licofelone alone) (Fig. 5C). To better investigate the role of EGFR reactiva-
vation in preventing Licofelone-induced apoptosis, we also treated HCA-7 cells with the EGFR endogenous ligand EGF, in presence/absence of Licofelone. As shown in Fig. 5E, EGF addition, in a concentration that significantly induced EGFR phosphorylation (Fig. 5D), reversed by about 50% the percentage of Licofelone-induced HCA-7 apoptotic cells.

Overall, these findings indicate that Licofelone induces apoptosis by inhibition of EGFR signalling, an effect reversed by the exogenous addition of oleic acid.

4. Discussion

A proper membrane lipid composition is essential for biological functions [25]. The relevance of lipids in the maintenance of cellular homeostasis is highlighted by some abnormalities in the lipid composition and structural organization identified in the cell membrane of oncologic patients [26]. Such alterations are an early and common feature of neoplastic transformation and lead to significant differences in many membrane properties, when compared to their normal counterpart [27,28]. In this regard, variations in membrane fluidity can be of pivotal relevance, as they can directly influence tumor cell proliferation, metastatization and resistance to chemotherapeutic agents [28].

Many reports have underlined the antitumoural efficacy of NSAIDs towards colon cancer [1] and the inhibition of COX-2 enzyme has been considered for a long time the only explanation for such property. During the last decade, however, additional mechanisms have emerged [2]. Among these, NSAIDs-induced variations in membrane fluidity are frequently evoked to explain...
the pharmacological effects of these drugs [29]. A significant indication about such ability is the well-known gastro-lesivity of many, if not all, NSAIDs, a side effect explainable not only in terms of COX-1 inhibition [29,30]. In the gastric mucosa, indeed, the lipid bilayer exerts an important barrier function and the changes in membrane physical–chemical properties induced by NSAIDs can result in an increased susceptibility to rupture and to form pores for protons that, moving from the lumen into the tissue, can cause gastrointestinal injury and ulceration [30].

Fluidity is one of the membrane properties mainly affected by the interaction between NSAIDs and plasma membrane [4]. Notably, whether membrane fluidity increases or decreases, greatly depends on the specific biophysical properties (i.e. pkₐ, hydrophobicity) of the NSAID employed [29]. In the present study, we found that the NSAID Licofelone decreased membrane fluidity in HCA-7 colon cancer cells. This effect was related to the ability of this drug either to rise membrane cholesterol level, or to induce a remarkable change in the relative proportions of some SFAs, MUFAs and PUFAs constituting HCA-7 phospholipid fraction. In particular, we found that Licofelone strongly increased the levels of the SFAs palmitic and stearic and decreased the percentage of the MUFAs oleic and of the PUFAs arachidonic and docosahexaenoic, thereby leading to an increase of HCA-7 membrane saturation index. Notably, a diminution of membrane saturation index has been observed in patients with primary and recurrent colon cancer, when compared with control subjects [31].

Changes in membrane fluidity are known to result in concomitant alterations in the localization and activity of proteins embedded in cell membrane, since lipids form transient or stable platforms for protein activity and protein–protein interactions by interacting dynamically each other [7]. Among membrane proteins, EGFR is a tyrosine kinase receptor frequently over-expressed in colon cancers and its upregulation has been reported to correlate with a more aggressive disease and poor prognosis [32]. Activation of the intrinsic tyrosine kinase is the first and crucial step for EGFR cascade and the loss of such activity leads to the depletion of most of its intracellular signalling events [33]. In the last two decades, several EGFR tyrosine kinase inhibitors and anti-EGFR monoclonal antibodies (MAb) have been developed to block the activation of this receptor in many human malignancies, including colon carcinoma [34]. Notably, either in colon cancer prevention or treatment, these molecules have revealed a greater efficacy when employed in combinatorial therapeutic strategies, in respect to mono-therapy regimens [19]. In particular, the simultaneous inhibition of EGFR and COX-2 by anti-EGFR MAb and NSAIDs has been reported to give encouraging results in colon cancer animal models [35,36].

Intriguingly, we here provide evidences that EGFR phosphorylation was significantly inhibited after the administration of the NSAID Licofelone, and was followed by the inhibition of both AKT and p44-42 MAPK cascades. A constitutive activation of these pathways has been detected in many human cancers, particularly in colon ones, and their block has been shown to inhibit tumour metastatization, angiogenesis and to induce apoptosis in several cancer types [9,10]. Consistent with this last finding, we observed that inhibition of the EGFR downstream p44-42 MAPK and AKT cascades by Licofelone triggered apoptosis in our experimental model, as also confirmed by experiments with U0126, LY294002 and Gefitinib inhibitors. On the other hand, the observation that EGF exogenous addition reversed the pro-apoptotic effect of Licofelone only partially, clearly indicates that EGFR inhibition is one of the mechanisms, but not the only, by which this drug triggers apoptosis in HCA-7 cells.

The finding that EGFR inhibition was unrelated to a direct binding of Licofelone to this receptor and was concomitant to a significant reduction of HCA-7 membrane fluidity, led us to hypothesize the involvement of membrane fluidity changes in Licofelone-induced EGFR inhibition. Indeed, membrane fluidity changes have been reported to regulate EGFR activation [20]. In its inactive form, EGFR is known to associate with lipid rafts [37,38], dynamic and specialized membrane microdomains functioning as platforms for the regulation of many cell signalling events [39]. Lipid rafts are primarily composed of tightly packed, liquid-ordered sphingolipids/cholesterol/saturated phospholipids [39]. This peculiar composition makes them less fluid than the remainder of the membrane and susceptible to modulation by fatty acids and cholesterol [40]. In particular, phospholipids with saturated acyl chains are known to promote lipid raft formation while cholesterol depletion results in lipid raft disruption [40]. This last phenomenon has been reported to associate with the release of EGFR from these microdomains, thereby allowing its activation [37,38,41]. Therefore, it can be hypothesized that Licofelone treatment, increasing SFAs and cholesterol levels, alters lipid raft structure, thereby preventing EGFR activation and signal transduction.

Interestingly, the effects elicited by Licofelone on HCA-7 membrane fluidity, EGFR signalling and apoptosis were found to be significantly reversed by combined treatment with oleic acid. Although it cannot be excluded that oleic acid can re-activate EGFR signalling through mechanisms independent from membrane fluidity modulation, it is worth to underline that such MUFAs is a well known membrane fluidizer [11]. This property is of pivotal relevance, since it reinforces the notion of the involvement of membrane fluidity changes in the pro-apoptotic mechanism of Licofelone. As a matter of fact, when HCA-7 cells where treated with this drug in presence of oleic acid, a significant recovery of membrane fluidity was concomitant to the recovery of EGFR signalling and was followed by the reduction of apoptotic cells. A possible explanation for such a phenomenon could be that the reduction of SFAs and cholesterol induced by oleic acid, parallel to the increase of UFAs, leads to lipid raft disruption in the membrane, thus resulting in EGFR re-activation and signal transduction recovery. On the other hand, it is conceivable that the membrane fluidity recovery induced by oleic acid could also affect the function of other membrane proteins involved in apoptosis, besides EGFR. This hypothesis is supported by the observation that oleic acid exogenous addition was more effective than EGF to rescue HCA-7 cells from apoptosis, when administered in combination with Licofelone.

Overall, the data obtained in the present study provide new insights in the complex scenario of NSAID pharmacological properties, suggesting modulation of membrane fluidity as an additional and effective mechanism by which these drugs can inhibit cell signalling events crucial for cancer onset and progression. Even if further studies are needed, these findings support the development and the employment of drugs targeting the dynamics of cell membranes in colon cancer treatment. In this context, due to the absence of gastrointestinal and cardiovascular side-effects described for classical NSAIDs [42,43], the novel dual 5-LOX/COX inhibitor, Licofelone, could represent a really promising candidate in this setting.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.canlet.2012.02.003.

References


