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Licofelone Inhibits Proliferation of Rat Hepatoma Cells

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Authors' Contributions

POV, EI and MGK designed the study, performed cell culture experiments and statistical analysis, GK established flow cytometric analysis. SK and RU helped in designing and writing the manuscript.

Key words

Licofelone, Anti-proliferation, Apoptosis, Hepatoma, *in vitro*.

ABSTRACT

Despite clinical treatments, hepatocellular carcinoma is one of the most common causes of cancer death. We tested whether licofelone, an inhibitor of both cyclooxygenase and lipoxygenase pathways of arachidonic acid metabolism, changes the survival rate and induces apoptosis in a hepatoma cell line (H4IIE). We determined *in vitro* survival rate with MTT assay, the morphological changes with an inverted microscope, apoptosis using flow cytometry and statistical significance with one way analysis of variance followed by Tukey's multiple comparison test. IC_{50} values of licofelone were determined as 171 µM for 24 h and 140 µM for 48 h applications. Used as a positive control, 250 µM 5-Fluorouracil decreased the cell survival by only 40 % after 48 h, but licofelone treatment with the same dose and time duration decreased the number of surviving cells by 87 %. Treatments with 150, 200 and 250 µM licofelone caused early apoptotic cell rates of 4, 15 and 24 % for 24 h and 6, 13 and 24 % for 48 h, respectively. In addition, 150, 200 and 250 µM of 5-Fluorouracil resulted early apoptotic cell values of 13, 12, and 11 % for 24 h and 18, 22 and 16 % for 48 h. This study revealed that licofelone possesses dose and time dependent anti-proliferative and apoptotic properties on hepatoma cells.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the third leading cause of cancer mortality (Clark *et al*., 2005) and the worldwide incidence is currently increasing (Altekruse *et al*., 2009). While acute inflammation is a part of body defense system, chronic inflammation can lead to diseases like cancer formation and development. Supporting studies indicate that chronic inflammatory disorders have an increased risk of cancer development and treatment with non-steroidal anti-inflammatory drugs decreases the incidence and the mortality of tumor cells (Leonardi *et al*., 2012). One of the major pathways that take role in inflammation is arachidonic acid (AA) metabolism. Regarding the relationship between AA and carcinogenesis, recent studies are revealing new molecular targets for cancer treatment (Ye *et al*., 2005). Activation of phospholipases (*e.g.* cytosolic phospholipase A_2) releases free AA that is esterified to membrane glycerophospholipids and makes it available for oxidative metabolism by several enzymatic systems. For example, the enzyme cyclooxygenase (COX) produces prostaglandins, thromboxane and prostacyclin from metabolism of AA (Zeldin, 2001). Lipoxygenase (LOX) converts AA, linoleic and other polyunsaturated fatty acids into biologically active metabolites called leukotrienes, hydroxyeicosatetraoneoic acids and lipoxins (Shureigi and Lippman, 2001). As a result, the metabolism of arachidonic acid produces oxidized products that behave as potent autocrine and paracrine regulators of cell function and modulate diverse physiologic and pathologic responses including growth and invasiveness of tumor cells (Sigal, 1991; Cao *et al*., 2000; Hyde and Missailidis, 2009). Overexpression of arachidonic acid metabolism in malignant conditions is associated to cell survival and protection against apoptosis (Tauler and Mulshine, 2008). Therefore, blocking arachidonic acid metabolism by inhibiting both COX and LOX pathways might be useful for cancer prevention and treatment.

Apoptosis is a controlled process that regulates cell population by removal of unwanted and atypical cells

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(Salseven and Dixit, 1999; Huerta *et al*., 2007). Because of the importance in development of many disease processes, including cancer, apoptosis has undergone extensive examination in the past 30 years. In apoptosis there is a dynamic interplay of several molecules with up-regulatory and down-regulatory properties. Stimulation of pro-apoptotic molecules or inhibition of apoptotic factors are dependent on the cell type and the form of insult. The onset of cancer usually results from uncontrolled cell proliferation or an inability of cells to undergo apoptotic cell death (Herr and Debatin, 2001). Based on outcomes, chemicals that trigger apoptosis of transformed cells may be promising drugs for the treatment of cancer.

Licofelone has analgesic, antipyretic, antiplatelet and anti-inflammatory effects (Cicero *et al*., 2005; Gao *et al*., 2010). By inhibiting both COX-2 and 5-LOX pathways, licofelone decreased the production of inflammatory chemicals like leukotrienes and prostaglandins (Gracia-Alvaro, 2004; Khurdayan and Matito, 2007). Licofelone had also anticancer properties on prostate (Narayanan *et al*., 2007), colon (Tavolari *et al*., 2008; Mohammed *et al*., 2011), breast (Liu *et al*., 2011; Kurkjian *et al*., 2007), fibroblast (Kabadere *et al*., 2014) and glial cancer cells (Kus *et al*., 2013). In addition, previous studies have shown that licofelone inhibits urothelial and pancreatic ductal adenocarcinoma tumor growth *in vivo* (Madka *et al*., 2014; Raol *et al*., 2015). Furthermore, Tavolari *et al*. (2008) showed that licofelone induced apoptosis via loss in mitochondrial membrane potential, cytochrome c release, caspase-9 and 3 activation and poly-(ADP-ribose) polymerase-1 cleavage. As an advantage, licofelone has no gastric toxicity when compared to the selective inhibitor drugs that take role in both arachidonic acid pathways (Narayanan *et al*., 2007). Since there is no encountered study examining the role of licofelone on hepatoma, we intended to reveal its possible role on rat hepotama cell line (H4IIE) *in vitro*.

MATERIALS AND METHODS

Drug preparation

Licofelone (Cayman) and 5-Fluorouracil (5-FU, Sigma) were first dissolved in dimethyl sulfoxide (DMSO, Sigma) and diluted further with Dulbecco's modified Eagle's medium (DMEM, Sigma) to obtain required final concentrations. 5-FU, an established antitumor agent, was used as a positive control for cytotoxicity and induction of apoptosis.

Cell culture

Obtained from American Type Culture Collection (USA), H4IIE cells were grown in a complete medium containing DMEM, 10% fetal calf serum (FCS, Sigma) and 1 % penicillin-streptomycin solution (Sigma) in a humidified atmosphere of 95 % O_2 and 5 % CO_2 in air at 37 ^{0}C. After achievement of confluence, the cells were detached from the bottom of flasks with 0.25 % trypsin-EDTA (Sigma), centrifuged at 1200 rpm, 4 ^{0}C for 5 min and then counted with a cell counter (CEDEX, Roche).

Experimental groups

Control group had only complete medium; DMSO group, a final concentration of 0.1 % DMSO (solvent) in complete medium; Licofelone group, treated with 10, 50, 100, 150, 200 and 250 µM licofelone for 24 or 48 h and 5-FU group, 10, 50, 100, 150, 200 and 250 µM 5-FU applied for 24 or 48 h.

Cytotoxicity tests

Before inoculation ($1x10^4$ cell/well) into 96 well plates, the cells were first cultured in flasks until confluence and then detached with trypsin. After 24 h incubation in plates with complete medium, the drugs were added and H4IIE cells were incubated further for 24 or 48 h. 3-[4,5-dimethylthiazol-2yl]-diphenyl tetrazolium bromide (MTT, Sigma) method was applied for the determination of cell number in the presence or absence of present drugs (Elsayed *et al*., 2016). Absorbance of formed formazan was measured by a microtiter-plate reader (Bio Tec) at 550 nm.

Cell morphology

In order to determine possible morphological changes, $1x10^6$ cells were seeded first to 25 cm^2 flasks with 5 mL complete medium and then doses of 150, 200 or 250 µM licofelone were added for 24 h period. Morphological effect of licofelone was observed with an inverted microscope (Olympus CK2, Japan).

Flow cytometric analysis

Based on MTT results, only higher three (150, 200 and 250 µM) doses of both drugs were tested for detection of the type of cell death in subsequent flow cytometric analysis. Apoptotic role of our drugs was determined by staining the hepatoma cells with fluorescein isothiocyanate (FITC)-labeled Annexin V (Invitrogen) and following flow cytometric tests (Engeland *et al*., 1998). Briefly, after incubation of the cells in 25 cm^2 flasks with the presence or absence of the drugs for 24 or 48 h, cell suspensions were spanned twice at 1200 rpm and 4 ^{0}C for 5 min in cold phosphate buffered saline (Sigma) and resuspended in binding buffer ($1x10^6$ cells in 100 µL). Five µL Annexin V with FITC and 10 µL propidium iodide were added to the cell suspension and then incubated in dark room with 23 ^{0}C for 15 min. After dilution with 400 µL binding buffer,

the samples were then analyzed by a FACS Calibur flow cytometer (Becton-Dickinson) and discriminated by Cell Quest data acquisition program.

Statistical analysis

One way analysis of variance followed by Tukey's multiple comparison tests were applied for the statistical significance and the data were expressed as the mean percent fraction of control ± standard error of mean. The p value less than 0.05 was taken to be significant and the apoptotic results were shown as percentage of cells. All results are the mean of at least three independent experiments.

RESULTS

Cell viability assay

Treatment of the cells with DMSO did not cause any significant change in cell viability and morphology in pilot studies. Treated both with the same doses and exposure time, the effect of licofelone on cell survival was clearly different than 5-FU (Figs. 1 and 2). The half maximal inhibitory concentration (IC_{50}) values of licofelone were 171 µM for 24 h and 140 µM for 48 h (Fig. 1).

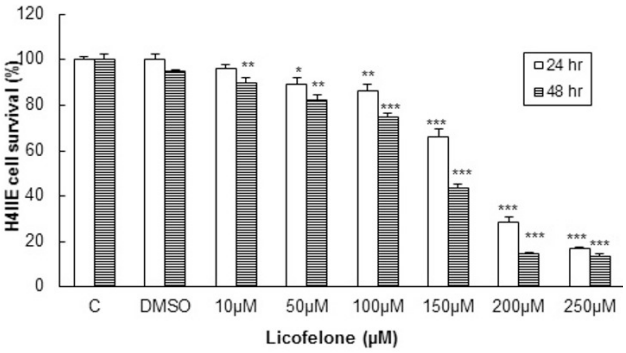

Fig. 1. Time and dose dependent effects of licofelone on survival rate of hepatoma cells after 24 and 48 h applications. C, Control (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

All experimented doses of 10 to 250 µM 5-FU showed (Fig. 2) decreases about 21 to 40 % ($p < 0.001$) in cell number, therefore IC_{50} value for 5-FU could not be calculated. While 10 µM licofelone did not induce any significant change in cell survival after 24 h, the same dose after 48 h lowered cell viability by 11 % ($p < 0.01$). The cell viability decreasing effect of licofelone after 24 h began with 50 µM dose (11 %, $p < 0.05$) and continued with 100 µM (13 %), 150 µM (34 %), 200 µM (72 %) and 250 µM (83 %, $p < 0.001$). However, doses of 50 to 250 µM licofelone after 48 h treatments, showed more decreases of about 18 % ($p < 0.01$), 26 %, 57 %, 85 % and 87 % ($p < 0.001$), respectively. The reduction in the number of surviving hepatoma cells in culture is dependent mostly on exposure time of 5-FU, but it is dependent on both exposure time and dose of licofelone.

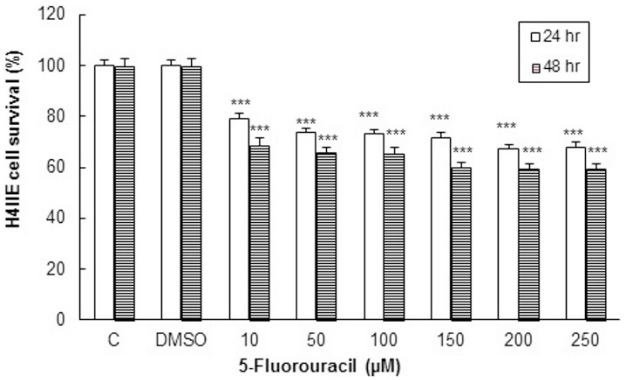

Fig. 2. The survival rates in percentage of hepatoma cells in culture after treatment with DMSO and different doses of 5-FU for 24 and 48 h.

Cell morphology

As in the control and DMSO groups, licofelone doses of 10, 50, and 100 µM did not affect cell morphology. Concentrations of 150, 200 and 250 µM licofelone changed the morphology and declined the number of cells dose dependently. Especially in 200 and 250 µM licofelone groups, alive cells moved away from each other and were quite rounded (Fig. 3).

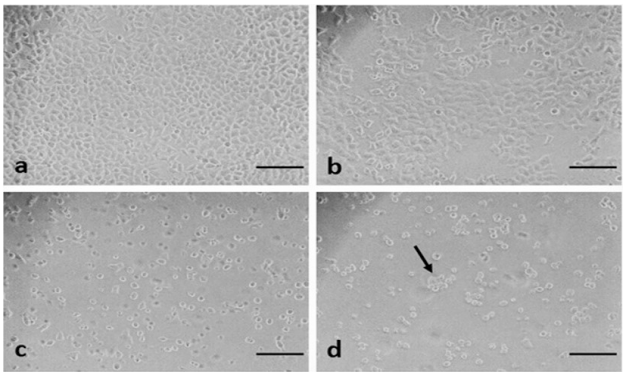

Fig. 3. Morphological appearance of H4IIE cells after treatment with three licofelone doses for 24 h (a, control; b, 150 µM; c, 200 µM; d, 250 µM; scale bar 50 µm; → shows rounded cells).

Flow cytofluorimetric analysis

To understand further the underlying mechanism of

reduction of cell survival detected with MTT assay, we examined the apoptotic role of the drugs on hepatoma cells using Annexin V/propidium iodide assay. Figures 4 and 5 represent flow cytometry results of licofelone applied for 24 and 48 h, respectively. A concentration dependent apoptosis of hepatoma cells, including early as well as late apoptotic cell death, was detected after the treatment with both drugs. The analysis of 24 h treatment with 150 µM licofelone demonstrated that 4 % of the cells underwent early apoptosis, whereas 13 % of the cells underwent early apoptosis with the same dose of 5-FU. The percentages of early apoptotic values of 200 and 250 µM drugs were about 15 and 24 % for licofelone and 12 and 11 % for 5-FU, respectively (Table I). When the exposure time expanded to 48 h, early apoptotic values of the three doses differed slightly and were as 6, 13, 24 % for licofelone, and as 18, 22 and 16 % for 5-FU, respectively. Comparing to the controls, all three doses of both drugs also elevated the percentage of late apoptotic/necrotic cells. Applications of 150, 200 and 250 µM licofelone caused about 41, 48 and 73 % late apoptotic/necrotic cell rates after 24 h and about 50, 62 and 75 % after 48 h, respectively. Same doses of 5-FU resulted in late apoptotic/necrotic cell values of 43, 44, and 48 % after 24 h, and 49, 50 and 58 % after 48 h.

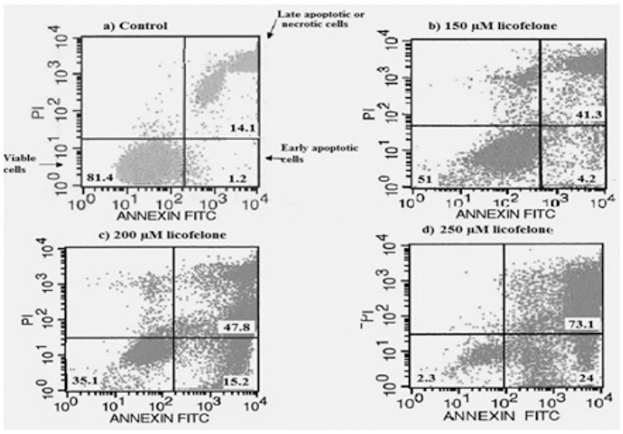

Fig. 4. Typical quadrant analysis of Annexin V-FITC/PI flow cytometry of hepatoma cells that were treated for 24 h with three higher doses of licofelone.

DISCUSSION

There are increasing number of studies about the relationship between licofelone and inflammation, but to date no data are available concerning the use of licofelone as an anti-proliferative drug in hepatoma treatment. We found that licofelone, a dual COX and LOX inhibitor, diminishes survival rate and induces apoptosis of hepatoma cells *in vitro*. The effects of licofelone on hepatoma cells was also compared with 5-FU, an established antitumor agent. Decreasing effect of 5-FU on cell survival was dependent on time but not on dose; however, licofelone had a dose and time dependent effect. Incubation of the cells for 48 h with the highest dose of licofelone (250 µM) decreased the number of living cells by 87 % that is the percentage of surviving cells was only 13 %. With the same dose of 5-FU, the amount of dead cells was calculated as 40 % after 48 h. The result is that licofelone was more cytotoxic to hepatoma cells than 5-FU.

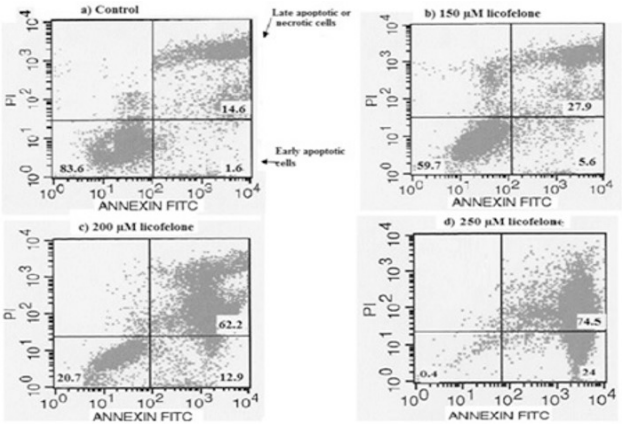

Fig. 5. Proportions of early apoptotic and late apoptotic/necrotic cells after exposure to high licofelone doses for 48 h.

We determined IC_{50} values of licofelone as 171 for 24 h and 140 µM for 48 h treatments. We also observed that licofelone doses deeply affected H4IIE cell morphology as seen on studies of Narayanan et al. (2007) and Tavolari et al. (2008). Therefore, three licofelone doses were studied further to discriminate the type of cell death in flow cytometric analysis. The highest percentage (24 %) of early apoptotic cells were detected with 250 µM licofelone treatment after 48 h (Table 1). Licofelone also increased both early and late apoptotic hepatoma cell rates up to 75 % as compared with the control.

Supporting present results, we previously found that 150, 200 and 250 µM doses of 5-FU and licofelone decreased live cell number and promoted apoptosis of H-Ras transformed fibroblast cells by rising caspase 3 level which was detected with qRT-PCR (Kabadere et al., 2014). In another our study, 150, 200 and 250 µM licofelone doses lowered the number of living glioma cells by 58, 88 and 93 %, respectively and induced apoptosis of the cells in a dose and time dependent manner. Licofelone also induced a 28 % early apoptosis of glioma cells in a time and dose dependent manner and was more apoptogenic than 5-FU and colchicine after 48 h exposures, and moreover it

Table I.- Determination of early or late apoptotic hepatoma cells in percent through Annexin V–FITC/PI staining after treatment with licofelone and 5-FU.

	Time (h)	Control (%)	150 µM (%)	200 µM (%)	250 µM (%)
Licofelone					
Viable cells	24	81.4 ± 4.5	51.0 ± 5.7	35.1 ± 9.0	2.3 ± 1.7
	48	83.6 ± 7.0	39.7 ± 4.5	20.7 ± 5.8	0.4 ± 0.2
Early apoptotic cells	24	1.2 ± 0.3	4.2 ± 0.9	15.2 ± 4.0	24.0 ± 3.7
	48	1.6 ± 0.2	5.6 ± 3.4	12.9 ± 6.4	24.0 ± 9.7
Late apoptotic or necrotic cells	24	14.1 ± 4.4	41.3 ± 6.3	47.8 ± 8.0	73.1 ± 2.4
	48	14.6 ± 6.1	49.9 ± 1.9	62.2 ± 3.9	74.5 ± 6.4
5-FU					
Viable cells	24	82.3 ± 4.2	41.7 ± 4.4	41.6 ± 2.3	39.0 ± 4.2
	48	84.0 ± 6.5	29.7 ± 3.6	24.2 ± 2.4	23.2 ± 3.0
Early apoptotic cells	24	1.8 ± 0.5	13.2 ± 1.4	11.5 ± 1.9	10.9 ± 1.6
	48	1.7 ± 0.3	17.9 ± 1.2	21.8 ± 3.3	15.6 ± 2.7
Late apoptotic or necrotic cells	24	13.8 ± 3.8	43.0 ± 3.3	44.2 ± 2.5	47.9 ± 4.9
	48	15.0 ± 6.3	49.1 ± 5.2	50.3 ± 4.3	57.8 ± 7.4

elevated caspase 3 level (Kus et al., 2013). Several other studies also support our results. Comparing licofelone with 5-FU and cisplatin, Liu et al. (2011) studied with breast cancer cell line and found that licofelone and its derivatives showed growth inhibitory effects. Narayanan et al. (2007) indicated that when applied to prostate cancer cells licofelone showed down regulation of COX-2 and 5-LOX pathways and prevented cell proliferation via apoptotic process. Licofelone also decreased viability of colon cancer cell line (HCA-7) and induced apoptosis by intrinsic pathway (Tavolari et al., 2008). In addition, *in vivo* administration of 150 and 300 µg/mL licofelone for 14 weeks significantly reduced total intestinal tumor size and multiplicity dose dependently in both male and female mice (Mohammed et al., 2011).

COX or LOX activities usually increase alone or together in tumor cells. Although we believe that dual inhibition is more effective on suppression of cell multiplication and stimulation of apoptotic mechanism, there are some studies indicating that blocking only one way of the arachidonic acid metabolism (COX or LOX) results in decreasing viability of cancer cells. For example, inhibiting only 5-LOX in colon (Melstrom *et al*., 2008) and only COX-2 in prostate cancer cells (Kamijo *et al*., 2001) resulted in reduction of cancer cell viability. Like 5-LOX, inhibition of 12-LOX also reduced the numbers of living HCC cells by increasing apoptosis and reducing proliferation (Xu *et al*., 2012). However, in support of our belief, Schroeder *et al*. (2007) inhibited simultaneously COX-2 and 5-LOX pathways of arachidonic acid in human lung cancer cells with drug combinations and concluded that COX and LOX together are more potent in suppression of proliferation and stimulation of cell death. In colon cancer cells, the inhibitory action of combining COX and LOX inhibitors on cell proliferation was again much higher than treatments of using either inhibitor alone (Ye *et al*., 2005). Furthermore, Zhang *et al*. (2008) concluded that a common inhibitor, DHDMBF30, of 5-LOX/COX-2 induces apoptosis and inhibits the proliferation of pancreatic cancer.

CONCLUSION

The present study reveals that licofelone, a dual inhibitor of COX and LOX pathways, decreases cell survival in a time and dose dependent manner. Furthermore, licofelone induces more late apoptotic/necrotic cells death than early apoptotic cell death in rat hepatoma cells. Although much work is needed, our data provides evidence of potential implications for the application of licofelone as a possible anti-proliferative drug against hepatoma.

Conflict of interest statement

We declare that we have no conflict of interest.

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