Anticancer Effects of Licofelone (ML-3000) in Prostate Cancer Cells

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Abstract. Background: Licofelone, a potent anti-inflammatory agent has been reported to interfere with the cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX) signaling pathways with few side-effects. However, the underlying mechanism of licofelone against human cancer is not understood. Materials and Methods: Human and mouse prostate cancer cells were exposed to licofelone in a time- and dose-dependent manner. Cell growth/cell viability, apoptosis, and expression of COX-2 and 5-LOX at the gene and protein levels were investigated. Results: For the first time, it was demonstrated that licofelone inhibited prostate cancer cell growth and significantly down-regulated COX-2 and 5-LOX expression. A weak inhibitory effect on COX-1 protein was also observed. Conclusion: Licofelone inhibited COX-2 and 5-LOX simultaneously and prevented overall cancer cell growth by enhancing apoptosis in both androgen-dependent and androgen-independent prostate cancer cells. Validating the dual role of licofelone in animal models of prostate cancer is critical for promoting its use as a potential chemopreventive or therapeutic agent.

Prostate cancer is one of the most common malignancies among men, and is the second leading cause of cancer death in the United States (1, 2). African-American men have far higher rates of prostate cancer incidence in the U.S. compared to their white counterparts (3). Most importantly, epidemiological reports have shown a decreased risk of prostate cancer among men who are regularly taking aspirin or other nonsteroidal anti-inflammatory drugs (NSAIDs) (4-7). Despite the potential benefits, side-effects due to the use of higher doses of cyclooxygenase-2 (COX-2) inhibitors are a major concern (8-9). A growing number of studies have indicated that the metabolism of arachidonic acid (AA) either by the COX or the lipoxygenase (LOX) pathways generates eicosanoids involved in the tumor promotion, progression and metastasis of prostate cancer (10, 11). It has been pointed out that inhibiting only one of these COX/LOX biosynthetic pathways cannot disrupt the metabolism of AA involved in human carcinogenesis (12). Increasing interest in the simultaneous blocking of the COX/LOX pathways by interfering with the production of both prostaglandins and the biosynthesis of leukotrienes (LTs) is emerging as a promising approach for cancer chemoprevention and treatment.

Licofelone ([2,2-dimethyl-6-(4-chlorophenyl)-7-phenyl-2,3-dihydro-1H-pyrrolizine-5-yl]-acetic acid), previously named as ML3000, (Figure 1) has been demonstrated to inhibit COX-2 and 5-LOX. This dual COX/LOX inhibitor has been shown to be an effective anti-inflammatory agent against carrageenan-arachidonic acid- and bradykinin-induced paws edema and bradykinin-induced mechanical hyperalgesia (13-17). Licofelone is currently under evaluation as a treatment for osteoarthritis (OA), the most common form of arthritis (OA), the most common form of arthritis, and has been shown to be effective when administered orally and well tolerated on single or repeated administration (15). The pharmacological profile of licofelone against arthritis has been well characterized based on the COX-1, COX-2 and 5-LOX enzyme activities (13, 18). Licofelone exhibits anti-inflammatory and analgesic activity comparable to that of conventional NSAIDs and has also shown an improved gastrointestinal safety profile (15, 19, 20). Based on these earlier findings, it was decided to test its effect against prostate cancer involving AA metabolism. To determine the chemopreventive efficacy of licofelone, cell viability was determined after the application of various concentrations...
All the experiments were repeated three times for confirmation. The effects of licofelone on human prostate cancer cells and cells derived from a transgenic adenocarcinoma of the mouse prostate (TRAMP). Its effect on COX-2, 5-LOX and VEGF (vascular endothelial growth factor) expression, both at the mRNA and protein level, and on other proinflammatory genes at the transcription level was examined.

Materials and Methods

Cell culture and treatments. The cancer cell types used in this study included human prostate cancer cells (PC-3) obtained from the American Type Culture Collection (Manassas, VA, USA) and human benign prostatic hyperplasia (BPH-1) cells, a gift from Hayward et al. (21). These cells were grown in RPMI (Gibco, CA, USA) with 5% fetal bovine serum (FBS) in standard cell culture conditions (22). The TRAMP-derived mouse prostate cancer cell line TR-75 used in the present study was established at Dr. Bhagavathi Narayan’s laboratory for ongoing studies (23). These cells were grown in DMEM (F12) high glucose W/L-glutamine media supplemented with 5% Nu-Serum IV, a growth medium supplement from BD Biosciences (San Jose, CA, USA) and 5% FBS, 5 μg/ml insulin, 25 units/ml of penicillin-streptomycin and 10^{-8} M dihydrotestosterone (23, 24). A uniform number of cells plated in T-25 and/or 35-mm cell-culture dishes were placed in a humidified incubator at 37°C with 5% CO_{2} before treatment. All the cells that reached 75% confluence were used for various treatments.

Dr. Mukundan Attur and Dr. Steven B. Abramson at the Hospital for Joint Diseases, New York University School of Medicine, provided the compound licofelone (ML-3000) for this study. The range of licofelone concentrations selected for this study was based on various previously reported doses used in several cell culture studies, namely human umbilical vein endothelial cells (14), rat basophilic leukemia cells (25), human osteoarthritic chondrocytes (26) and gastric parietal cells (27). The licofelone was dissolved in DMSO and added to the cell culture media to arrive at the final concentrations and incubated with the cells for various lengths of time. Profiling the pharmacologically effective dose of licofelone on COX-2 and 5-LOX enzyme activity was not an objective of this study, since the main focus was to demonstrate the anticancer effect of licofelone and its ability to modulate genes and proteins associated with prostate cancer cell growth. Controls received 1% DMSO only. All the experiments were repeated three times for confirmation.

Cell viability determination. Cell viability after licofelone treatment was determined by the trypan blue (0.4%) exclusion assay (22). After 6, 12, 24, and 48 h exposure to 2.5, 5, 10, 20, 30, 40 or 50 μM licofelone, adherent and floating cells were harvested by trypsinization and recovered by centrifugation. Trypan blue staining of the cells enabled easy identification of dead cells because they took up the dye and appeared blue with uneven cell membranes. By contrast, the living cells repelled the dye and appeared retractile and colorless. The viable cells from three parallel sets of experiments were counted to determine the % of cell survival, and to compare the rate of cell growth inhibition between the control (DMSO) and licofelone treatment.

Detection of apoptosis by DAPI and annexinV staining. The rate of apoptosis induced by licofelone in prostate cancer cells was assessed first with DAPI staining of the nuclear material. Briefly, cells grown in 35-mm dishes were treated with licofelone 10 μM (IC_{50}) (determined by a dose-response study) for 48 h, and then the floating and adherent cells were fixed in 10% formalin for 15 min. After washing with phosphate-buffered saline (PBS), the cells were treated with 0.1% Triton X-100, 4 M HCl, and sodium tetraborate. Each treatment was performed for 15 min and was followed by a PBS wash. The cells were then stained with DAPI in 80% methanol for 30 min and again washed with PBS, and were viewed under a fluorescence microscope as described previously by Narayan et al. (22). In order to detect early apoptotic cells induced by licofelone, parallel experiments with annexin V staining of the membrane for PS (phosphatidylserine externalization) were performed in the control and licofelone-treated cells. The cells showing positive staining for annexin V with the characteristic morphological changes of apoptosis were detected using high power microscopy and quantified using 100 cells per field for 10 fields.

Immunohistochemical detection of COX-2 and 5-LOX in TRAMP tissues. Paraffin-embedded, 5-μm thickness dorsolateral (DL) prostate tissue sections of TRAMP mice were used for all the immunohistochemical analyses. Following rehydration, the antigens were retrieved by a process which involved microwaving with antigen-unmasking fluid (Vector Laboratories CA, USA) twice for 5 min with a 3-min interval. After 15 min at room temperature the sections were washed and blocked with 10% normal horse serum. The sections were then incubated for 1 h with primary mouse anti-COX-2 (Cayman, Ann Arbor, MI, USA), and anti-5-LOX (Biomole, Philadelphia, PA, USA) antibodies at room temperature. The overall expression levels of COX-2 and 5-LOX were detected using a universal labeling kit (Hrp/DAB) from Ventana Medical Systems (Tucson, AZ, USA). The Image Pro software program (Media Cybernetics, Silver Spring, MD, USA) was used to quantify the total number of positively stained cells in a minimum of using 100 cells per field for 10 fields at x40 magnification (28).

Immunofluorescence detection of COX-2 and 5-LOX in TR-75 cells of TRAMP. In order to determine the effect of licofelone on COX-2 and 5-LOX expression, immunofluorescence detection procedures were used with TR-75 cells treated with 10 μM licofelone. Briefly, cells grown in 35 mm dishes treated with licofelone for 48 h were washed in PBS, fixed in 10% formalin, and pretreated with 0.1% Triton X-100 for 15 min each. After blocking with 1% bovine serum albumin (BSA) for an hour,
COX-1, COX-2 and 5-LOX expression was detected after incubating the cells with the specific antibody conjugated to FITC (Cayman, Ann Arbor MI, USA) for 1 h at room temperature. Green fluorescence signaling of COX-1, COX-2 and 5-LOX expression with reference to DNA staining with DAPI was viewed under an Olympus AX-70 epifluorescence microscope (Olympus America Inc., Center Valley, PA, USA) as described previously (29). Quantification of the green fluorescence was used to measure the level of expression.

RNA isolation and quantitative real-time PCR analysis. A two-step real-time reverse transcription-polymerase chain reaction (RT-PCR) was carried out using the total RNA extracted from the TR-75 cells as described previously (23). Briefly, 5 µg of the total RNA extracted from cells treated with 10 µM licofelone for 24 h were subjected to real-time RT-PCR analysis using gene-specific primer sequences for COX-1, COX-2, prostaglandin E receptor 2 (PTGER2), 5-LOX, 12-LOX, VEGF, NF-κBp65, TNFα, with amplification of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) used as the internal control. All of the templates were initially denatured for 2 min at 94°C, and the amplification was extended at a final temperature of 72°C for 7 min. Real-time RT-PCR analysis using a PCR reaction mix containing Syber green intercalating dye (Bio-Rad Laboratories, Hercules, CA, USA) was carried out to evaluate the mRNA expression. PCR amplification was performed using Cepheid Smart Cycler II (Cepheid, Sunnyvale, CA, USA).

Western blot analysis. Prostate cancer cells TR-75 and PC-3 treated with 10 µM licofelone for 48 h were harvested by trypsinization. The total protein was isolated with protein extraction buffer containing 150 mM NaCl, 10 mM Tris (pH 7.2), 5 mM EDTA, 0.1% Triton X-100, 5% glycerol and 2% sodium dodecyl sulphate (SDS), in addition to a mixture of protease inhibitors (Boehringer Mannheim, GmbH, Mannheim, Germany). Equal amounts of protein (50 µg/lane) were fractionated on 10% SDS-polyacrylamide gel electrophoresis (PAGE) gels and transferred to PVDF (polyvinylidene difluoride) membranes. The Western blot procedure was carried out as described previously (29). The 5-LOX and VEGF antibodies were purchased from Biomole (Philadelphia, PA, USA) and Santa Cruz Biotechnology, (Santa Cruz, CA, USA), respectively. The antibodies for COX-1 and COX-2 were purchased from Cayman Chemicals (USA). The reactive protein bands developed were detected using chemiluminescence reagents (ECL from GE Healthcare Amersham Bio-Sciences, Piscataway, NJ, USA). Densitometric analysis for quantification of the protein bands was performed with the software Gel-Pro Analyzer (Media Cybernetics).

Statistical analysis. The significant differences in the cellular effects induced by licofelone including cell growth inhibition, apoptosis, and expression of COX-2 and 5-LOX between control and licofelone-treated cells were compared using one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparisons procedure (30).
Results

Licofelone inhibits prostate cancer cell growth. Although cell viability assays were performed to determine the effect of licofelone against prostate cancer cell growth for various time-points, the results are presented only for 48 h. Phase-contrast microscopic observations revealed a dose-dependent effect on growth inhibition in the TR-75 cells with an IC50 of 10 μM (Figures 2 and 3). Significant growth inhibition was observed at 5, 10 and 20 μM concentrations (p<0.01). However, at higher concentrations of 30, 40 and 50 μM, fewer adherent, but more floating cells were observed. In addition, PC-3 and BPH-1 cells also revealed a similar trend in cell growth inhibition with licofelone treatment (Figure 3).

Licofelone induces apoptotic cell death. In order to confirm whether licofelone-induced cell growth inhibition was mediated through apoptosis, the rate of apoptosis was measured by annexin V staining, which determines early apoptotic cells characterized by phosphatidylserine externalization (PS). All the three prostate cancer cells (TR-75, PC-3 and BPH cells) treated with 10 μM licofelone for 48 h showed a higher number of early apoptotic cells compared to that of the control. A semi-quantification of apoptotic cells revealed a significant increase in the rate of apoptosis in all three cell types (p<0.001) and the results are presented in Figure 4.

TRAMP tissues express COX-2 and 5-LOX. COX-2 and 5-LOX expression in TRAMP tissues was confirmed by immunohistochemical detection using specific antibodies. As shown in Figure 5, a higher expression of both COX-2
Figure 5. COX-2 and 5-LOX expression in TRAMP tissues. A, H&E staining of paraffin-embedded prostate tissue sections indicating the cellular changes, associated with PIN and adenocarcinoma stages of TRAMP (A1, A2). The immunohistochemical detection shows the COX-2 (A3, A4) and 5-LOX (A5, A6) expression in TRAMP tissues. B, The chart illustrates the quantification of the mean number of positively stained cells per field for COX-2 and 5-LOX expression in the PIN and adenocarcinoma of the TRAMP at x40 magnification.
and 5-LOX was evident in the TRAMP tissue than in the PIN, suggesting that the cells derived from TRAMP tumor were suitable for testing the effect of licofelone against prostate cancer. Haematoxylin and eosin-stained prostate tissue sections indicated the cellular changes associated with the PIN and adenocarcinoma stages of the mouse prostate (Figure 5).

Licofelone inhibits COX-2 and 5-LOX in TRAMP cells. Immunofluorescence detection indicated that cells exposed to 10 μM licofelone for 48 h significantly (*p* < 0.001) reduced COX-2 and 5-LOX expression (Figure 6) compared to that in the control cells. Our results indicated that there is no significant effect on COX-1 expression in the TR-75 cells with licofelone treatment.

Licofelone alters the transcription levels of proinflammatory genes. In order to determine the impact of licofelone on key pro-inflammatory gene targets at the transcription level, a two-step real-time RT-PCR was carried out with total RNA extracted from the TR-75 cells. RT-PCR was performed as described earlier (23). As shown in Figure 7, licofelone-treated TR-75 cells showed down-regulation of COX-2, PTGER2, 5-LOX, 12-LOX, NF-κBp65 and VEGF, while indicating a small change in the expression levels of GAPDH and COX-1 compared to the level in the untreated control. These preliminary findings suggest a definite anti-inflammatory effect of licofelone against prostate cancer. However, the lack of impact on TNFα may be indicative of yet another mode of action.

Discussion

Chemopreventive agents of both natural and pharmaceutical sources are known to play a critical role in prostate cancer prevention (31-36). In this study licofelone clearly demonstrated anti-inflammatory activity by blocking the 5-LOX and COX-2 expression and inhibited human and mouse prostate cancer cell growth in

![Figure 6](image_url). Cellular localization of COX-2 and 5-LOX in TR-75 cells. The bar graph represents the percentage of immunofluorescent cells for COX-1, COX-2 and 5-LOX in TR-75 cells exposed to licofelone (10 μM) for 48 h. DAPI was used for the DNA staining as control. FITC conjugated COX-2 and 5-LOX mouse monoclonal antibodies were used to detect the levels of protein expression in the cytoplasm and/or in the nucleus.
a dose-dependent manner. By using both androgen-dependent TR-75 and androgen-independent PC-3 prostate cancer cells, overall cancer cell growth inhibition induced by licofelone has been demonstrated for the first time. These findings indicate its potential role in preventing early and metastatic prostate cancer, whether it is driven by hormone dependent or independent factors. Most importantly, our findings suggest that the effect of licofelone could be mediated by enhancing the apoptosis mechanism and abrogating the antiapoptotic influence of COX-2 and 5-LOX, but sparing the COX-1 expression. Further analysis of down-regulated pro-inflammatory genes including COX-2 and 5-LOX along with other mediators of inflammation in TR-75 cells, clearly showed the effect of licofelone as an anti-inflammatory agent against prostate cancer.

Figure 7. RT-PCR. A two-step real-time RT-PCR was carried out with 5 µg of total RNA extracted from TR-75 cells treated with 10 µM of licofelone for 24 h, as described in the Materials and Methods section. Real-time RT-PCR amplification of COX-1, COX-2, prostaglandin E receptor 2 (PTGER2), 5-LOX, 12-LOX, VEGF, TNFα and NF-κBp65 were determined using gene-specific primers. Amplification of GAPDH was used as the internal control. Real-time PCR amplification of the gene products were indicated by the Syber green signal intensity, as described by Narayanan et al. (21). Note: The number of PCR cycles is inversely related to the amount of mRNA.
The debate on whether selective inhibition of COX-2 with or without concomitant inhibition of COX-1 or whether selective inhibition of the lipoxygenase pathway could affect the reproductive tissues is still inconclusive. The role of COX/LOX inhibitors, such as licofelone, present major advantages when compared to the selective COX-2 inhibitors in that they act on two major pathways, moreover associated with AA metabolism; the dual inhibitors are reported to have no gastric toxicity (37, 38). Most importantly, however, all in vivo data must be taken into account to understand the comparative efficacy of individual agents and safety as indicated by Clark (39) before choosing dual inhibitors for clinical use. Although significant progress has been made in understanding the role of bioactive lipids in cancer prevention, many questions remain unanswered. Ensuring an appropriate risk-to-benefit ratio remains a significant challenge for the development of viable chemopreventives targeting inflammatory pathways. As the uncertainty over cardiovascular complications with selective COX-2 inhibitors indicates the need for alternative agents, validating the dual role of licofelone in animal models of prostate cancer is very critical for promoting its use as a potential chemopreventive or therapeutic agent for

Figure 8. Western blot analysis. COX-2 and 5-LOX protein expressions were analyzed using specific antibody as described in the Materials and Methods section. A, TR-75 and B, PC-3 cells showing reduced COX-2 and 5-LOX protein expression and bar graph representing the quantification of the respective protein expression levels.
prostate cancer. Most of the present data on the effect of licofelone on several of the molecular targets has been investigated using TR-75 cells because of our future interest in testing the efficacy of licofelone in the TRAMP model assay for prostate cancer.

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References


