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Original Article

7-Farnesyloxycoumarin Exerts Anti-cancer Effects on a Prostate Cancer Cell Line by 15-LOX-1 Inhibition

Saffiyeh Saboormaleki, MSc[1]; Hamid Sadeghian, PhD[2]; Ahmad Reza Bahrami, PhD[1,3]; Ala Orafaie, MSc[1]; Maryam M. Matin, PhD[1,3*]

[1]Department of Biology, Faculty of Science, Ferdowsi University of Mashhad, Mashhad, Iran
[2]Department of Laboratory Sciences, School of Paramedical Sciences, Mashhad University of Medical Sciences, Mashhad, Iran
[3]Novel Diagnostics and Therapeutics Research Group, Institute of Biotechnology, Ferdowsi University of Mashhad, Mashhad, Iran

Abstract

Background: Prostate cancer is one of the leading causes of cancer related deaths in males worldwide. Overexpression of 15-lipoxygenase-1 (15-LOX-1) enzyme and high activity of its metabolic pathway is reported to be a driver for prostate cancer malignancy. Farnesyloxycoumarin derivatives (3f, 4f and 7f) inhibit lipoxygenase enzyme. We hypothesized that farnesyloxycoumarins may exert an anti-cancer effect on prostate cancer cells due to their 15-LOX-1 inhibitory potential.

Methods: The enzyme inhibitory activity of 3f, 4f and 7f was initially evaluated on PC-3 and DU145 prostate cancer cell lines. MTT assay was performed on cancer cell lines and HFF3 cell line to assess cytotoxicity of the compounds. The apoptotic morphology of cells after treatments was assessed by DAPI staining and single cell gel electrophoresis. Propidium iodide staining was also performed to detect cell cycle variations after treatment.

Results: 7f inhibited 15-LOX-1 at IC_{50}=4.3 µg/mL, while 3f and 4f did not show high inhibitory activity. 7f reduced cell viability in PC-3 cells at IC_{50}=22-31 µg/mL, however, no significant cytotoxicity was revealed on normal cells. DAPI staining and comet assay confirmed apoptosis and DNA damage in PC-3 cells after 7f treatment, while flow cytometry results revealed G1 arrest in PC-3 cells.

Conclusion: The results are indicative of a distinctive cytotoxic mechanism for 7f compared to other coumarins, possibly due to its 15-LOX-1 inhibitory potential. Thus, this compound is valued for further assessments with the aim of developing a promising targeted therapy for prostate cancer patients.

Keywords:15-LOX-1 enzyme, Farnesyloxycoumarin derivatives, Prostate cancer, Umbelliprenin

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Introduction

Prostate cancer is the most common cancer diagnosed in males and the second leading cause of cancer-related deaths in males worldwide.[1] Various approaches are employed to eradicate this disease including radical prostatectomy, hormone therapy, radiotherapy and chemotherapy. Limitations and drawbacks in these methods have led to a crucial need for targeted therapies.[2,3]

It has been suggested that excessive use of animal fats and vegetable oils is a risk factor for prostate cancer.[4,5] Arachidonic acid and its precursor, linoleic acid, are the main fatty acids present in animal and vegetable fats which can be metabolized by lipoxygenase (LOX), cyclooxygenase (COX) and P450 epoxygenase (P450 EOX) pathways.[6] Several studies have reported the association of the LOX pathway with some cancers especially prostate cancer.[7–14] 15-lipoxygenase-1 (15-LOX-1) is a lipid peroxidizing enzyme, active in the LOX pathway.[15] This enzyme metabolizes linoleic acid, as the main substrate, to 13-hydroxyoctadecadienoic acid (13-(S)HODE).[16] A high expression of 15-LOX-1 enzyme and high degrees of its metabolite, 13-(S)HODE, have been reported in prostate cancer tissues and cells.[17] This correlates with prostate cancer invasiveness.[18]

The LOX pathway is also active in plants.[19–21] It has been reported that farnesyloxycoumarin derivatives (3-farnesyloxycoumarin (3f), 4-farnesyloxycoumarin (4f) and 7-farnesyloxycoumarin (7f)), which are synthesized prenylated coumarins, can inhibit soybean LOX.[22] Among these derivatives, 7f (umbelliprenin) is a natural coumarin compound present in various *Ferula* species.[23,24] This compound has diverse biological properties including anti-bacterial and anti-fungal activities.[23–25] Its anti-inflammatory and immunomodulatory properties have been recently shown in immune cells.[26] Its anti-proliferative, anti-tumor[27] and apoptosis inducing activities[28,29] have also been reported in some cancerous cell lines. The aim of this study was to investigate the inhibitory activity of 3f, 4f and 7f on 15-LOX-1 in prostate cancer cells, and subsequently to determine their cytotoxicity and anti-cancer effects *in vitro*.

Our results indicated that among these three

***Corresponding Author:** Maryam M. Matin, PhD; Department of Biology and Institute of Biotechnology, Ferdowsi University of Mashhad, Park Square, Mashhad, Iran. Tel: +98-51-38805514, Fax: +98-51-38796416, Email: matin@um.ac.ir.

compounds, 7f was most effective at inhibiting 15-LOX-1 enzyme in PC-3 prostate cancer cell line, and selectively reduced the viability of cells in a dose-dependent manner. DNA damage assays and cell cycle analysis also revealed that PC-3 cells displayed apoptotic properties after 7f treatment, and were arrested at G1 phase of the cell cycle. Although 7f had similar effects to cisplatin, which is a commonly used drug for cancer therapy, it did not influence the viability of normal HFF3 cells. These results indicate that 7f could be considered as a potential anti-cancer agent in future *in vitro* and *in vivo* studies.

Materials and Methods

Cell Culture

Prostate cancer cells (PC-3 and DU145) were purchased from Pasteur Institute (Tehran, Iran) and cultured in Roswell Park Memorial Institute medium (RPMI-1640) (Gibco, Scotland), supplemented with 10% fetal bovine serum (FBS, Gibco, Scotland).[30] Human Foreskin Fibroblast (HFF3) normal cells were obtained as a generous gift from Royan Institute (Tehran, Iran) and cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Scotland) containing 15% FBS.[31] Cells were incubated at 37°C and 5% CO_2 in a humidified atmosphere, and sub-cultured using trypsin-ethylenediaminetetraacetic acid (EDTA) (1X) (Gibco, Scotland) when required.

Enzyme Activity Assessment

PC-3 and DU145 cell lysates were prepared by suspension of cells in Tris buffer (1.5 mL; 0.1 M, pH=7.2) and sonication (20 kHz, 3 minutes) (Bandelin-Sonopuls, Germany).[32] The LOX activity was assessed using kinetic method, according to the literatures.[33,34] The slope of absorbance increasing at 235 nm after addition of linoleic acid (final concentration: 100 μM) to the mixture of the cell lysate (100 μL), Tris buffer (1900 μL; 0.1 M, pH=7.2) and inhibitors (3f, 4f and 7f; final concentrations 1-20 μg/mL) during 3 minutes, was defined as LOX activity. The inhibitory potential of compounds was reported by IC_{50} values calculated from sigmoidal dose response curves.

Umbelliprenin Synthesis

Farnesyloxycoumarin derivatives were synthesized as previously described.[22] Briefly, a mixture of 7-farnesyloxycoumarin (5 mmol), farnesyl bromide (6 mmol) and anhydrous potassium carbonate (0.70 g; 5 mmol) in dry acetone (3 mL) was refluxed for 12 hours and then cooled. The mixture was diluted with water (10 mL) and then extracted with ether (2–20 mL). The combined extracts were washed with 10% NaOH (2–10 mL) and dried with anhydrous sodium carbonate. After removal of the solvent the products were purified by crystallization from methanol.

Preparation of Compounds

Farnesyloxycoumarin derivatives (3f, 4f and 7f) were dissolved in DMSO (dimethyl sulfoxide). 15-LOX-1 inhibitor, 4-MMPB (4-methyl-2-(4-methylpiperazinyl) pyrimido[4,5-b]benzothiazine) was purchased from Cayman Chemicals (Estonia) and dissolved in HCl. Cisplatin was obtained from Sigma-Aldrich (Germany). Serial dilutions were prepared to obtain the applied concentrations.

MTT Assay

MTT assay was based on the protocol described for the first time by Mosmann,[35] and was optimized for cell lines used in this study. Briefly, 24, 48 and 72 hours after incubation of DU145, PC-3 and HFF3 cells with various concentrations (3, 6.25, 12.5, 25 and 50 μg/mL) of different compounds (3f, 4f, 7f, 4-MMPB and cisplatin), cells were incubated for 4 hours with 5 mg/mL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich, Germany). The purple formazan crystals formed by live cells were dissolved in DMSO after removing the MTT solution. Absorbance of the DMSO solutions was then recorded at 545 nm using an enzyme-linked immunosorbent assay (ELISA)-reader (Awareness, USA). The half maximal inhibitory concentration values (IC_{50}) were evaluated via sigmoidal dose-response curves. All treatments were carried out in triplicate. Cisplatin and 4-MMPB (a selective 15-LOX-1 inhibitor[36]) were used as positive controls in this study.

Morphological Analysis

PC-3 and HFF3 morphological alterations were observed and photographed by means of an invert microscope (ZEISS Axiovert S100, Germany) after treatment with IC_{50} concentrations of the compounds and equivalent controls (0.25% DMSO and 0.25% HCl).

DAPI Staining

Seventy-two hours after treating PC-3 cells with IC_{50} concentrations of 7f, 4-MMPB and controls, cells were fixed with paraformaldehyde (PFA; Sigma-Aldrich, Germany) incubated with triton-X-100 (8 min, Merck, Germany) and stained with DAPI (4',6-diamidino-2-phenylindole) (Sigma-Aldrich, Germany).[37] Cells were then observed under a fluorescent microscope (Olympus, Japan). 600 to 700 cells were photographed per treatment group and the percentage of cells with chromatin condensation was evaluated. Each treatment was performed in triplicate.

Single Cell Gel Electrophoresis

Seventy-two hours after treatment of PC-3 and HFF3 cells with IC_{50} concentrations of 7f, 4-MMPB and controls, treated and untreated cells were subjected to alkaline version of comet assay according to the literatures.[38–40] 300 to 350 cells were photographed per treatment group and analyzed using Tri-Tek Comet Score 1.5 software. The treatments were repeated three times, and the percentage of DNA damage in each treatment was evaluated.

Cell Cycle Analysis

In order to investigate variations in PC-3 cell cycle, DNA contents were assessed by staining the cells with propidium iodide (PI) (Sigma-Aldrich, Germany),[41] 72 hours after treatments (39 µg/mL 7f and 0.25% DMSO control). Cells were then monitored by FACSCalibur (Becton Dickinson, USA) and the percentages of cells in the G1, S and G2/M phases of the cell cycle were determined using WinMDI 2.9 software.

Statistical Analysis

Statistical analysis was applied using one-way analysis of variance (ANOVA) (Tukey's multiple comparison test) in GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego CA). *P*-value of <0.001 was considered statistically significant. 15-LOX-1 inhibitory activity, cell viability, chromatin condensation and DNA damage were presented graphically in the form of histograms using GraphPad Prism version 4.00 for Windows.

Results

In order to assess the inhibitory effects of 3f, 4f and 7f on 15-LOX-1 activity in prostate cancer, DU145 and PC-3 cell lines were studied for their enzyme activity, and PC-3 cells with 20-fold higher 15-LOX-1 activity were chosen for further experiments. 3f, 4f and 7f inhibitory effects were examined on PC-3 cell lysate. In the range of concentrations used in this study (1–20 µg/mL), no IC_{50} values could be obtained for 3f and 4f (data not shown). On the other hand, IC_{50} value for 7f as evaluated from sigmoidal dose-response curve, was calculated as 4.3 µg/mL (11.8 µM) (Figure 1).

The cytotoxic effects of various concentrations (3, 6.25, 12.5, 25 and 50 µg/mL) of compounds were assessed on PC-3 and HFF3 cells after 24, 48 and 72 hours, using MTT assay. As shown by MTT assay, 3f did not exhibit any significant cytotoxic effects on PC-3 or HFF3 cell lines and no IC_{50} values were obtained (data not shown). On the other hand, 4f had a dose-dependent reduction in cell viability in both cancerous and normal cell lines. The IC_{50} concentrations of 4f on PC-3 cells were 32, 30 and 22 µg/mL, while IC_{50} concentrations on HFF3 cell line were 22, 16 and 18 µg/mL at 24, 48 and 72 hours after treatments, respectively (Figure 2, a and b). Furthermore, 7f caused a dose-dependent decrease in PC-3 cell viability with IC_{50} values of about 37, 35 and 39 µg/mL (101, 95, 106 µM) after 24, 48 and 72 hours of treatments, respectively. However, 7f did not have any significant cytotoxic effects on HFF3 normal cells (Figure 2, c and d). The effects of 7f on PC-3 cells were similar to cisplatin as assessed by MTT assay (data not shown). The 4-MMPB IC_{50} values obtained for PC-3 cell line were 25, 16 and 13 µg/mL, and for HFF3 cells were calculated as 80, 41 and 31 µg/mL after 24, 48 and 72 hours, respectively (Figure 2, e and f).

Morphological assessment of PC-3 cells treated with IC_{50} concentrations of 7f revealed significant cell death and cytoplasmic granulation, whereas no morphological changes were observed in HFF3 cells treated with the same concentrations of 7f (Figure 3).

DAPI staining revealed apoptotic properties as indicated by condensed chromatin and/or fragmented nuclei in 7f and 4-MMPB treated cells, and the percentages of chromatin condensation formed in these cells had a significant difference with the control groups ($P < 0.001$) (Figure 4). Comet assay indicated large amounts of DNA strand breaks generated after incubation of PC-3 cells with either compound, however, treating HFF3 cells with 7f induced insignificant DNA strand breaks, as analyzed by the standard comet assay. Comet moments formed in PC-3 cells treated with 7f and 4-MMPB (40.61% and 43.40% DNA in tail, respectively) had a significant difference compared to control samples and HFF3 cells ($P < 0.001$) (Figure 5).

In order to investigate changes in PC-3 cell cycle after treatments (39 µg/mL 7f and 0.25% DMSO) flow cytometry was performed after PI staining. As can be observed in Figure 6, the results were indicative of G1

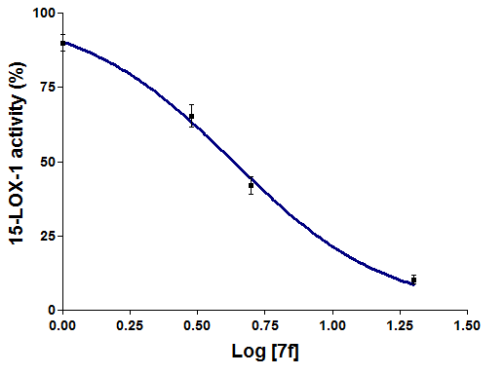

Figure 1. Dose-Response Curve of 15-LOX-1 Inhibition by 7f in PC-3 Cells. Enzyme activity was assessed after affecting a 7f concentration range of 1-20 µg/mL on cell lysate. The IC_{50} value of 7f was calculated as 4.3 µg/mL by Prism 4.0 software.

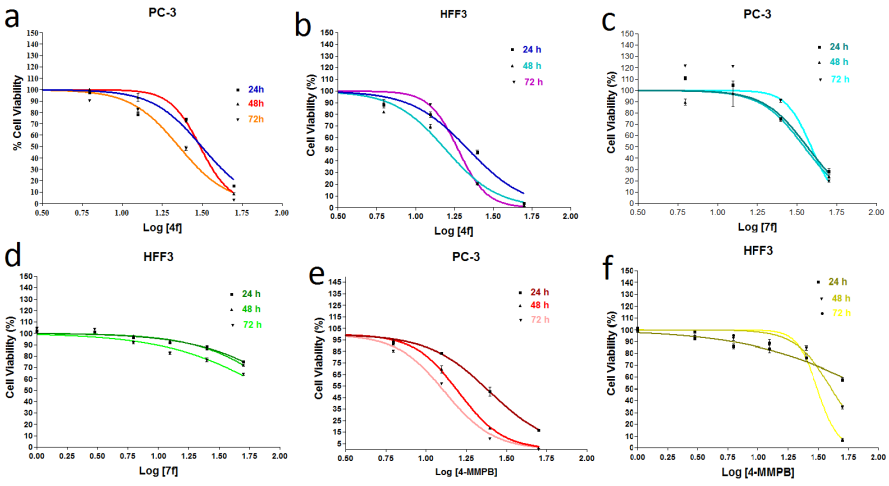

Figure 2. Cancerous and Normal Cell Dose-Response Curves for Each of Farnesyloxycoumarin Agents After MTT Assay. Sigmoidal graphs exhibit 4f (**a and b**), 7f (**c and d**) and 4-MMPB (**e and f**) effects on PC-3 and HFF3 cell lines, respectively, 24, 48 and 72 h after treatments. IC_{50} values were obtained by means of Prism 4.0 software. Results are expressed as mean ± SD for IC_{50} values obtained from three to five repeats for each individual compound.

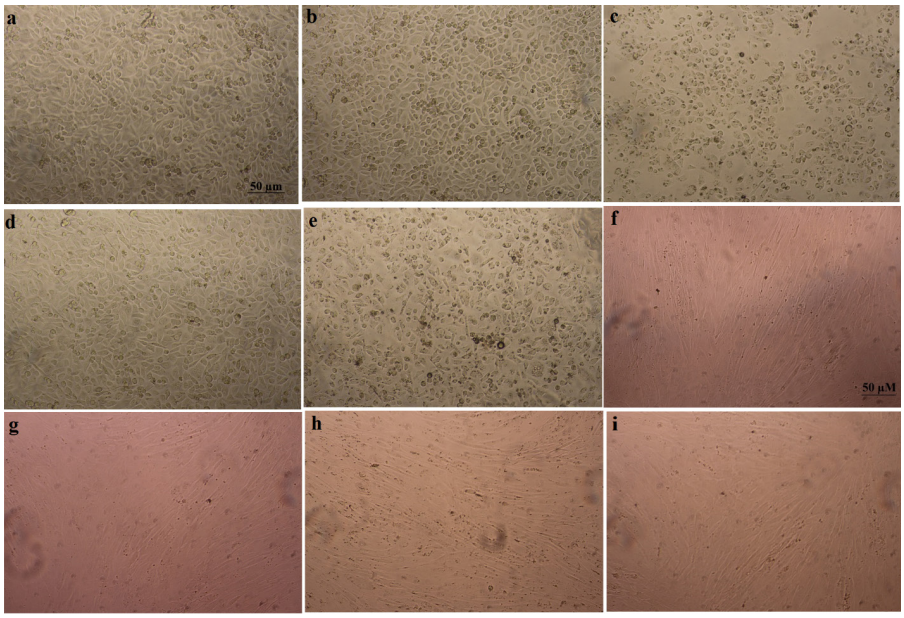

Figure 3. Representative Photomicrographs Showing the Morphological Changes of PC-3 and HFF3 Cells in Response to Various Treatments. PC-3 (**a-e**) and HFF3 (**f-i**) cells were photographed 72 h after treatments. Untreated cells (**a and f**), cells treated with 0.25% DMSO (**b and g**), cells treated with 39 µg/mL 7f (**c and h**), cells treated with 0.25% HCl (**d**), cells treated with 13 µg/mL 4-MMPB (**e**) and 31 µg/mL 4-MMPB (**i**) are indicated.

arrest in cell cycle after 7f treatment.

Discussion

Several studies have shown a correlation between the LOX pathways and various cancers especially prostate cancer.[8,12,42–47] High degrees of 12/15-LOX mRNA is reported to have a positive correlation with invasion in prostate cancer cells.[8] Overexpression of 12/15-LOX in PC-3 cells generated more invasive tumors in mice.[12] Moreover, Timar et al reported high expression of 12/15-LOX in metastatic prostate cancer cell lines.[14] In this study, since enzyme assay revealed a 20-fold higher activity of 15-LOX-1 in PC-3 cells compared to DU145, further experiments were carried out on PC-3 cell line. Spindler et al have also reported a high expression of 15-LOX-1 in prostate cancer tissues and likewise in PC-3 and LNCap cell lines. Furthermore, they reported a higher degree of 13-(S)HODE (15-LOX-1 product) in PC-3 cells compared to LNCap cells.[17] Additionally, Kelavkar et al reported that the amount of 15-LOX-1 expression has a positive correlation with the invasiveness of prostate cancer cells.[18] Since the overexpression of LOX pathway

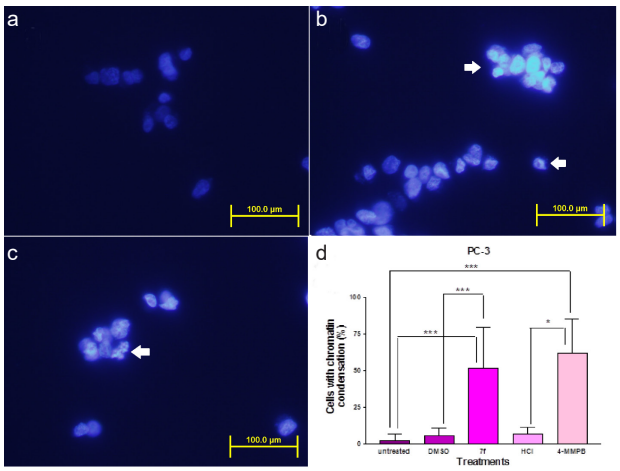

Figure 4. DAPI Staining of Nuclei Indicating Chromatin Condensation in PC-3 Cells. Figures indicate untreated PC-3 cells (**a**), cells treated with 39 µg/mL 7f (**b**), and cells treated with 13 µg/mL 4-MMPB (**c**). Chromatin condensation is indicated by arrows. **d**) Histogram of mean percentage of cells with chromatin condensation after each treatment. Data are shown as mean ± SD. Chromatin condensation percentages were compared using one-way ANOVA (Tukey's multiple comparison test) in Prism 4.0 software.

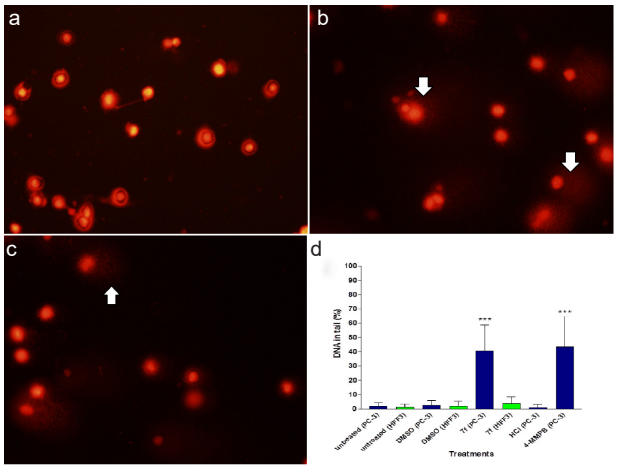

Figure 5. DNA Damage in PC-3 Cells as Revealed by Alkaline Comet Assay. Indicated are untreated cells (**a**), cells treated with 39 µg/mL 7f (**b**) and cells treated with 13 µg/mL 4-MMPB (**c**) (arrows indicate damaged DNA in comet tail). (**d**) Histogram of DNA damage in cells 72 h after treatments. Data are shown as mean ± SD and were compared using one way ANOVA (Tukey's multiple comparison test) in Prism 4.0 software.

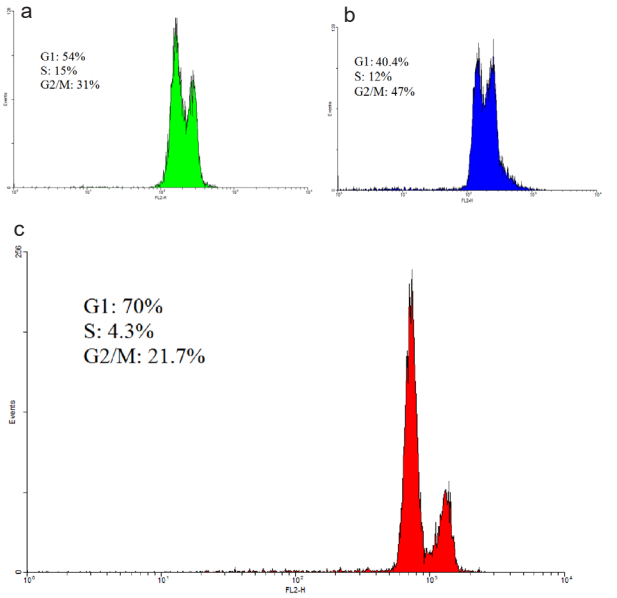

Figure 6. Cell Cycle Profile of PC-3 Cells After Treatment with Umbelliprenin. Percentage of cells in cell cycle phases are indicated after 72 h in untreated cells (**a**), cells treated with 0.25% DMSO (**b**) and cells treated with 39 µg/mL umbelliprenin (**c**). Note that the higher percentage of cells in G1 phase after umbelliprenin treatment indicates G1 arrest in cell cycle.

mediators results in invasiveness of cancer cells, some studies have focused on mediators' inhibition in prostate cancer. The inhibition of 5-LOX pathway in prostate cancer cell lines, caused cell growth inhibition in PC-3 and LNCap cell lines,[9,10] and also induced apoptosis in PC-3 cells.[7] In addition, 12-LOX inhibition in PC-3 and DU145 cells resulted in decreased proliferation, apoptosis and G0/G1 arrest in these cells.[13] *In vitro* and *in vivo* studies of 15-LOX-1 inhibition in PC-3 cell line have also revealed a decrease in cell proliferation.[48] 15-LOX-1 product, 13(S)HODE, is known to be a ligand for peroxisome proliferator-activated receptor gamma (PPARγ).[49,50] 13(S)HODE activates MAP-Kinase pathway in prostate cancer, which can reduce the activity of PPARγ[51,52] leading to PC-3 and DU145 proliferation.[53–55] 13(S) HODE can also increase IGF-1R expression, resulting in high proliferation and migration in prostate cancer.[56] These studies indicate that by inhibiting 15-LOX-1 enzyme, cell proliferation could be decreased. In this study, 15-LOX-1 inhibitory potency of three farnesyloxycoumarins (3f, 4f and 7f) was investigated in prostate cancer cells. Results indicated enzyme activity inhibition of 7f at IC_{50}=11.8 µM, exceeding 3f and 4f inhibitory activity. This method has also been used to inhibit soybean LOX activity with several synthetic prenylated coumarins.[22]

3f did not show any significant cytotoxic effects on either PC-3 or HFF3 cells whereas 4f exhibited cytotoxicity on both cancerous and normal cells. Unlike 3f and 4f, 7f had a selective cytotoxic effect on these cells, since no significant decrease in HFF3 cell proliferation was detected after 7f treatment as shown in the photomicrographs (Figure 3). 7f exhibited similar cytotoxic results on PC-3 cells as compared to cisplatin which was used as a positive control in this study. To note, cisplatin has been used as an anti-cancer agent and also as a positive control in several studies.[57–60] 5-farnesyloxycoumarin and 8-farnesyloxycoumarin have shown similar cytotoxic properties in prostate cancer cells.[61,62] Cytotoxic effects of 7f (umbelliprenin) have

been tested on various cell lines such as M4Beu (metastatic melanoma), QU-DB and A549 (lung cancer), Jurkat (leukaemia), SK-MEL-28 (melanoma) and CH1 (ovarian carcinoma), which in all cases indicated a cytotoxic and anti-tumor activity.[27–29,63] Umbelliprenin encapsulated by nanoliposome has also revealed high cytotoxicity on 4T1 (mouse breast cancer) cell line.[64]

7f and 4-MMPB-treated PC-3 cells had a high percentage of chromatin condensation as revealed by DAPI staining, showing a significant difference with controls ($P < 0.001$). To note, chromatin condensation and nuclear fragmentation have long been reported as the main characteristics of apoptosis.[65–67] DAPI staining has been used to detect apoptosis in other studies which apply anti-cancer agents such as cisplatin,[40, 68–72] etoposide,[69] vincristine,[73–77] vinblastine, doxorubicin[73] and paclitaxel.[78] DNA damage in cells was assessed by comet assay on PC-3 and HFF3 cell lines. PC-3 cells treated with 7f and 4-MMPB indicated high amounts of DNA damage compared to cells treated with control solvents and also normal HFF3 cells ($P < 0.001$), indicating a selective genotoxic effect of 7f on cancerous cells. Comet assay is a sensitive genotoxicity test[39] that has been used to detect DNA damage in cancer cells in many studies.[40,71,79–83] These data suggest that 7f can be assumed as an anti-cancer and apoptosis inducing compound.

The effects of 7f on cell cycle revealed G1 arrest in PC-3 cells 72 h after treatment. 7f affects gastric cancer cells in a similar manner.[84] Likewise, coumarin and its derivatives e.g. scopoletin, 7-hydroxycoumarin and decursin also cause G1 arrest in treated cells.[85–88] Studies have declared that cisplatin also exerts its cytotoxic effects via cell cycle arrest at G1 phase.[89,90] The reason for G1 arrest is still controversial, but studies have shown reduced *RAS* and *Myc* gene expression by use of coumarins.[91,92] G1 arrest is also suggested to be due to activation of P53 and on-going activation of WAF1/CIP1, a cyclin dependent kinase inhibitor.[93,94] In a study, the amounts of cyclin dependent kinase inhibitors such as CIP/P21 and KIP/P27 were elevated after treatment with decursin.[88] Other studies also claimed that treating HL-60 and A427 cells with 7-hydroxycoumarin and esculetin respectively, could result in downregulation of cyclin D1.[95,96] Chuang et al stated that coumarin could decrease the amounts of cyclin D1, Cdk2, P15 and Cdc25A whereas it could increase P21 and P53 proteins.[85] Oxypeucedanin also caused a decrease in Cdc25C, Cyclin A, Cyclin B1 and Cdc-2 in DU145 cells.[97] All these studies suggest that coumarin compounds exert their effects by activating P53 pathway. Similarly, a study has reported upregulation of P53 and p21/WAF1/CIP1 after treatment with cisplatin, and has claimed that cisplatin may have a P53 dependent mechanism,[90] thus 7f might have similar effects to cisplatin. Moreover, some studies claim that 7f induces G1 arrest through a caspase dependent manner[28] which is due to its prenyl group on the umbelliferone ring.[24,82] 7f is also reported as an MMP (matrix metalloproteinase) inhibitor,[98] which might be the reason for its anti-cancer effects.

In conclusion, our results indicated that 7-farnesyloxycoumarin inhibited 15-LOX-1 activity and exerted selective cytotoxic and anti-cancer effects on PC-3 cells. We further showed that 7f had similar cytotoxic effects on PC-3 cells as compared to cisplatin, which is extensively used in the clinic. Considering the fact that 4-MMPB (a selective 15-LOX-1 inhibitor) had similar effects on PC-3 cells, the anti-cancer effects of 7f might be due to its 15-LOX-1 inhibitory activity. Nevertheless, to understand the exact mechanism underlying this event, further studies are required. It is noteworthy that 7f could be extracted from various *Ferula* species,[23,24] making it a natural potential anti-cancer agent which is valued for future studies.

Authors' Contribution

SSM: Data collection, data analysis and drafting the manuscript. MMM: Study supervision and revision of final manuscript. HS and ARB: Supervision of the study. AO: Assistance in data collection.

Conflict of Interest Disclosures

The authors declare no conflict of interest.

Ethical Statement

This study was approved by the ethics committee at Ferdowsi University of Mashhad (FUM).

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