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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

5-farnesyloxycoumarin: a potent 15-LOX-1 inhibitor, prevents prostate cancer cell growth

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Abstract In this study, the activity of 15-LOX-1 was first evaluated in two prostate cancer cell lines PC3 and DU145. Considering the enzyme inhibitory potency of 5-farnesyloxycoumarin (5f), the cytotoxic effects of this compound was studied in PC3, DU145 and HFF3 (normal cell line) by MTT assay. Since 5f was more effective on PC3 cells, this cell line was used for further explorations. The chromatin condensation and DNA damage were studied by DAPI staining and comet assay, respectively and cell cycle analysis was performed by propidium iodide staining. Results indicated that 15-LOX-1 activity was very low in DU145 cells. Moreover, PC3 cells were more sensitive to 5f as compared to DU145 cancerous cells, while no significant effect was observed on normal HFF3 cells. Interestingly, the IC_{50} values of 5f on PC3 cells were similar to cisplatin. DAPI staining showed that 5f induced chromatin condensation in 63 % of PC3 cells. Comet assay also demonstrated 53 % "DNA in tail" for PC3 cells. Finally, 5f induced G_0/G_1 arrest in PC3 cell cycle. These findings suggest that cytotoxic effects of 5f might be due to the inhibition of 15-LOX-1 in PC3 cell line and, it can be introduced as a potent anticancer compound.

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Keywords 5-farnesyloxycoumarin · 15-LOX-1 · PC3 · DU145

Introduction

Tumorigenesis is a multi-factorial sequential process, which usually takes many years to progress. To date, the greatest challenge in cancer prevention and treatment still lies in identifying the multitude of complex and partially interconnected pathways critical to malignant cell survival, proliferation, tumor metastasis and neo-angiogenesis. Prostate cancer (PCa) is the most common form of malignancies and the second leading cause of cancer-related death in men in the United States (Ilic et al. 2013). Usually, PCa initially responds to androgen ablation therapy, however, androgen-independence almost always develops and eventually metastasizes to distant organs. The diagnosis and control of metastases after androgen deprivation therapy are major problems in treatment of PCa. Nearly no treatment will be effective against metastatic, androgen-independent, PCa. Thus, investigating the factors involved in development of PCa would be useful to improve the therapeutic approaches and early detection of metastasis in PCa patients (Meng et al. 2013).

Among the vast number of factors involved in tumor progression, arachidonic acid (AA) and its metabolites have recently generated a heightened interest due to growing evidence on their significant roles in cancer biology (Hyde and Missailidis 2009). AA is a polyunsaturated fatty acid that is present in the phospholipids (Burke and Dennis 2009). Free arachidonic acid can be metabolized by two key enzyme families: cyclooxygenases (COXs) and

lipoxygenases (LOXs). LOXs are a group of closely related non-heme iron-containing dioxygenases, which are classified depending on their site of oxygen insertion on AA into 5-LOXs, 8-LOXs, 12-LOXs and 15-LOXs (Yamamoto et al. 1999). Within the 15-LOX pathway, two isoforms have been identified (Brash et al. 1997), where 15-LOX-1 preferentially metabolizes linoleic acid into 13S-HODE, while 15-LOX-2 is mainly responsible for the production of 15S-HETE from 15S-HpETE (Murakami et al. 2005; Shappell et al. 2001). Studies have shown a functional role of LOXs and their metabolite derivatives in carcinogenesis (Comba and Pasqualini 2009). Opposing actions have been reported for 15-LOX-1 (Wang and Dubois 2010) and 15-LOX-2 (Schneider and Pozzi 2011) in carcinogenesis. However, the exact role of LOX isoforms in the pathology of human cancers remains unknown (Daurkin et al. 2011).

The interest in the function of 15-LOX-1 in PCa has increased during the past two decades. Spindler et al. (1997) first suggested a carcinogenic role for 15-LOX-1 metabolites by detecting high levels of 13-HODE, a linoleic acid derivative, in both human PCa specimens and PCa cell lines. Concomitantly, the presence of indeterminate 15-LOX was documented in these cell lines. When athymic nude mice were injected with PCa cell lines overexpressing 15-LOX-1, much larger prostatic tumors were generated as compared to those injected with PCa cell lines with normal expression of 15-LOX-1 (Kelavkar et al. 2001).

The inhibitors of AA cascade, have mainly been of interest in the treatment of inflammatory conditions. Eleftheriadis et al. (2015) have described structure activity relationships for 6-benzyloxysalicylates as inhibitors of human 15-LOX-1. In addition, inhibitory activity of a series of flavonoids has been studied on soybean 15-LOX-1 (Ribeiro et al. 2014). Moreover, a series of imidazo [2,1-*b*] thiazol-5-amines have been synthesized as 15-LOX inhibitors. Among these compounds, the 2,4,4-trimethylpentan-2-yl derivative 5i was the most active compound (Tehrani et al. 2014).

An increasing number of investigations support the role of 15-LOX-1 inhibitors in chemoprevention of cancers such as PCa, although the precise molecular mechanisms that link levels of AA, and its metabolites, with cancer progression still need to be elucidated (Hyde and Missailidis 2009).

Coumarins form an elite class of naturally occurring compounds that possess promising therapeutic perspectives. These belong to the flavonoid class of plant secondary metabolites and have a variety of biological activities, usually associated with low toxicity (Borges et al. 2005, 2008). Various coumarin-based natural and synthetic derivatives are found to have anticancer activities (Riveiro et al. 2008). Coumarins have been also used as inhibitors of LOX in the AA cascade (Bilgin et al. 2011; Napagoda et al. 2014).

Umbelliprenin (7-trans,trans-farnesyloxycoumarin), one of the farnesyloxycoumarin derivatives, has been examined for its in vitro antioxidant activity, in vitro inhibitory activity against soybean LOX, and in vivo anti-inflammatory activity. This compound exhibited a remarkable and potent inhibition against soybean 15-lipoxygenase (15-sLOX-1) (Iranshahi et al. 2009). We also showed the inhibiting effects of 5-farnesyloxycoumarin (5f) (Fig. 1) on 15-LOX-1activity in vitro (Iranshahi et al. 2012).

In present study, 15-LOX-1 activity of two prostate cancer cell lines PC3 and DU145 was first studied and the relationship between 15-LOX-1 inhibitory effect of 5-farnesyloxycoumarin (5f) and cell death was examined in these cell lines.

Materials and methods

Cell lines

Human PCa cell lines PC3 and DU145 were obtained from Pasteur Institute (Tehran, Iran) and human normal fibroblast HFF3 cells were a generous gift from Royan Institute (Tehran, Iran). The cell lines maintained in humidified atmosphere of 5 % CO_2 at 37 °C and cultured in Roswell Park Memorial Institute (RPMI) 1640 or Dulbecco's modified Eagle's medium (Gibco, Scotland) supplemented with 10 % fetal bovine serum (Gibco, Scotland) and 1 % penicillin–streptomycin.

Enzyme preparation

In order to study the 15-LOX-1 activity in two PCa cell lines, PC3 and DU145, phosphate buffered saline (PBS) suspended cells were centrifuged (1000 *g*, 8 min) and 1 mL of Tris buffer (0.1 M, pH 7.2) was added to sediments. The cells were sonicated (20 kHz, 2 min) for cell lysing and enzyme release.

Enzyme activity and inhibitory assessment

A linoleic acid solution (the substrate of 15-LOX-1 enzyme) was prepared by mixing 5 mg of linoleic acid (Sigma, Germany) in 3 mL water and then diluting with KOH

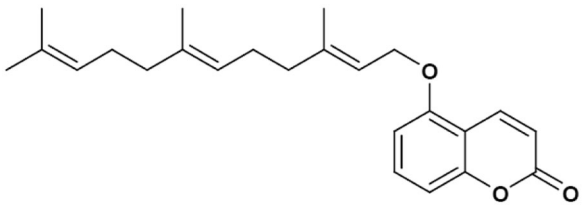

Fig. 1 The chemical structure of 5-farnesyloxycoumarin

(100 mM) to a final volume of 5 mL. To determine the LOX activity and 5f inhibitory potency, linoleic acid (45 µL) was added to mixture of lysed cells (100 µL), 5f (final concentrations: 2–20 µg/mL) and Tris buffer (1855 µL); the slopes of the absorbance increase were then recorded at 234 nm during 300 s.

Cytotoxicity test

5-farnesyloxycoumarin was prepared as described previously (Iranshahi et al. 2012). 4-methyl-2-(4-methylpiperazinyl) pyrimido [4,5-b] benzothiazine (4-MMPB) (the selective 15-LOX-1 inhibitor) and cisplatin were obtained from Caymann and Sigma, respectively. 5f was dissolved in dimethyl sulfoxide (DMSO) (Sigma, Germany) and stored at 4 °C until use, while 4-MMPB was dissolved in HCl (0.3 N). Cytotoxicity was determined by MTT assay according to the manufacturer's instructions. In this method the tetrazolium dye, 3-(4,5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide, is reduced by living cells, and this reaction is used as the end point in a rapid drug-screening assay. PC3, DU145 and HFF3 cells were incubated with increasing concentrations of 5f and 4-MMPB (0, 3, 6.25, 12.5, 25, and 50 µg/mL) for 24, 48, and 72 h, separately. Since the inhibitors were dissolved in DMSO and HCl, which are both cytotoxic solutions themselves, DMSO and HCl controls (0.25 %), equivalent to their inhibitor solutions, were used as concentration of 0.00 in our experiments. In addition, the same concentrations of cisplatin were tested (Figs. 2, 3 and 4).

Demonstration of apoptotic morphology

PC3 cells were stained using a dye specific for the detection of apoptotic cells, namely 4,6- diamidino-2-phenylindole (DAPI, Merck, Germany). Briefly, a monolayer of untreated, control and treated cells (5f = 26 µg/mL (71.03 µM); 72 h) were washed with PBS and fixed in 4 % (w/v) paraformaldehyde (Sigma, Germany) for 30 min at room temperature. These cells were then washed with PBS and centrifuged (1000 *g*, 3 times), permeabilized with 0.1 % triton X-100 (Merck, Germany) in PBS and incubated with 2 µg/mL DAPI at 37 °C for 10 min. Cells were washed three times with PBS and viewed using a fluorescent microscope. About 700 cells from each treatment were examined and counted. Apoptotic cells were confirmed by identification of intensely stained, fragmented nuclei and condensed chromatin.

Comet assay

For estimation of DNA damage, comet assay was performed with single cell gel electrophoresis, as per protocol described elsewhere (Rassouli et al. 2011). Briefly, untreated, control and treated PC3 cells (5f = 26 µg/mL; 72 h) were suspended in 25 µL PBS and mixed with 75 µL of 0.75 % (w/v) low melting point agarose (LMA, Fermentas, Germany) and layered over a frosted microscopic slide precoated with a layer of 1 % (w/v) normal melting agarose (Helicon, Russia); covered with a coverslip and kept for 20 min at 4 °C for solidification. The coverslips were then removed, 100 µL of 0.75 % (w/v) LMA was added to each slide, covered with a coverslip and kept for another 20 min at 4 °C. Three slides were prepared for each sample. Slides were then immersed in freshly prepared ice-cold lysing buffer (2.5 M NaCl; 100 mM Na_2EDTA; 10 mM Tris; 2 % (v/v) triton X-100; pH 10) and incubated 4 h at 4 °C for lysis of cells and nuclear membranes. Slides were then

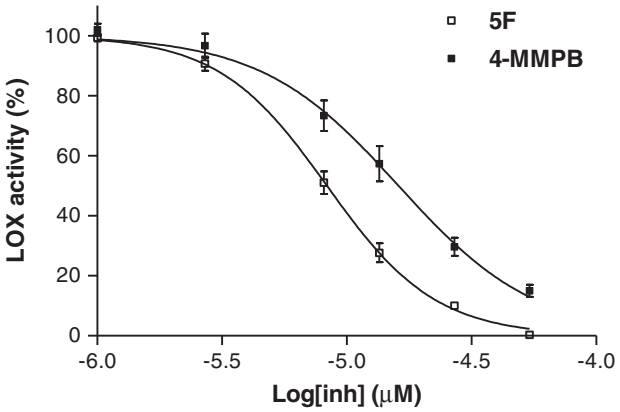

Fig. 2 Sigmoidal dose-response curves of 5f and 4-MMPB against PC3 lipoxygenase activity as determined by kinetic procedure

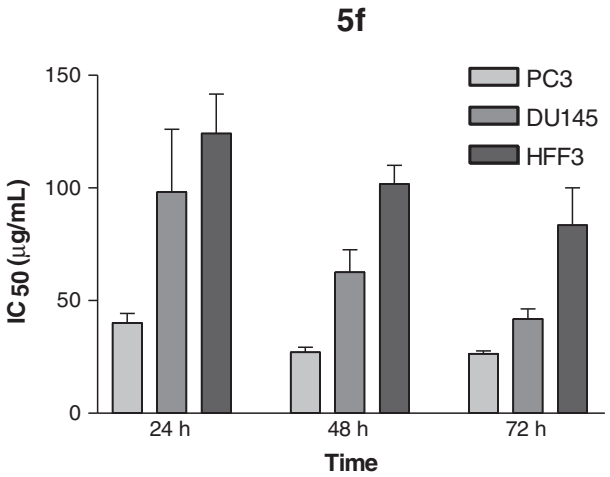

Fig. 3 The cytotoxic effects of 5f on the three cell lines as assessed by MTT assay. In all cell lines, 5f produced a concentration and time-dependent decrease in cellular proliferation, following 24, 48 and 72 h incubation. Bars indicate mean ± SD, $n = 3$

washed with cold distilled water, placed in an electrophoresis chamber filled with freshly prepared cold alkaline electrophoresis buffer (1 mM EDTA; 0.3 N NaOH; pH 13) and incubated for 30 min at 4 °C in order to unwind DNA. Electrophoresis was then carried out for 20 min (25 V; 300 mA) at 4 °C. Slides were then washed three times with ice-cold neutralizing buffer (0.4 M Tris—HCl buffer; pH 7.5), dried with 96 % ethanol, stained with ethidium bromide (final concentration of 40 µg/mL) and examined under a fluorescent microscope attached to a CCD camera. Comets were scored with computer aided image analysis system TriTek Cometscore version 1.5 software. percentage of DNA damage was determined from analysis of tail length.

Cell cycle analysis

For cell cycle analysis, PC3 cells were treated with 26 µg/mL of 5f for 72 h. The cell layer was trypsinized and washed with cold PBS and then fixed with 70 % ethanol. A solution including 350 µL of cold PBS, 50 µL triton X-100 (1 %), 20 µL RNase (0.2 mg/mL) (Sigma, Germany) and 30 µL propidium iodide (100 mg/mL) (Sigma, Germany) was added to the cell suspensions and the cells were kept on ice for 15 min in a dark place. Then the samples were analyzed with a FACSCalibur flow cytometer. The percentage of cells in sub-G_1, G_0/G_1, S and G_2/M phases of cell cycle was determined and analyzed using WinMDI 2.9 software.

Statistical analysis

Statistical analyses were performed using the statistical software Prism 3.0. Significant differences were ascertained by one way ANOVA, followed by Tukey multiple comparison tests. Values are expressed as mean ± SD. *P*-values of <0.05 in Tukey test and <0.001 in one way ANOVA tests were considered as significant.

Results

Based on the discovery of 15-LOX-1 inhibition by 5f (Iranshahi et al. 2012) and the reported role of the enzyme expression in prostate cancer cells proliferation (Kelavkar et al. 2004), the effects of the compound on two steroid dependent prostate cancer cell lines (PC3 and DU145) were studied for the first time.

To study the effect of 15-LOX-1 inhibition on cell growth, the enzyme assay was first performed by using the kinetic procedure (measurement of the enzyme activity within 300 s after addition of the linoleic acid as the substrate) (Sadeghian et al. 2008, 2009). The results showed no enzyme activity in the absence of the lysed cells. 15-LOX-1 activity was observed in PC3, while low enzyme activity was recorded in DU145 cells. The 15-LOX-1 inhibitory potency of 5f and 4-MMPB (as a selective and standard 15-LOX-1 inhibitor) was assessed on PC3 cell lysates. The LOX activity was reduced by 5f and 4-MMPB with IC_{50} values of 8.32 ± 0.56 µM and 16.1 ± 1.25 µM, respectively.

In order to study the cytotoxicity effects of 5f and 4-MMPB, these compounds were incubated with three human-derived cell lines, two carcinoma (PC3 and DU145) and a normal cell line (HFF3), and their anti-proliferative effects were determined using MTT assay. Treatment of PC3 and DU145 human PCa cells with different concentrations of both 15-LOX-1 inhibitors for 24, 48 and 72 h, resulted in a dose-dependent and time-dependent decrease in the survival of the cells. The IC_{50} values were calculated using Prism 3.0 software (Tables 1 and 2). Based on both dose-response curves and the IC_{50} values calculated, it appears that PC3 cells were more sensitive than DU145 for both inhibitors. Moreover, MTT assay results, revealed that 5f had no significant anti-proliferative profile on the normal cells (Table 1).

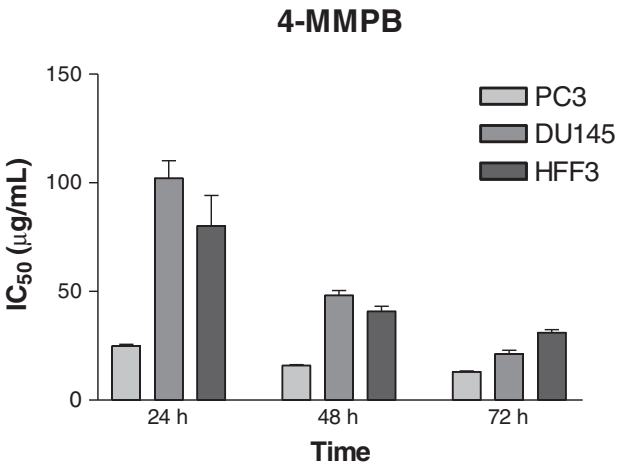

Fig. 4 The cytotoxic effects of 4-MMPB on the three cell lines as assessed by MTT assay. In all cell lines, the compound produced a concentration and time-dependent decrease in cellular proliferation, following 24, 48 and 72 h incubation. Bars indicate mean ± SD, $n = 3$

Table 1 Anti-proliferative activity of 5f on PC3, DU145 and HFF3 cells, as determined by MTT assay. IC_{50} values are shown as mean ± SD ($n = 3$)

Time (h)	PC3 IC_{50} (µg/mL)	DU145 IC_{50} (µg/mL)	HFF3 IC_{50} (µg/mL)
24	40.1 ± 7.9	98.14 ± 48.3	124.02 ± 30.3
48	27.05 ± 3.9	62.5 ± 17.3	101.7 ± 14.5
72	26.43 ± 2.1	41.85 ± 7.8	81.42 ± 28.7

The effects of cisplatin (a general and well-known cytotoxic compound) on PC3 and DU145 were also studied. The results indicated that cisplatin has similar cytotoxic effects in both cancerous cell lines. Moreover, the IC_{50} values of cisplatin were close to 5f on PC3 cells (Table 3).

In order to determine the mechanism involved in cytotoxic effects of 5f, chromatin condensation and DNA damage were analyzed by DAPI staining and alkaline version of comet assay, respectively. Demonstrating apoptotic morphology by DAPI staining revealed that 64 % of PC3 cells treated with 26 µg/mL of 5f, presented condensed chromatin, which was significantly ($P < 0.001$) higher than control and untreated cells (Fig. 5). Moreover, Fig. 6 represents photomicrographs of DNA damage in untreated and control cells in comparison with cells incubated with the inhibitor. Results obtained from comet assay revealed that combination of 26 µg/mL 5f induced approximately 54 % DNA damage, significantly ($P < 0.001$) higher than control and untreated cells.

In the next step, the status of the cell cycle of PC3 cells treated with 5f (26 µg/mL; 72 h) was analyzed. As shown in Fig. 7, 5f induced cell cycle arrest in G_0/G_1 phase.

Table 2 Anti-proliferative activity of 4-MMPB on PC3, DU145 and HFF3 cells, as determined by MTT assay. IC_{50} values are shown as mean ± SD ($n = 3$)

Time (h)	PC3 IC_{50} (µg/mL)	DU145 IC_{50} (µg/mL)	HFF3 IC_{50} (µg/mL)
24	24.96 ± 1.2	102.1 ± 13.9	80.18 ± 24.3
48	15.92 ± 0.6	48.27 ± 4	40.78 ± 4.1
72	13.08 ± 0.7	21.28 ± 2.3	31.06 ± 2.3

Table 3 Anti-proliferative activity of cisplatin on PC3, DU145 and HFF3 cells, as determined by MTT assay. IC_{50} values are shown as mean ± SD ($n = 3$)

Time (h)	PC3 IC_{50} (µg/mL)	DU145 IC_{50} (µg/mL)	HFF3 IC_{50} (µg/mL)
24	32.01 ± 3.2	35.22 ± 1.9	33.2 ± 1.6
48	25.47 ± 1.9	27.84 ± 2.22	20.45 ± 7.2
72	18.97 ± 2.8	19.52 ± 4.92	12.83 ± 5.8

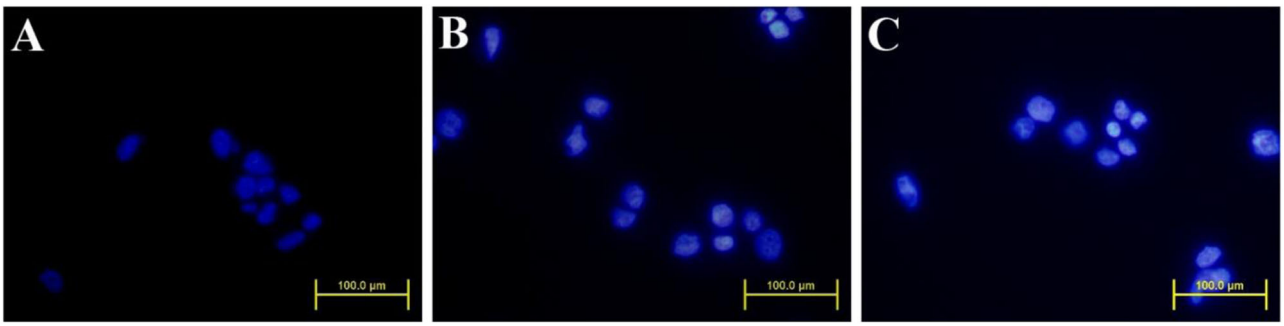

Fig. 5 Chromatin condensation assessment of control and treated PC3 cells by DAPI staining. **a** untreated cells **b** cells treated with 0.25 % DMSO **c** cells treated with 26 µg/mL 5f for 72 h. Apoptosis was determined by chromatin condensation and nuclear fragmentation, as appeared by intensive staining in **c** in comparison to **a** and **b**

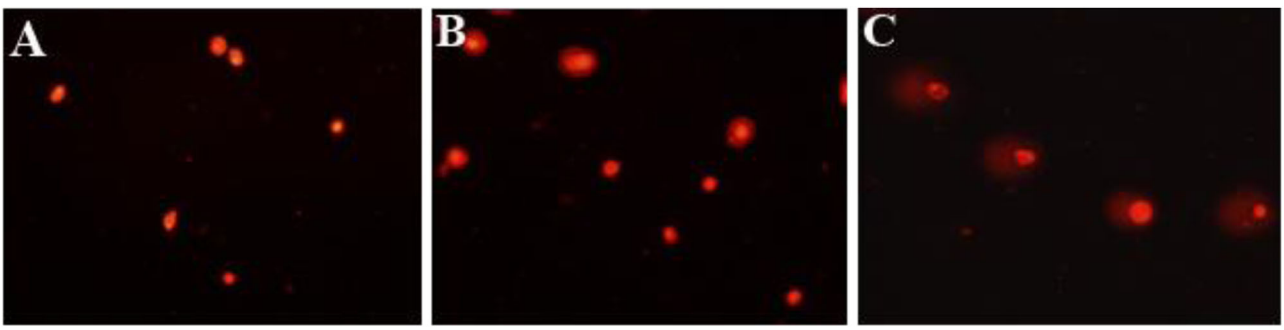

Fig. 6 DNA damage inducing effects of 5f on PC3 cells as determined by comet assay **a** untreated PC3 cells **b** PC3 cells treated with 0.25 % DMSO **c** cells treated with 26 µg/mL 5f after 72 h

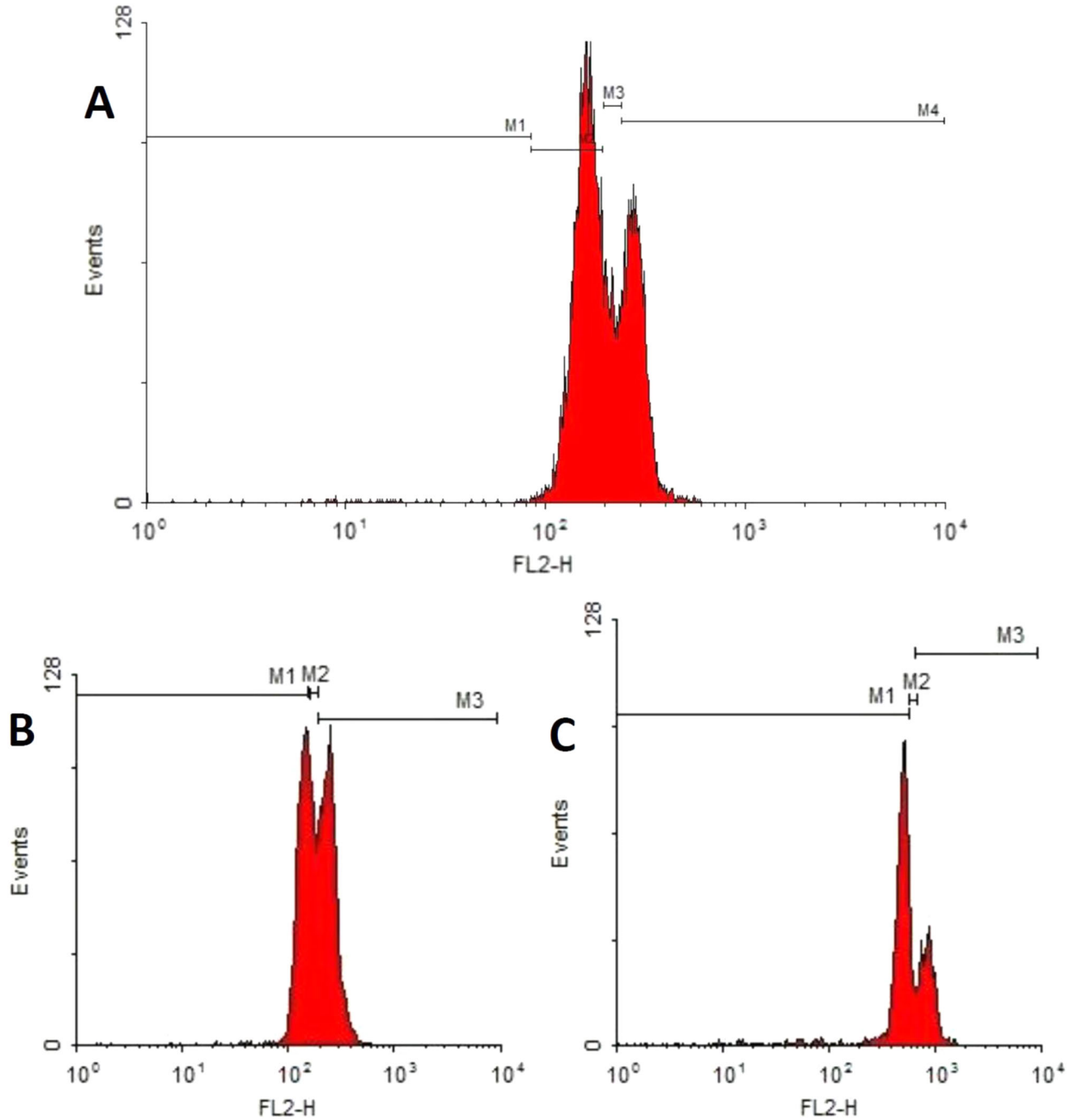

Fig. 7 5f induces cell cycle arrest at G_1 phase, as shown by PI staining. **a** untreated PC3 cells **b** cells treated with 0.25 % DMSO **c** cells treated with 26 µg/mL 5f for 72 h

Discussions

The critical role of the 15-LOX-1 metabolite, 13-(S)-hydroxyoctadecadienoic acid (13-HODE), in the progression of PCa, and inhibition of 15-LOX-1 activity for apoptosis induction in PC3 cells have been demonstrated (Kelavkar et al. 2006, 2001). Here we describe a selective anti-proliferative potential of 5f, a synthetic coumarin, on PC3 and DU145 cells. This compound has an ability to inhibit 15-LOX-1 enzyme. In this study, we observed a higher level of 15-LOX-1 activity in PC3 cells in comparison with DU145 cell line. In addition, the 15-LOX-1 inhibitory potency of 5f was tested in PC3 cell lysate. In order to investigate the relationship between 15-LOX-1

inhibition and cell death induction, 4-MMPB (a selective 15-LOX-1 inhibitor) was also used. Moreover, to highlight the potential advantages of 5f, comparative studies were carried out using one of the best known anti-cancer drugs, cisplatin.

Initial anti-proliferative studies were carried out to determine the IC_{50} values for the two inhibitors and cisplatin, using three human-derived model cell lines, two prostate cancer cell lines and one normal fibroblastic cell line. As presented in Tables 1 and 2, results show that both 15-LOX-1 inhibitors, were more effective on PC3 cells with higher 15-LOX-1 activity, in comparison with DU145 cells. Furthermore, these compounds displayed both concentration- and time-dependent anti-proliferative effects on the cell lines tested (Figs. 2–4). Interestingly, our synthetic coumarin (5f) appeared to be a potent agent, as it had an IC_{50} value, which was approximately similar to cisplatin (Table 3) while, it had no significant anti-proliferative activity on normal cells.

We next examined the cell death mechanism of 5f on PC3 cells. To do so, two techniques were performed. Firstly, the cells were stained with DAPI. Results presented in Fig. 5 clearly show that 5f could induce morphological changes, which are consistent with the induction of apoptotic cell death. Then, analysis of DNA damage in PC3 cells was performed using alkaline comet assay and a significant increase in DNA breakage was observed after 5f treatment of the cells (Fig. 6).

To date, many studies have shown the chromatin condensation and DNA damage induction by different coumarins. For instance, combination of mogoltacin (32 µg/mL) with cisplatin (1 µg/mL) after 24 h, induced chromatin condensation in 83 % of 5637 cells (a TCC subline) and increased the apoptotic effects of cisplatin on 5637 cells via DNA lesion by 44 % (Rassouli et al. 2011). Moreover, 7-isopentenyloxycoumarin (65 µg/mL; 72 h) increased the amount of chromatin condensation and DNA damage in 5637 cells by 58 and 33 %, respectively (Haghighi et al. 2014). Our results indicated that 5f (26 µg/mL; 72 h) could induce condensed chromatin in 63 % of PC3 cells and caused 54 % DNA breakage in these cells.

The status of the cell cycle of PC3 cells treated with 5f (26 µg/mL; 72 h) was also analyzed. As shown in Fig. 7, exposure of PC3 cells to 5f caused cell cycle arrest in G_0/G_1 phase. According to many studies, most of investigated coumarins could induce cell cycle arrest at G_0/G_1 phase. It was shown that 7,8-diacetoxy-4-methylcoumarin (DAMC) and one of its derivatives, induced the G_0/G_1 arrest in A549 cells (lung cancer) via inhibition of MAPK signaling pathway and activation of NF-κB (Goel et al. 2009). Esculetin (100 µM) also induced G_0/G_1 arrest in HL-60 cell line (human leukemia) (Wang et al. 2002). In addition, umbelliprenin (12.5 µM or 4.6 µg/mL) arrested the M4Beu cell cycle in G_0/G_1 phase (Barthomeuf et al. 2008). Gulappa et al. (2013), studied a furanocoumarin on two drug resistant PCa cell lines (PC3 and C4-2B). Their results indicated that this coumarin arrests the cell cycle in G_0/G_1 stage, via inhibition of cyclin/cdk complexes and induction of P21 and P27 (cdk inhibitors) expression. Cyclins and their related kinases (cdk) are essential factors for eukaryotic cell cycle and passing the cells from G_1 to S phase (Lee et al. 2008).

Based on our experimental results, it could be concluded that 5f induces apoptosis in PC3 cells via inhibition of 15-LOX-1 activity. This study is the first report about the anti-proliferative activity of 5f and its possible cell death mechanism. Since 5f has the selective anti-proliferative effects on cancer cells, this work could be an initiation point for introducing new chemotherapiutic agents.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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