# 8-Farnesyloxycoumarin induces apoptosis in PC-3 prostate cancer cells by inhibition of 15-lipoxygenase-1 enzymatic activity

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**Prostate cancer is the second most common cancer in men worldwide. Overexpression of 15-lipoxygenase-1 (15-LOX-1) has been reported in prostate cancer patients. This study aimed to investigate the cytotoxic and anticancer effects of 8-farnesyloxycoumarin (8f), a prenylated coumarin, by inhibition of 15-LOX-1 activity, in prostate cancer cells. The activity of 15-LOX-1 and the inhibitory effects of 8f on this enzyme were first assessed in PC-3 and DU145 prostate cancer cells. The MTT assay was used to examine the cytotoxicity effects of 8f on PC-3 cells following 15-LOX-1 inhibition. To determine the type of cell death, chromatin condensation and DNA damage were examined by DAPI staining and comet assay, respectively. Furthermore, the effects of 8f on the cell cycle were evaluated by PI staining and flow cytometry. The activity of 15-LOX-1 was determined to be higher in PC-3 compared with DU145 cells; thus, this cell line was selected for further experiments. 8f induced cell death in PC-3 cells in a dose-dependent and time-dependent manner, with $IC_{50}$ values similar to cisplatin, which was used as a control. However, 8f did not significantly affect the viability of HFF3, human foreskin fibroblast cells, under identical conditions. The appearance of apoptotic cells after 8f treatment was confirmed by the presence of PC-3 cells containing condensed chromatin as shown by DAPI staining. The comet assay indicated the induction of DNA damage in cancerous cells compared with normal cells. In addition, 8f induced a potent G1 cell-cycle arrest in PC-3 cells. Our results showed that the antitumor effects of 8f on PC-3 cells were promoted by apoptosis induction, probably via inhibition of 15-LOX-1 activity, thus suggesting that 8f may have therapeutic value in prostate cancer treatment.** ***Anti-Cancer Drugs*** **27:854–862** 

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## Introduction

Carcinoma of the prostate is one of the most commonly diagnosed visceral malignancies in more developed countries and the fifth leading cause of cancer-related deaths in men. In 2012, an estimated 1.1 million cases and 307 000 deaths were diagnosed in men worldwide [1]. Generally, the highest rates are recorded in wealthy countries, where screening is more available and prostate-specific antigen testing is common. Similarly, in less developed countries, prostate cancer incidence rates are increasing, which might be due to changes in their life style. Prostate cancer is typically diagnosed in the sixth and seventh decades of life, and the importance of age is highlighted by its epidemiology. Different factors implicated in the pathogenesis of this disease include age, race and ethnicity, diet, androgen secretion, and activated oncogenes [2].

Some evidences show that lifestyle and environmental factors, including dietary habits, may play important roles in the development and/or prevention of prostate carcinoma [2]. The metabolism of dietary fatty acids is of particular interest in prostate cancer. It has been shown that aberrant expression of the enzymes involved in the oxidative metabolisms of unsaturated fatty acids, arachidonic acid and linoleic acid (LA), contributes significantly toward the development of prostate cancers [3]. One family of these enzymes includes lipoxygenases (LOXs). More emerging evidences suggest that LOXs play many important roles in human diseases including cancers [4,5]. It was shown that the expression of 15-lipoxygenase-1 (15-LOX-1) is strongly correlated with the degree of malignancy in prostate cancers [6]. Correlating with these results, the upregulation of human 15-LOX-1 enhanced the proliferation of cells and its inhibition resulted in apoptosis in cultured cell lines such as PC-3 and explanted human prostate cancer cells [3]. 15-LOX-1 converts LA, its preferred substrate, into 13-S-hydroxyoctadecadienoic acid (13-HODE) and other metabolites. It is becoming increasingly clear that these metabolites alter cellular signaling pathways and thus the inappropriate expression of 15-LOX-1 may contribute toward tumor development. For example, vascular homeostasis, cell growth, and differentiation are altered by LA

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metabolites [6]. Therefore, finding a strategy that inhibits the expression or the activity of 15-LOX-1 and its metabolites could be useful in prostate cancer treatment.

As prostate cancer is a chronic disease, chemotherapy can be an attractive strategy for its control. A continuous increase in the incidence of cancer and failure of conventional chemotherapy against advanced prostate cancers have led to the search for natural and synthetic compounds as novel cancer chemopreventive agents to treat and prevent this malignancy [7,8]. Chemopreventive agents are used to prevent induction and inhibit or delay progression of cancers. Coumarins are a group of natural and synthetic compounds, which have been shown to induce apoptosis in prostate cancer cells [7,9]. The anticancer effects of some coumarins are related to their inhibitory effects on LOXs [10]. For example, reports have shown that umbelliprenin, a natural coumarin derivative, not only has antiproliferative and significant cancer chemoprevention activity but also shows inhibitory activity against soybean lipoxygenase enzyme [11,12]. Moreover, the inhibitory activity of O-prenylated derivatives of coumarin has been shown against soybean 15-LOX and human 15-LOX-1 [13]. In the current study, we investigated the inhibitory effects of 8-farnesyloxycoumarin (8f) (Fig. 1), a prenylated derivative of coumarins, on 15-LOX-1 activity in human prostate cancer cells. Then, we evaluated its toxic effects on PC-3 and HFF3 cell lines using MTT assay and to assess chromatin condensation and DNA damage in these cells, DAPI staining and a comet assay were used, respectively. In addition, PI staining was also carried out for cell-cycle analysis. In this study, the effects of 8f were compared with 4-MMPB, a selective inhibitor of 15-LOX-1 [14], and cisplatin, a routine drug used in cancer treatment.

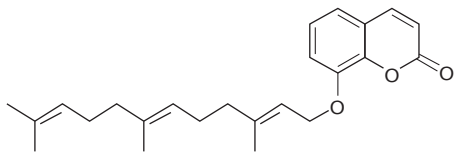
## Materials and methods

### Cell culture and materials

8f used in this study was synthesized as described previously by Iranshahi *et al.* [13]. 4-MMPB and cisplatin were obtained from Caymann and Sigma (Sigma-Aldrich, Darmstadt, Germany), respectively.

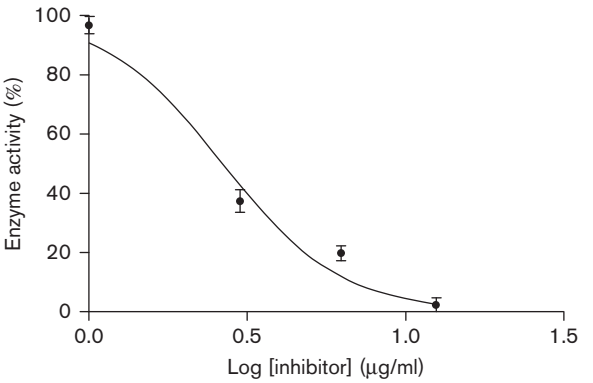
Cell lines PC-3 and DU145 were obtained from Pasteur Institute (Tehran, Iran) and HFF3 cells were kindly provided by Royan Institute (Tehran, Iran). PC-3 and DU145 cells were grown in Roswell Park Memorial Institute (RPMI-1640) medium and HFF3 cells were cultured in high-glucose Dulbecco's modified Eagle's medium (HG-DMEM; Gibco, Paisley, Scotland, UK), supplemented with 10% fetal bovine serum (Gibco) for prostate cancer cells and 15% fetal bovine serum for HFF3 cells in a humidified atmosphere of 5% $CO_2$ at 37° C. Cells were passaged using 0.25% trypsin-1 mmol/l EDTA (Trypsin-EDTA; Gibco) when required.

**Fig. 1**




Chemical structure of 8-farnesyloxycoumarin.

**Fig. 2**




Sigmoidal dose–response curve of 8f against PC-3 lipoxygenase activity (values given are mean ± SD; $n = 3$).

### 15-LOX-1 enzyme activity in PC-3 cells

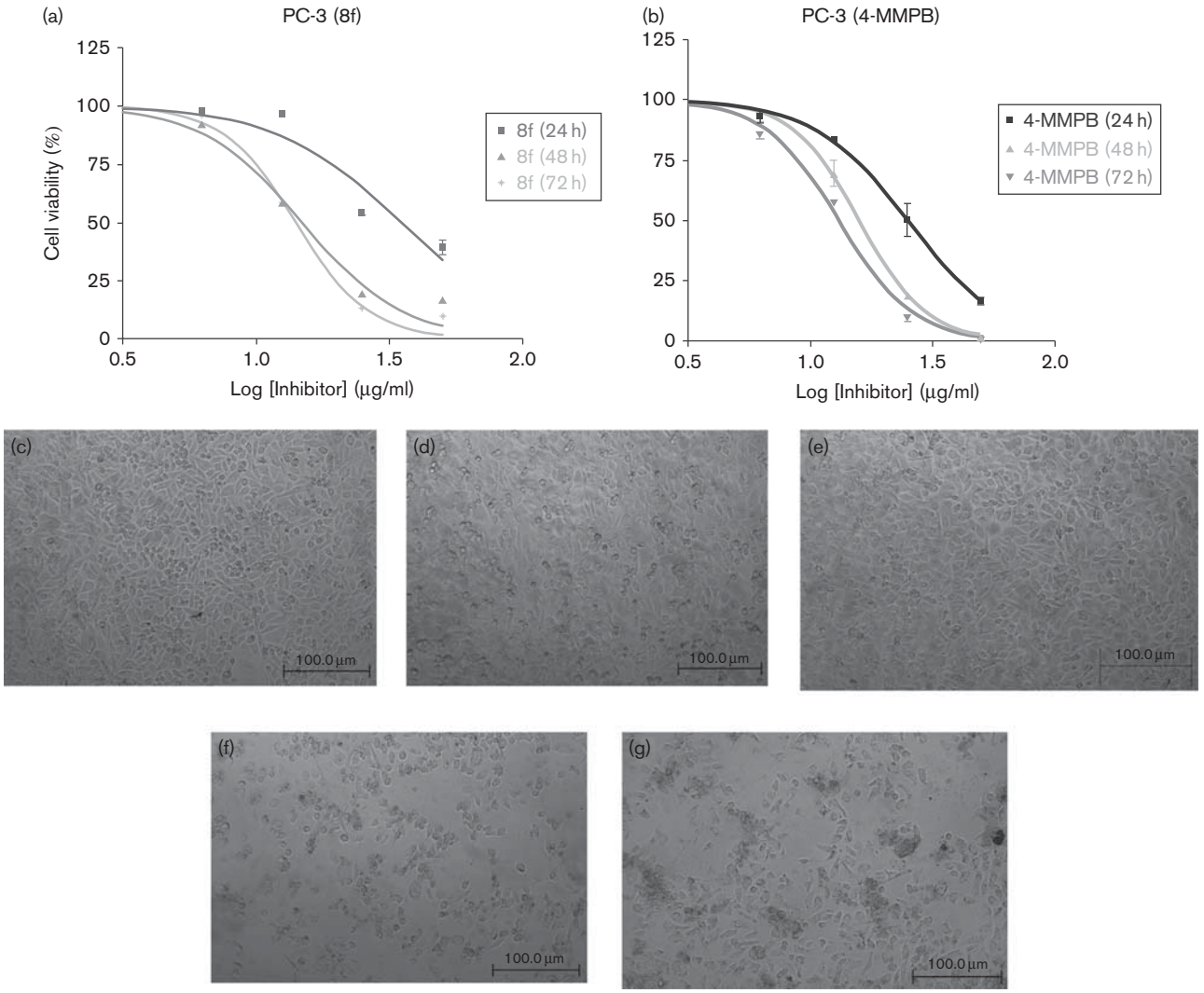
The activity of 15-LOX-1 in PC-3 and DU145 cells was estimated spectrophotometrically. For this assay, cells were harvested and then 1 ml of Tris buffer (0.1 mol/l; pH 7.2) was added to pellets. The cell suspension was sonicated at 20°C for 2 min.

To evaluate the LOX activity, cell homogenates were incubated with an LA solution (final concentration: 100 µmol/l) in 2 ml Tris buffer (0.1 mol/l pH 7.2). Evaluation of the inhibitory effect of 8f was performed as described above, but the reaction mixture contained variable concentrations of 8f (2–20 µg/ml). Finally, the absorbance of each sample was measured at 234 nm for 300 s.

### MTT assay

The MTT assay is a well-documented cell viability assay and has been modified by several investigators since it was first developed by Mosmann [15]. It is considered to be based on the transformation of tetrazolium salt by mitochondrial succinic dehydrogenases in viable cells, yielding purple formazan crystals that are not soluble in an aqueous solution. Cell survival was measured using the MTT assay as described previously [16]. Briefly, after treatment with various concentrations of 8f (3, 6.25, 12.5, 25, and 50 µg/ml in 0.25% DMSO) (DMSO; Merck, Darmstadt, Germany) for 24, 48, and 72 h, cells were incubated with 10 µl MTT (Sigma-Aldrich) per well and

**Fig. 3**




Cytotoxic effects of 8f (a) and 4-MMPB (b) on PC-3 cells. PC-3 cells were treated with different concentrations (3–50 µg/ml) of 8f and 4-MMPB, and the MTT test was performed at 24, 48, and 72 h to measure cell viability (data are expressed as mean ± SD). Morphological changes were monitored 72 h after treatments. Photomicrographs of PC-3 cells are presented without any treatment (c), treated with 0.25% DMSO (d) and HCl (0.25%) (e), cells treated with 14 µg/ml 8f (f), and 13 µg/ml 4-MMPB (g) after 72 h.

were kept for 4 h to allow MTT metabolization. Removal of the medium was followed by the addition of DMSO. The optical density (OD) was measured spectrophotometrically at 545 nm. The morphological changes in the treated cells were also observed under a light inverted microscope (Olympus, Tokyo, Japan) after 24, 48, and 72 h of treatments.

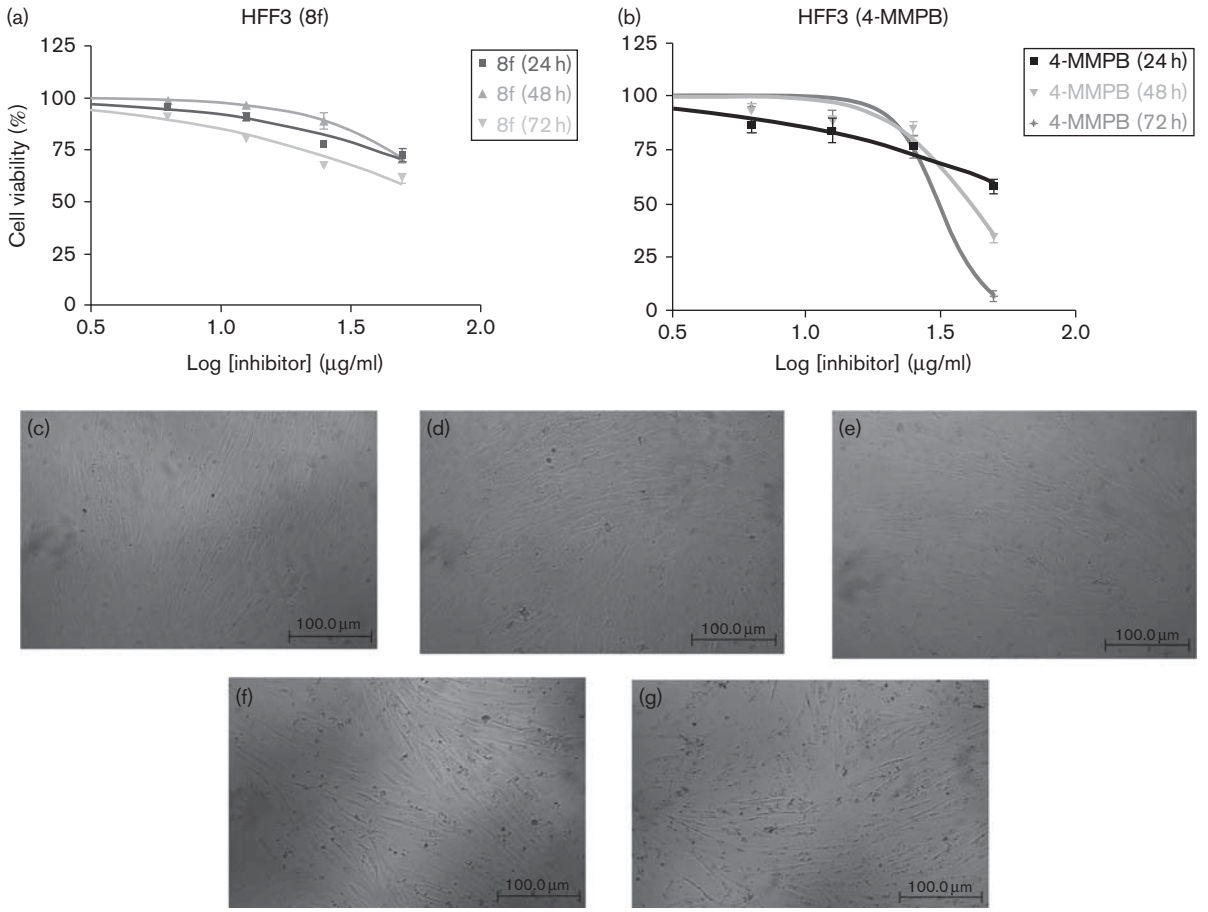
### 4,6-Diamidino-2-phenylindole, dihydrochloride staining

The apoptosis-inducing effects of 8f were assessed by fluorescence microscopic analysis of cells with condensed chromatin and fragmented nuclei following staining with DAPI as described before [17]. PC-3 cells were exposed to $IC_{50}$ concentrations of 8f (14 µg/ml or 38.58 µmol/l) and 4-MMPB (13 µg/ml or 42.33 µmol/l) and controls relevant to DMSO (0.25%) and HCl (0.25%) for 72 h. As 8f did not have any $IC_{50}$ value on HFF3 cells in the concentration range of 3–50 µg/ml, for this assay, these cells were treated with the highest amount of 8f. Then, cells were harvested and fixed with 4% paraformaldehyde. Subsequently, cells were permeabilized with Triton X-100 and stained by incubation with DAPI (Merck). Finally, cells with condensed and fragmented chromatin (apoptotic cells) were counted under a fluorescence microscope.

### Alkaline comet assay

The single-cell gel electrophoresis (Comet) assay is a relatively simple method for the measurement of DNA damage [18]. This assay was performed as described

**Fig. 4**




Cytotoxic effects of 8f (a) and 4-MMPB (b) on HFF3 cells. HFF3 cells were treated with different concentrations (3–50 μg/ml) of 8f and 4-MMPB, and the MTT test was performed at 24, 48, and 72 h to measure cell viability (data are expressed as mean ± SD). Morphological changes were monitored 72 h after treatments. Photomicrographs of HFF3 cells are presented without any treatment (c), treated with 0.25% DMSO (d) and HCl (0.25%) (e), cells treated with 14 μg/ml 8f (f), and 13 μg/ml 4-MMPB (g) after 72 h.

**Table 1 The $IC_{50}$ values of 8f and 4-MMPB on prostate cancer cells and nontumor cells (24, 48, and 72 h after treatments)**

| | The $IC_{50}$ values (μg/ml) of 8f | | | The $IC_{50}$ values (μg/ml) of 4-MMPB | | |
|---|---|---|---|---|---|---|
| Cell lines | 24 h | 48 h | 72 h | 24 h | 48 h | 72 h |
| PC-3 | 34.98 (95.57) | 14.92 (40.76) | 14.12 (38.58) | 24.90 (79.55) | 15.89 (50.77) | 13.25 (42.33) |
| HFF3 | 125.9 (343.99) | 77.00 (210.38) | 77.38 (211.42) | 80.93 (258.56) | 42.41 (135.50) | 31.97 (102.14) |

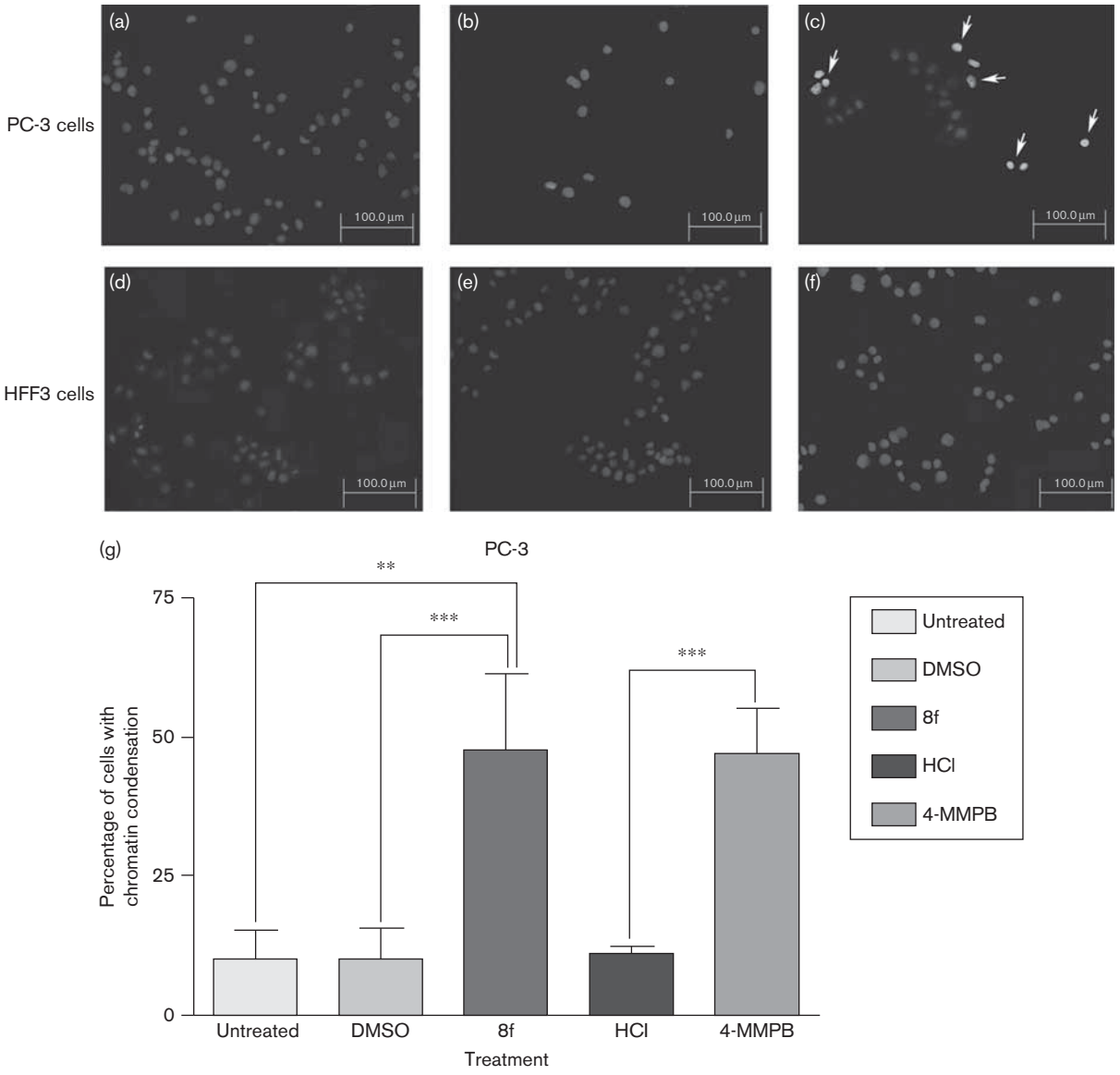
Values given are mean (±SD); $n = 3$. The values written in parentheses represent the equivalent amounts calculated as μmol/l.

previously [16]. In summary, PC-3-treated and HFF3-treated cells were embedded in a layer of low melting point agarose (1.5%, w/v; Fermentas, St Leon-Rot, Germany) on frosted slides and immersed in a lysis buffer at 4°C. Subsequently, the slides were incubated in freshly prepared alkaline buffer and then electrophoresed at 25 V and 300 mA. Then, the slides were neutralized and stained with ethidium bromide. A total of 200 nuclei were scored per slide using the image analysis system Tri Tek Comet Score, version 1.5 (TriTek Corp., Sumerduck, Virginia, USA). The DNA in the tail, defined as the percentage of DNA migrating from the head of the comet into the tail, was measured for each nucleus scored.

### Flow cytometric analysis of cell cycle with propidium iodide

For cell-cycle analysis, 72 h after treatment with 8f (14 μg/ml or 38.58 μmol/l) and its relevant DMSO control, PC-3 cells were harvested and washed with PBS. Cell pellets were then resuspended in a staining solution containing Triton X-100 (0.1%, v/v), sodium citrate (0.1%, w/v), RNase (Fermentas), and propidium iodide

**Fig. 5**




8f-induced chromatin condensation and nuclear fragmentation in PC-3 cells as observed by DAPI staining. Representative images from untreated cells (a, d), DMSO controls (b, e), and 8f-treated cells (c, f) after 72 h are shown. Arrows point to apoptotic cells with condensed chromatin. Columns (g) indicate the percentage of cells with chromatin condensation in untreated, 8f-treated cells, and control groups. Each data point represents the mean ± SD (***$P < 0.001$, **$P < 0.01$).

(Sigma-Aldrich). The samples were analyzed using a flow cytometer (FACSCalibure; Becton Dickinson, Franklin Lakes, New Jersey, USA) [16].

### Statistical analyses

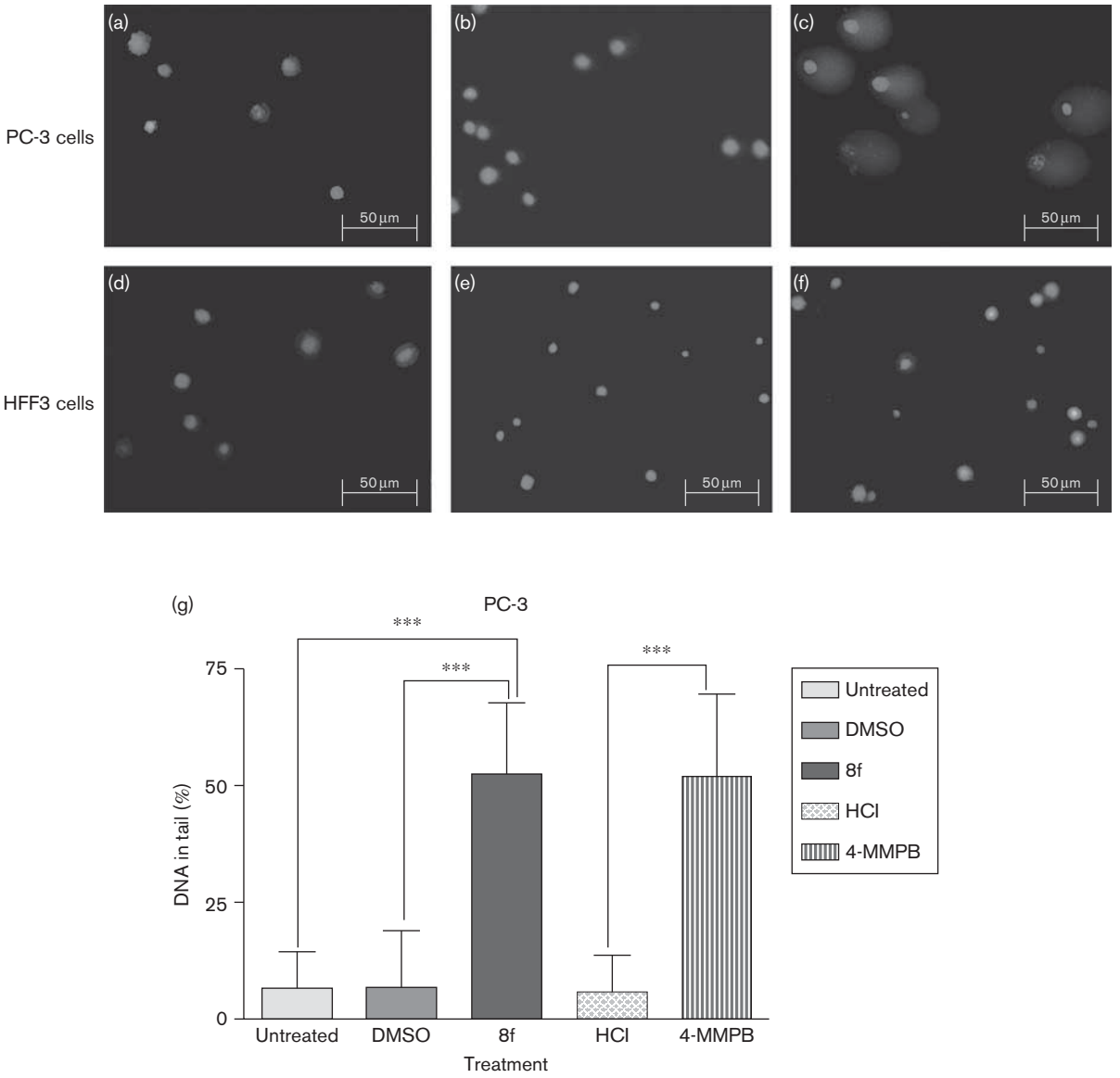
Results were analyzed by one-way analysis of variance-Tukey and Student's *t*-test (two tailed) for normal data and the Kruskal–Wallis test was used for analysis of data for variables not showing a normal distribution using GraphPad Prism 3.0 (GraphPad Software, Inc., San Diego, California, USA). Results were expressed as mean ± SD and differences were considered significant at $P$ values less than 0.01 and $P$ values less than 0.001.

## Results

### 8f decreased 15-LOX-1 activity in PC-3 cells

For evaluating the activity of 15-LOX-1 and inhibitory effects of 8f on this enzyme, two prostate cancer cell lines, PC-3 and DU145 cells, were used for enzyme assay. Among the two cell lines, low enzyme activity was observed for DU145 cells. Thus, 8f was evaluated for inhibition of LOX activity in PC-3 cells. The enzyme activity was measured in

**Fig. 6**



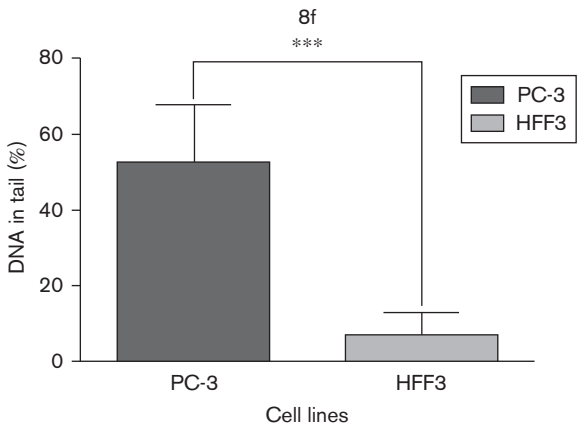

Alkaline comet assay shows DNA damage induced by 8f. Representative images of the alkaline comet assay of untreated cells (a, d), 0.25% DMSO-treated cells (b, e), and cells after exposure to 8f (c, f) for 72 h are shown. Columns (g) indicate the percentages of DNA in tails in 8f-treated cells and the control groups. Each data point represents the mean ± SD (***$P < 0.001$).

the absence and presence of LA. In samples without this substrate, no enzyme activity was recorded, while the activity was observed after the addition of LA. A remarkable and significant LOX inhibition was observed in the presence of 8f ($IC_{50} = 2.64 \pm 0.36$ µg/ml) (Fig. 2).

### MTT results showed the cytotoxic and anticancer effects of 8f

The effects of 8f on the viability of PC-3 and HFF3 cells were examined using the MTT assay at 24, 48, and 72 h after treatments. As shown in Fig. 3, treatment with 8f and 4-MMPB induced a time-dependent and dose-dependent decrease in the cell viability of PC-3 cells compared with untreated cells and relevant controls. Interestingly, our data showed that 8f did not exert any significant cytotoxic effects on HFF3 normal cells at testing concentrations (Fig. 4). The results were analyzed using GraphPad Prism 3.0 and the $IC_{50}$ values of compounds on each cell line are shown in Table 1. The effects of 8f on PC-3 and HFF3 cells were also confirmed by morphological observations. In this study, the effects of cisplatin, a routine drug used in the clinic, were also evaluated using the MTT assay, as a positive control. The results showed that the $IC_{50}$ values of 8f and cisplatin were very similar on PC-3 cells (data not shown).

**Fig. 7**




Percentages of DNA migrating in the tails of comets compared between PC-3 and HFF3 cells 72 h after treatment with 8f. Each data point represents the mean ± SD (***$P < 0.001$).

### 8f increases condensed chromatin and fragmented nuclei

For evaluation of the mechanism by which 15-LOX-1 inhibition causes cell cytotoxicity, DAPI staining was carried out on PC-3 and HFF3 cells that were treated with 8f and 4-MMPB. The nuclear morphology of cells in each sample was assessed to determine whether the effects of 8f and 4-MMPB were attributable to apoptotic changes. DAPI staining showed a significant nuclear fragmentation and chromatin condensation in PC-3 cells after treatment with $IC_{50}$ concentrations of both compounds compared with the controls. These changes were not observed in HFF3 cells that were treated with 8f (Fig. 5).

### Alkaline comet assay shows that 8f induces DNA damage

To confirm whether 15-LOX-1 inhibition with 8f indeed induced DNA damage, PC-3 and HFF3 cells were further subjected to an alkaline comet assay. We measured the percentages of DNA migrating in the tails of comets, an indicator of DNA damage, after treatment with 8f and 4-MMPB compared with the untreated cells and the relevant controls. Statistical analysis indicated that in PC-3 cells, 14 μg/ml (38.58 μmol/l) of 8f induced ~52% DNA damage, which was significantly ($P < 0.001$) higher than that induced by 0.25% DMSO (Fig. 6), and it was close to the 4-MMPB results. However, in HFF3 cells, treatment with 8f caused only 7% DNA damage, which shows significant differences ($P < 0.001$) between cancerous and normal cell lines (Fig. 7).

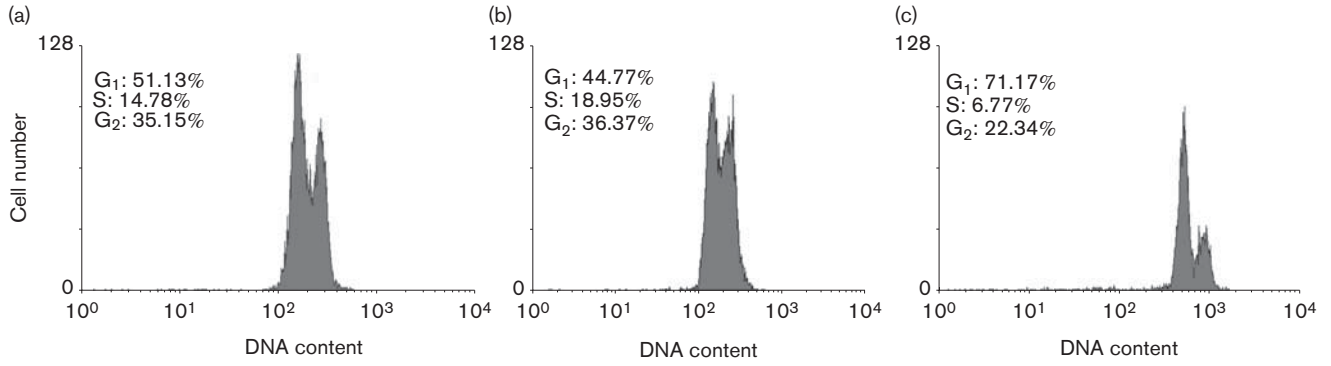
### 8f induces cell-cycle arrest in PC-3 prostate cancer cells

To determine whether the 8f-mediated 15-LOX-1 inhibition in PC-3 cells was attributable to cell-cycle arrest, PI staining was used to analyze the cell-cycle distribution. As shown in Fig. 8, treatment with 8f resulted in a significant increase in the number of cells at G1 and a decrease in the number of cells at the S and G2 phases of the cell cycle. The increasing amount at G1 was ~20% from untreated cells to cells that were exposed to 14 μg/ml (38.58 μmol/l) 8f. No significant changes in cell-cycle distribution were observed in 0.25% DMSO-treated cells, suggesting that the equivalent amount of DMSO alone exerted no effect on the cell-cycle distribution in PC-3 cells.

## Discussion

Coumarins are one of the most important classes of naturally occurring phenolic substances that are synthesized by numerous green plant species, some bacteria, and fungi. They are found to possess versatile biological properties including antiviral, antibacterial, anti-inflammatory, and antitumor activity [11,12,19,20]. For instance, coumarin (the simplest compound of these substances) and 7-hydroxycoumarin exert antiproliferative effects against a number of human malignant cell lines [21,22]. Furthermore, previous reports have introduced some coumarin derivatives as different enzyme inhibitors. It was shown that some coumarins constituted a completely new class of inhibitors of the zinc metalloenzyme carbonic anhydrase [23]. In addition, other studies reported the synthesis and anticancer activity of two series of coumarins as inhibitors of human NAD(P)H quinone oxidoreductase-1 (NQO1), which is overexpressed in several types of tumor cells [24]. Umbelliprenin is a natural prenylated coumarin that has also shown antitumor effects and inhibitory activity against the soybean LOX enzyme [11,12]. Similarly, in a recent study, mono O-prenylated coumarins were synthesized and their inhibitory potency against human 15-LOX-1 was determined [13]. Such reports show that these compounds can be used to induce cell death by specific enzyme activity inhibition in abnormal cells with uncontrolled growth and proliferation. In the current study, we hypothesized that 8f, a prenylated coumarin, decreased the viability of prostate cancer cell lines by a 15-LOX-1-inhibitory effect. As 15-LOX-1 is overexpressed in prostate tumors and plays important roles in the development of prostate cancer [6], its inhibition may be useful in the treatment of this disease. Therefore, we used two prostate cancer cell lines, PC-3 and DU145, and measured their 15-LOX-1 activity. Analysis by enzyme assay indicated that the activity of 15-LOX-1 in PC-3 cells was significantly higher than DU145 cells. Therefore, the PC-3 cell line was selected for further experiments. For evaluating the effects of 8f on these cells, MTT assay, DAPI staining, comet assay, and PI staining were performed. Moreover, human foreskin fibroblast cells (HFF3) were used as a control group for cytotoxicity tests. HFF3 cells are normal cells with a high potential for proliferation and have been used as control cells in other studies investigating the genitourinary system [25–29]. The results of the MTT assay indicated

**Fig. 8**




8f induces cell-cycle arrest in PC-3 cells. Flow cytometric DNA histograms after PI staining of untreated (a), 0.25% DMSO-treated (b), and 8f-treated cells (c).

that 8f significantly reduces the viability of PC-3 cells and these data are close to 4-MMPB (a selective 15-LOX-1 inhibitor) results, which confirmed that 8f cytotoxicity effects probably occurred because of 15-LOX-1 inhibition. In addition, the $IC_{50}$ values of 8f on PC-3 cancerous cells were similar to a very well-known anticancer drug, cisplatin. Importantly, we found that 8f was not toxic on HFF3 normal fibroblast cells. As shown in Table 1, a significant difference was observed between $IC_{50}$ values of 8f on PC-3 and HFF3 cells ($IC_{50}$ values were three to five times higher for HFF3 cells). The ability of this compound to promote prostate cancer cell death by inhibiting 15-LOX-1 activity in prostate cancer cells without affecting the growth of normal fibroblast cells indicates that it may be a promising candidate for prostate cancer therapy. The results of DAPI staining also indicated that treatment with 8f and 4-MMPB causes chromatin condensation and nuclear fragmentation in PC-3 cells. In addition, the percentages of DNA in tails of comets clearly confirmed the presence of DNA damage in these cells when they were subjected to an alkaline comet assay. However, apoptotic nuclear morphology and DNA damage were not observed in HFF3-treated cells. Our in-vitro data also showed that treatment of PC-3 cells with 8f induces G1 arrest, indicating that inhibition of cell-cycle progression is one of the events that probably occurred after 15-LOX-1 inhibition.

Studies have shown that some coumarin compounds exert their anticancer efficacy by induction of cell-cycle arrest at the G1 phase. Chuang *et al.* [30] showed that coumarin exerted cytotoxic effects and caused G0/G1 arrest and apoptosis on human cervical cancer HeLa cells. This study showed that coumarin treatment decreased the expression of G0/G1-associated proteins such as cyclin D1, Cdk2, and Cdc25A, which may have led to the G0/G1 arrest. In another report, G1-phase cell-cycle arrest was also associated with downregulation of Cdk2 and cyclin D1 expression as well as upregulation of p21 expression [31]. Decursin (another coumarin derivative), consistent with G1 arrest in prostate cancer cells, strongly increased protein levels of Cip1/p21, but showed a moderate increase in Kip1/p27 with a decrease in cyclin-dependent kinases (CDK), CDK2, CDK4, CDK6, and cyclin D1, and inhibited CDK2, CDK4, CDK6, cyclin D1, and increased binding of the CDK inhibitor (CDKI) with CDK [32]. The observation that 8f induced cell-cycle arrest in the G1 phase allows one to propose a similar mechanism of action for this effect. The cell-cycle arrest in G1 may involve the activation of the inhibitory cell-cycle regulatory proteins p21/or p27 with a decrease and inhibition in cyclin and CDKs expression and activity.

Several studies have suggested that the specific mechanism behind 15-LOX-1-mediated proliferation is through the selective inhibition of a specific peroxisome proliferator-activated receptor γ (PPARγ), which could act as a tumor suppressor. It was reported that the 15-LOX-1 product, 13-HODE, upregulates EGF/IGF-initiated MAP kinase (a central regulator of cell growth) activity and as a result, MAP kinase-dependent PPARγ phosphorylation is increased. This causes a downregulation or loss of PPARγ transcriptional activity and growth stimulation [33]. In addition, 15-LOX-1 also stimulated the expression of a putative angiogenic factor, vascular endothelial growth factor, in PC-3 cells [3]. Therefore, inhibition of 15-LOX-1 activity and its metabolite production may reduce MAPK signaling pathway activity and subsequently PPARγ phosphorylation and thus induces apoptosis in prostate cancer cells. Further investigations are required to determine the exact mechanism involved in 15-LOX-1 inhibition-mediated apoptosis.

The findings of the present study show that the observed anticancer activity of 8f *in vitro* could be attributed to the potent inhibitory effects of this compound on 15-LOX-1 activity, which reduces the formation of LOX-carcinogenic products. Here, we report the anticancer activity of 8f for the first time. Further in-vivo studies are

required to determine whether 8f could be an effective chemotherapeutic agent for the management of prostate cancer in animal models.

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### Conflicts of interest

There are no conflicts of interest.

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