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Identification of 7,8-Diacetoxy-3-Arylcoumarin Derivative as a Selective Cytotoxic and Apoptosis-inducing Agent in a Human Prostate Cancer Cell Line

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Abstract. *Background/Aim: Coumarins are a member of the benzopyrone family of compounds with diverse and interesting pharmacological properties. In the present study, we report the in vitro cytotoxicity evaluation of 7,8-Diacetoxy-3-arylcoumarin derivatives (5a-h) in human prostate (PC-3) and breast (MDA-MB-231) cancer cell lines. Materials and Methods: The cytotoxic activity was evaluated using crystal violet dye-binding assay. Furthermore, the most active compound in vitro cytotoxic activity in human non-cancerous cell line and its effect on the cell-cycle phases, apoptosis proteins expression, mitochondrial membrane potential (MMP), reactive oxygen species (ROS) production and Glutathione (GSH) level were performed. Results: Among the eight compounds that were evaluated, 7,8-Diacetoxy-3-(4-methylsulfonyl phenyl)coumarin (5f) was the most active derivative with highest cytotoxic activity and selectivity against the PC-3 cell line vs. the non-cancerous WPE1-N22 cell line. The cytotoxic action of compound 5f in PC-3 cells is associated with the cell-cycle arrest at –G0/G1 phase, apoptosis, loss in mitochondrial membrane potential (MMP), induced reactive oxygen species (ROS) production and depletion of Glutathione (GSH) level. Conclusion: The result indicates that the presence of p-methylsulfonylphenyl group on compound 5f is critical in modulating selective cytotoxic activity and induction of apoptosis via the mitochondrial apoptotic signaling pathway that is independent of cytochrome c release.*

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Key Words: 3-Arylcoumarin, anticancer, cell cycle, apoptosis, oxidative stress.

Coumarins belong to the benzo-α-pyrone family of oxygen-heterocyclic compounds and are classified as naturally-occurring coumarins (NOCs) and synthetic coumarins (SCs) (1, 2). The NOCs represent one of the largest phytochemicals occurring in the fruits, seeds, roots and leaves of many plant species; functioning as growth regulators, controllers of respiration, bacteriostats, fungistats, as well as prophylactics against infection (3). They are classified as simple coumarins, furanocoumarins, pyranocoumarins and pyrone-substituted coumarins based on the chemical structure of the compounds (3, 4). On the other hand, SCs have been the focus of chemical modification of the coumarin skeleton, in order to synthesize novel analogs with improved therapeutic activity and reduced toxicity (5, 6). SCs are classified as mono-, di- and poly-substituted, and fused coumarin derivatives (7). Chemically, SCs can be synthesized by various methods including the Pechmann reaction, Knoevenagel condensation, Claisen rearrangement, Perkin, Wittig, Reformatsky, Baylis Hillman and catalytic cyclization reactions (1, 8).

Coumarins, whether NOCs or SCs, exhibit a diverse array of pharmacological and biochemical activities such as anti-cancer, anti-coagulant, anti-HIV, anti-inflammatory, anti-microbial, anthelmintic and anti-oxidant properties (9-11). The structural diversity of coumarins arising from the nature of the substituent and its pattern of substitution on the core coumarin molecule influence these biological activities (8, 10, 12, 13). For example: (i) acetoxycoumarins *e.g.* 7,8-Diacetoxy-4-methylcoumarin (DAMC, Figure 1; 1) showed anti-cancer and pro-oxidant activities in different human tumor cell lines (14, 15) and (ii) hydroxycoumarins *e.g.* 7-Hydroxy-4-methylcoumarin (7-OHC, Figure 1; 2) displayed important pharmacological effects such as anti-cancer, anti-bacterial, anti-tubercular, anti-fungal, anti-neoplastic, anti-HIV, and anthelmintic activities (16-18). In addition to their biological activities, coumarins are used as additives in food and cosmetics, and as optical brightening agents (19, 20). These interesting biological properties of coumarins have made them attractive for synthesis and therapeutic application in the treatment of various diseases.

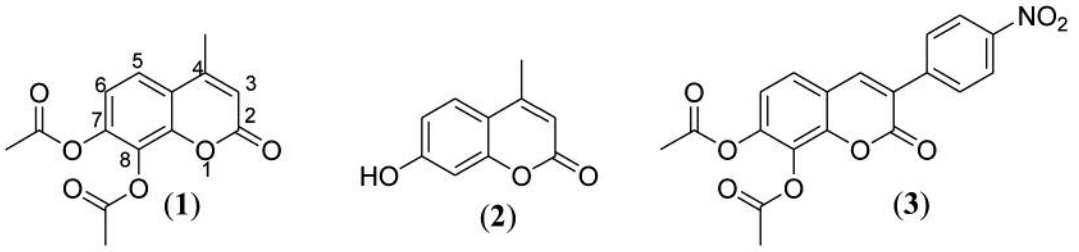


Figure 1. *Structures of 7,8-Diacetoxy-4-methylcoumarin (DAMC, 1), 7-Hydroxy-4-methyl-coumarin (7-OAC, 2) and 7,8-Diacetoxy-3-(4-nitrophenyl)coumarin (3).*

Recent studies have shown that the incorporation of an acetoxy group on the coumarin core structure played an important role in identifying potential targets for effective new therapeutic anti-cancer drugs (21-24). Most recently, we have demonstrated that 3-arylcoumarins bearing 7,8-diacetoxy group on the benzenoid ring: (i) 7,8-Diacetoxy-3-(4-nitrophenyl)coumarin (Figure 1; 3) exhibited non-selective cytotoxic activity in A549, MDA-MB-231 and PC3 cancer cell lines and (ii) 7,8-Diacetoxy-3-(4-methylsulfonylphenyl) coumarin (5f; Table I) exhibited selective cytotoxic activity in A549 (25, 26). Previous investigations have also demonstrated that the presence of a 7,8-diacetoxy group on the coumarin molecule enhances drug activity such as anti-cancer, antioxidant and radicals scavenging properties (14, 15). These findings aroused our interest in the cytotoxicity studies of 7,8-Diacetoxy-3-arylcoumarins in PC-3 and MDA-MB-231 cancer cell lines.

Prostate and breast cancers are the most common type of cancers among men and women, and there has been a growing research interest in the use of naturally occurring photochemicals such as coumarins to slow down the carcinogenesis process. As part of our ongoing investigation involving 7,8-Diacetoxy-3-arylcoumarins as potential anticancer agents, we herein report the *in vitro* cytotoxicity evaluation of 7,8-Diacetoxy-3-arylcoumarins (5a-h, Table I) in PC-3 and MDA-MB-231 cancer cell lines, and the most active compound cytotoxic activity in a non-cancerous cell line and cytotoxic mode of action in a cancer cell line.

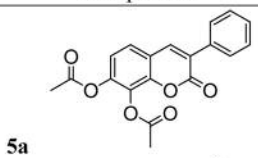
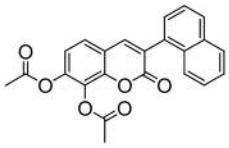
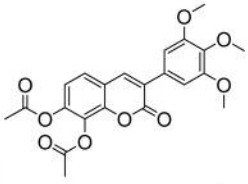
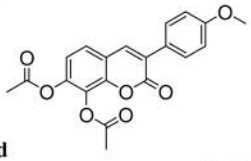
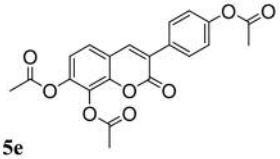
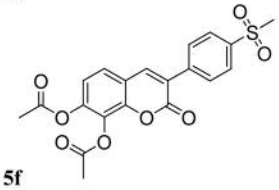
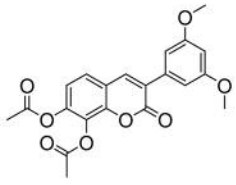
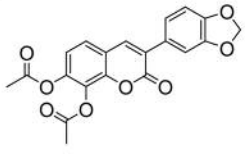
Materials and Methods

Chemicals. Medium (F12K and RPMI1640), penicillin-streptomycin anti-biotic solution (100×), fetal bovine serum (FBS), Trypsin-EDTA solution (1×), phosphate buffer (PBS), 25% glutaraldehyde, crystal violet, IGEPAL CA-630, propidium iodide, 2',7'-Dichlorofluorescin diacetate (DCFDA), Tetramethyl Rhodamine Methyl Ester (Rhodamine 123), Tamoxifen and RNase were obtained from Sigma Aldrich (St. Louis, MO, USA). Keratinocyte-SFM (1X) Serum Free medium was obtained from Gibco Life Technologies (Grand Island, NY, USA). The potassium phosphates, EDTA, Triton X-100 were obtained from Thomas Scientific Company (Swedesboro, NJ, USA). Human Apoptosis Arrays were obtained from RayBiotech, Inc. (Norcross, GA, USA). GSH-Glo™ Glutathione Assay Kit was obtained from Promega Corporation (Madison, WI, USA). Z-DEVD-fmk (caspase 3), Z-IETD-fmk (caspase 8), and Z-LEHD-fmk (caspase 9) inhibitors were obtained from MP Biomedicals, LLC (Solon, Ohio, USA). BID Antibody (Human Specific), Caspase-3 (3G2) Mouse mAb, Caspase-8 (1C12) Mouse mAb, Smac/Diablo Mouse mAb, XIAP (3B6) Rabbit mAb, Anti-rabbit IgG HRP-linked Antibody and Anti-mouse IgG HRP-linked Antibody were obtained from Cell Signaling Technology (Danvers, MA, USA). The compounds (5a-h) stock solutions were made up in DMSO and stored at 4˚C.

Cells and treatments. The human cell lines (PC-3, MDA-MB-231 and WPE1-NA22) were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured as per the guidelines supplied. The cells were maintained in F12K (PC-3) or RPMI 1640 (MDA-MB-231) or Keratinocyte-SFM (1X) (WPE1-NA22) medium containing 100 units of penicillin/ml, 100 μg of streptomycin/ml, and 10% FBS (F12K and RPMI media) in T-75 cm^2 flasks at 37˚C in a 5% CO_2 incubator. The cells were plated at a density of 5×10^4 cells per well in a polystyrene, flat bottom 24-well microtiter plate (Corning Costar, Rochester, NY, USA) in complete medium and allowed to stabilize overnight in a 5% CO_2 incubator at 37˚C. Afterwards, the cells were treated with compounds 5a-h at different concentrations (0, 10, 25, 50, 75 and 100 μM) in a final volume of 1 ml per well in triplicate wells for each treatment for 48 h at 37˚C in a 5% CO_2 incubator. The untreated cells (0 μM) were taken as control cells. All studies were repeated at least two times.

Evaluation of cell viability. The cell viability was evaluated using the crystal violet dye uptake assay according to our previously reported method (25, 26). Glutaraldehyde (400 μl of 0.25%) was added to each well and incubated for 30 min at room temperature (RT). The plates were rinsed with water to wash off the dead cells and dried under airflow inside a laminar hood for 5-10 min. Crystal violet (400 μl of 0.1%) was added to each well, incubated for 15 min, washed and dried. Finally, 1 ml of 0.05 M sodium phosphate solution (monobasic) in 50% ethyl alcohol was added to each well to solubilize the dye, and the plates were read at 540 nm in a plate reader (Bio-Tek EL800 Plate Reader, Winooski, VT, USA). The mean absorbance value of the control was considered as 100% and the treated sample percentages were calculated by comparing the treated samples absorbance with the mean absorbance of the control.

Table I. *The CC_{50} values (μM) for compounds 5a-h tested in PC-3 (prostate) and MDA-MB-231 (breast) cancer cell lines for a 48-h treatment. Data are represented as mean±SD, n=3.*

Compounds	CC_{50} (μM) mean±SD	
	PC 3	MDA-MB-231
 **5a**	68.70 μM±0.69	>100
 **5b**	43.30 μM±0.20	>100
 **5c**	67.80 μM±0.62	>100
 **5d**	41.10 μM±1.97	31.27 μM±1.75
 **5e**	78.73 μM±1.41	66.67 μM±1.44
 **5f**	26.43 μM±1.38 (a)	67.89 μM±0.78
 **5g**	47.20 μM±0.10	>100
 **5h**	36.80 μM±0.70	>100
TAMOXIFEN	29.97 μM±0.78	15.13 μM±0.09

[1]Data represent the average of triplicate values at various concentrations; [2]The cytotoxic concentration (CC_{50}) value was determined from the graph where the live and dead cells line graphs meet in the Graph pad Prism; [3]Drug effects were determined after 48 h exposure; [4](a): The most active compound (5f) showed no cytotoxic activity in normal prostate (WPE1-NA22) cell line (inactive; CC_{50} >100 μM).

Cell cycle analysis. The cell cycle analysis was evaluated using C6 Accuri flow cytometer (Accuri Cytometers, Ann Arbor, MI, USA) according to our previously reported method (25, 26). At the end of incubation, cells were trypsinized, pelleted, washed with PBS and resuspended in 1 ml of Vindelov's reagent (PBS 1× containing Ribonuclease A (10 μg/ml), Propidium Iodide (7.5 μg/ml) and IGEPAL CA- 630 (1 μl/ml). The cells were stained at 4˚C overnight and analyzed using a flow cytometer for the cells analysis at different phases at a low flow rate of ~150 cells/second or less.

Western blot analysis. The control and treated PC3 cell pellets were suspended in 100 μl of total protein cell lysis buffer (AMRESCO, Solon, OH, USA) containing EZBlock protease inhibitor cocktail (BioVision, Milpitas, California , USA) and incubated on ice for 30 min with periodic vortexing. The tubes were centrifuged at 14,000 g for 20 min at 4˚C. The supernatant was transferred to a fresh tube and stored at –80˚C freezer till further use. The protein concentration of cell lysates was quantitatively measured according to the kit manual using the Pierce BCA Protein Assay kit from Life Technologies (Grand Island, NY, USA). The protein (30 μg) from each sample in 1X SDS gel loading buffer was loaded into 15% SDS PAGE, run at 80 V and electrotransferred to a nitrocellulose membrane at 30 V for 16 h. The nitrocellulose membrane was washed with 25 ml of Tris buffered saline with 0.5% Tween 20 (TBS/T) buffer for 5 min at RT. The membrane was incubated in 10 ml of blocking buffer for 1 h at RT, and incubated with a primary antibody (1:1000 dilution) in 10 ml primary antibody dilution buffer with gentle agitation overnight at 4˚C. Later, the membrane was washed with 15 ml of TBS/T for 5 min each (3X), incubated with the appropriate HRP-conjugated secondary antibody (1:2,000) in 10 ml of blocking buffer with gentle agitation for 1 h at RT and then washed for 5 min each (3X) with 15 ml of TBS/T. The membrane was then incubated with 10 ml LumiGLO® (Cell Signaling Technology, Danvers, MA, USA) (0.5 ml 20X LumiGLO®, 0.5 ml 20X Peroxide and 9.0 ml Milli-Q water) with gentle agitation for 5 min at RT. The excess developing solution was drained from the membrane, wrapped in plastic wrap and exposed to x-ray film or to a ChemiDoc machine. The density of the bands on the blot was quantified using the Un-Scan-It get TM program (Silk scientific, Inc., Orem, UT, USA).

Measurement of Caspases 3, 8 and 9 activity. The involvement of Caspases 3, 8, and 9 in cytotoxic activity was determined using Caspase inhibitors. The cells were plated at a density of 5×10^4 cells per well in a polystyrene, flat bottom 24-well microtiter plate (Corning Costar, Rochester, NY, USA) in complete medium and allowed to stabilize overnight in a 5% CO_2 incubator at 37˚C. The cells were pre-treated with 20 μM Z-DEVD-fmk (Caspase 3), Z-IETD-fmk (Caspase 8), and Z-LEHD-fmk (Caspase 9) inhibitors for 1 h and then treated with compound 5f at different concentrations (0, 15 and 30 μM) in a final volume of 1 ml per well in triplicate wells for each treatment for 48 h at 37˚C in a 5% CO_2 incubator. After 48 h, the viability of cells was measured using the crystal violet dye viability assay.

Measurement of mitochondrial membrane potential (MMP). The loss of MMP was evaluated using rhodamine-123 fluorescent dye according to our previously reported method (25). The cells were plated at a density of 5×10^4 cells per well in a polystyrene, flat bottom 24-well microtiter plate (Corning Costar, Rochester, NY, USA) in complete medium and allowed to stabilize overnight in a 5% CO_2 incubator at 37˚C. Afterwards, the cells were treated with different concentrations (0, 10, 25, 50, 75 and 100 μM) of compound 5f in a final volume of 1 ml per well in triplicate wells for each treatment for 24 h. At the end of the incubation, cells were fixed with 400 μl of 0.25% aqueous glutaraldehyde containing 1 μM rhodamine-123 for 30 min at RT. The cells were rinsed with tap water and dried in air-flow hood for 10 min. The plates are then read with the excitation filter set at 485 nm and the emission filter at 538 nm in a Tecan Infinite F200 Pro plate reader (Tecan, Seestrasse, Männedorf, Switzerland).

Measurement of reactive oxygen species (ROS) production. The ROS production was evaluated using DCFDA dye according to our previously reported method (25). The cells at a concentration of 20,000 cells/ well in a final volume of 80 μl of PBS were plated in a 96-well black plate. Then 10 μl of DCFDA (1 mM) was added to each well and incubated for 30 min and then treated with 0, 10, 25, 50, 75, and 100 μM of the compound 5f for 30 min. The fluorescence was read in a Tecan Infinite F200 Pro plate reader (Tecan, Seestrasse, Männedorf, Switzerland) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

Measurement of glutathione (GSH) level. The GSH level in the PC-3 cells was quantitatively measured using GSH-Glo™ Glutathione Assay Kit (Promega, Madison, WI, USA). The experiment was performed following manual instructions. The cells at a concentration of 20,000 cells/well in a final volume of 90 μl of medium were plated in a 96-well white plate. After 30 min incubation with compound 5f, the cells were centrifuged at 1000 rpm for 5 min in a Hettich Universal 320 R centrifuge. Then, the medium was removed and 100 μl of GSH-Glo™ reaction buffer was added and incubated for 30 min in an incubator. Following this step, 100 μl of luciferin detection reagent was added and again incubated for 30 min in an incubator. The luminescence in the well of a plate was read in Tecan Infinite F200 Pro plate reader (Tecan, Seestrasse, Männedorf, Switzerland).

Statistical analysis. The data were presented as mean±standard deviation (SD, n=3). All data from treated cells were presented as percentage values in comparison to the untreated control (100%). The data were analyzed for significance by one-way ANOVA, and then compared by Dunnett's multiple comparison tests, using the GraphPad Prism Software, version 3.00 (GraphPad Software, Inc., San Diego, CA, USA). Differences with the respective untreated control were considered statistically significant when $p<0.05$. The viability and cytotoxic concentration (CC_{50}) graphs were plotted using the Prism 3.00 software (GraphPad Software, Inc., San Diego, CA, USA). The CC_{50} (cytotoxic concentration at which 50% of the cells die) value was calculated from the graph where the live and dead cells line graphs meet using the Prism 5 Software. In the apoptosis array analysis, protein expression in cell lysate of the treated samples with 30% more or less protein expression than the control cell lysate was considered significant.

Results

Cytotoxic effect of compounds 5a-h in cancer cell lines. We evaluated the *in vitro* cytotoxic activity of 7,8-Diacetoxy-3-arylcoumarins (5a-h) at different concentrations (0, 25, 50, 75 and 100 μM) in PC-3 and MDA-MB-231 cells after 48 h

treatment using crystal violet dye binding assay. The CC_{50} values for compounds 5a-h are listed in Table I and indicated that compounds 5a-h showed cytotoxic activity (CC_{50}=26.43 μM to 78.73 μM) in PC-3 cell line, while compounds 5d-f showed cytotoxic activity (CC_{50}=31.27 μM to 67.89 μM) in MDA-MB-231 cell line with respect to untreated control cells. Overall, compound 5f (CC_{50}=26.43 μM) is the most active compound based on its cytotoxic activity in the PC-3 cell line. However, comparison of the cytotoxic activity of compound 5f with Tamoxifen (TAM), a currently used anti-cancer drug, revealed that (i) compound 5f and TAM showed similar cytotoxicity in PC-3 cell line, and (ii) compound 5f showed twofold decrease in cytotoxicity compared to TAM in MDA-MB-231 cell line (Table I). The *in vitro* cytotoxicity study also revealed that compound 5f did not show cytotoxic activity (CC_{50}>100 μM) in the non-cancerous human prostate (WPE1-NA22) cell line (Table I).

Compound 5f affects cell cycle. To explore the mechanisms leading to the higher cytotoxic activity of compound 5f (the most cytotoxic and selective compound) in PC-3 cells, we evaluated the effect of this compound on cell cycle progression for 24 h treatment. The control and compound 5f treated cells were stained with propidium iodide (PI) and the percentage of cells in the cell cycle phases were analyzed using a flow cytometer. Cell cycle analysis indicated that the percentage of PC-3 cells treated with compound 5f was increased significantly (p<0.05) in G_0/G_1 phase at 20 μM (4.15%±0.01) and 40 μM (15.56%±0.02) in a concentration-dependent manner with respect to the untreated control cells (Figure 2).

Compound 5f induces apoptosis in PC-3 cancer cells. To determine whether apoptosis contributes to compound 5f induced cell death in a cancer cell line, PC-3 cells were treated with 20 μM of compound 5f for 24 h and protein expression associated with apoptosis was evaluated using RayBio® Human Apoptosis Antibody Array. The array analysis results indicated an up-regulation of apoptotic proteins (30% more expression than the control untreated PC-3 cells) of which Smac/DIABLO (Second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low isoelectric point) was the most significant up-regulated protein (data not shown). Additionally, no statistically significant change in Cytochrome *c* release was observed. The array analysis results were further validated using western blot analysis. The results showed an up-regulation of Caspases 3/8, Bid and Smac/DIABLO proteins expression, and down-regulation of Cytochrome *c* and XIAP proteins expression (Figure 3). Interestingly, Bid and Smac/DIABLO proteins were the most expressed apoptotic proteins (Figure 3).

Compound 5f induces apoptosis by the activation of Caspases -3, and -8. To further confirm the involvement of Caspase(s) in compound 5f induced apoptosis, PC-3 cells were pretreated with Caspase 3 inhibitor (Z-DEVD-fmk), Caspase 8 inhibitor (Z-IETD-fmk), and Caspase-9 inhibitor (Z-LEHD-fmk) for 1 h, followed by compound 5f for 48 h treatment. In this investigation, it was observed that the cells viability increased with Caspases 3 (12.48%±1.032) and 8 (18.35%±1.164) inhibitors pretreatment, while the cells viability did not increase with Caspase 9 inhibitor pretreatment (Figure 4).

Compound 5f decreases mitochondrial membrane potential (MMP). To evaluate whether compound 5f induced apoptotic cell death by affecting mitochondria function, PC-3 cells were treated with the compound 5f for 24 h, and then stained with Rhodamine-123 dye. In this investigation, it was observed that the percentage of MMP decreased at 10 μM (5.55%±3.002), 25 μM (39.94%±3.244), 50 μM (58.43%±3.221), 75 μM (64.60%±1.736), and 100 μM (62.12%±0.600) respectively in compound 5f treated cells in comparison to the untreated control cells (Figure 5).

Compound 5f induces oxidative stress (ROS production). In order to evaluate whether compound 5f induced ROS production as well, PC-3 cells were treated with the compound 5f for 30 min and intracellular ROS level was measured using ROS detecting fluorescence dye, DCFDA. In this investigation, it was observed that treatment of PC-3 cells with the compound 5f resulted in an increased fluorescence intensity at 10 μM (48.38%±6.952), 25 μM (230.72%±15.118), 50 μM (365.79%±20.522), 75 μM (397.99%±13.641), and 100 μM (404.43%±19.013) respectively in comparison to the untreated control cells (100%) (Figure 6).

Compound 5f depletes glutathione levels. In order to evaluate whether compound 5f induced apoptotic cell death in PC-3 cells was the result of excessive ROS production that led to induction of oxidative stress, GSH levels were measured in control and compound 5f treated PC-3 cells using GSH-Glo™ Glutathione Assay Kit (Promega, Madison, WI, USA). The results indicated a concentration-dependent decrease in GSH level after 30 min with the compound 5f treatment in comparison to the control cells (100%). Furthermore, it was observed that compound 5f treated PC-3 cells showed a slight increase in GSH level at 10 μM (101.86%±2.493), followed by significant GSH depletion at 25 μM (88.34%±2.413), 50 μM (78.19%±0.654), 75 μM (71.55% ±3.336), and 100 μM (71.28%± 1.364) (Figure 7).

Discussion

Coumarins have attracted considerable interest over the years due to their diverse pharmaceutical activities. As part of our ongoing investigation on coumarins, we herein report the *in vitro* cytotoxicity evaluation of 7,8-Diacetoxy-3-arylcoumarins

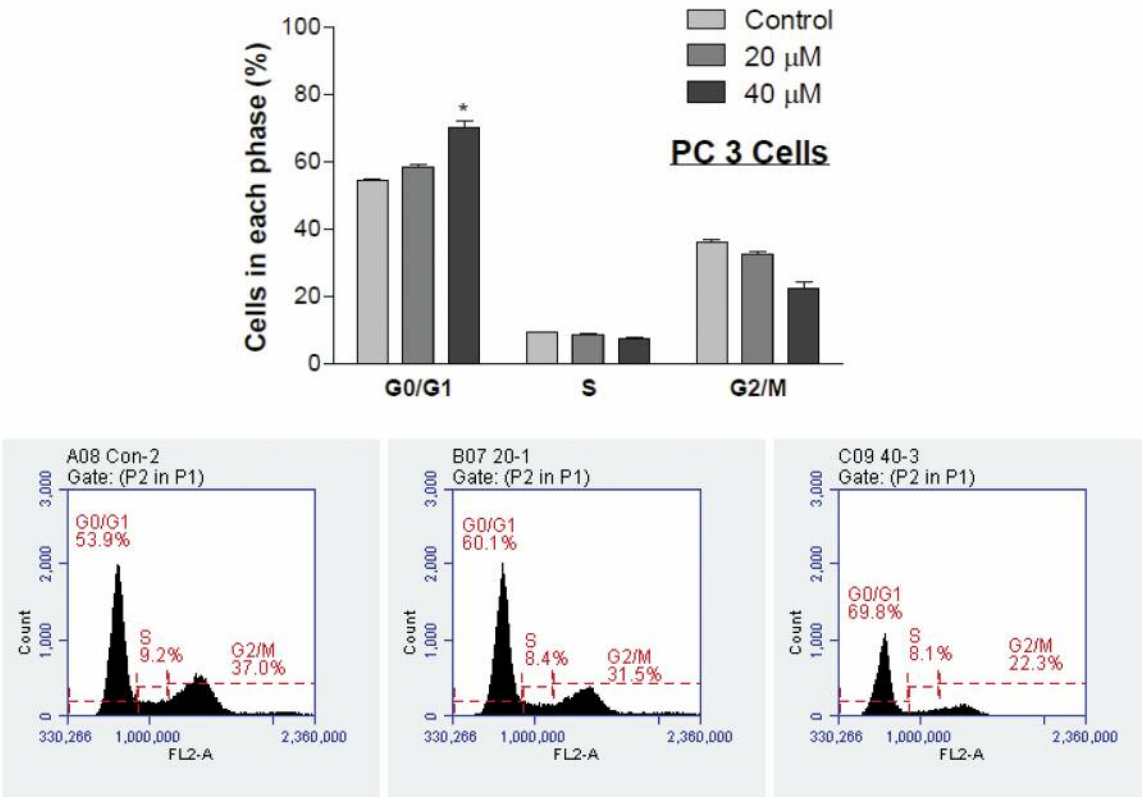


Figure 2. *Effect of compound 5f on cell-cycle progression in PC3 cells for 24 h treatment. Data are represented as mean±SD, n=3.*

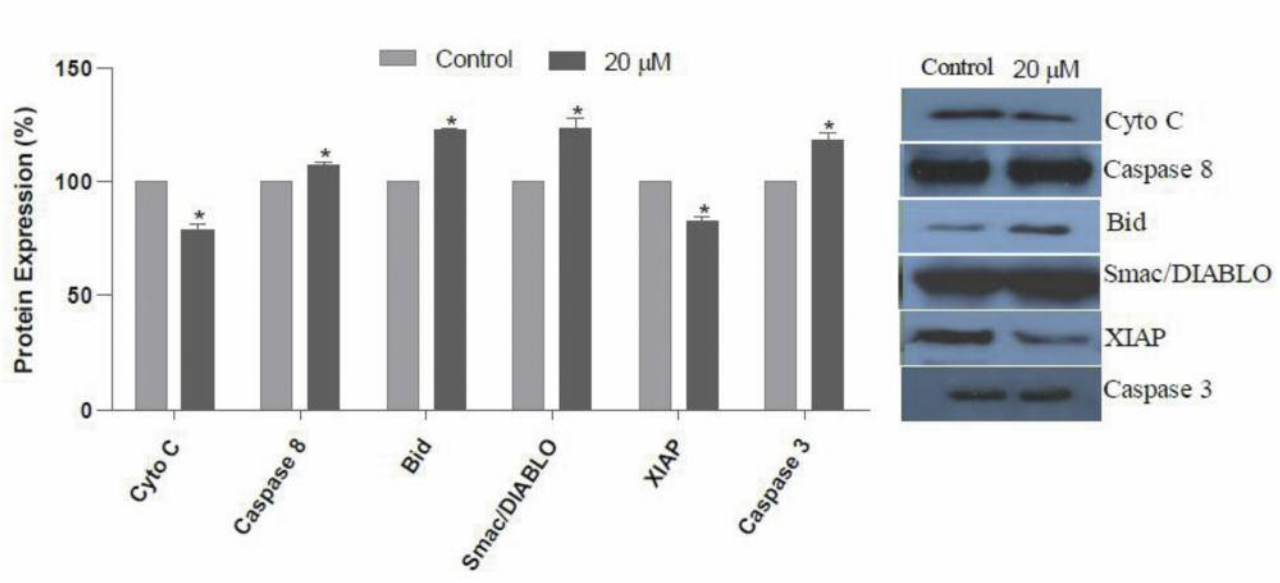


Figure 3. *Effect of compound 5f on Caspases 3/8, Bid, Smac/DIABLO, XIAP and Cytochrome c release proteins expression in PC3 cells after 24 h treatment using western blotting. Data are represented as mean±SD, n=3.*

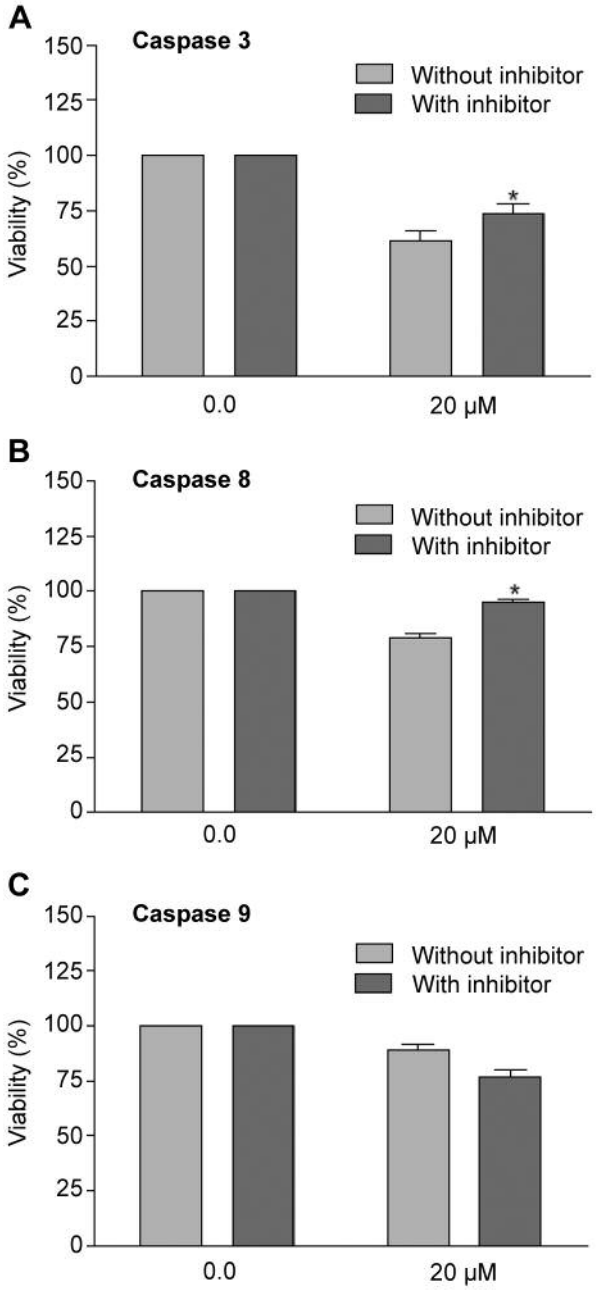


Figure 4. *Effect of Caspase 3 inhibitor (Z-DEVD-fmk), Caspase 8 inhibitor (Z-IETD-fmk) and Caspase-9 inhibitor (Z-LEHD-fmk) on compound 5f induced cell death of PC-3 cell for 48 h treatment. Data are represented as mean and SD, n=3.*

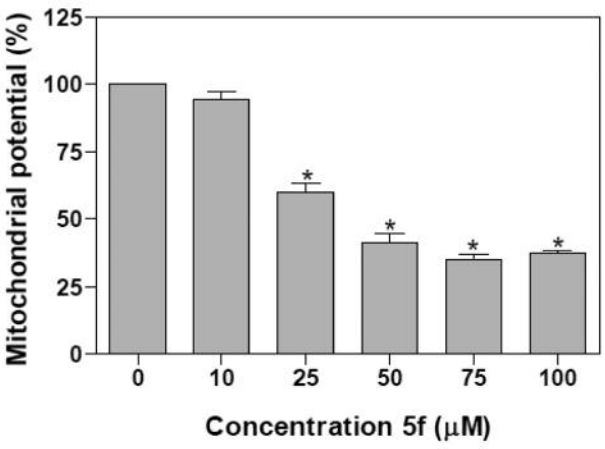


Figure 5. *Effect of compound 5f on mitochondrial membrane potential (MMP) in PC-3 cells for 24 h treatment. Data are represented as mean and SD, n=3.*

(5a-h) in PC-3 and MDA-MB-231 cells. Our *in vitro* cytotoxicity results indicated that compound 5f containing *p*-(methylsulfonyl)phenyl group at the C-3 position showed higher cytotoxic activity (26.43 μM±1.38) in PC-3 cell line, while compound 5d containing *p*-methoxyphenyl group at C-3 position showed higher cytotoxic activity (31.27 μM±1.75) in MDA-MB-231 cell line (Table I). Structure–activity relationship (SAR) study clearly indicates that the observed cytotoxic activity of 7,8-Diacetoxy-3-arylcoumarins (5a-h) depends on the cell line and aryl group substituted at the C-3 position (Table I). For example, the presence of phenyl, naphthalenyl, 3, 4, 5-trimethoxyphenyl, 3, 5-dimethoxy- phenyl and benzo[d][1,3]dioxol-6-yl groups at the C-3 position did not enhance the cytotoxic activity of 7,8-Diacetoxy-3-arylcoumarins (5a-c and 5g-h; CC_{50}>100 μM) in MDA-MB-231 cell line. Overall, compound 5f (CC_{50}=26.43 μM) was the most active compound based on its cytotoxic activity in PC-3 cell line and also did not show cytotoxic activity (CC_{50}>100 μM) in the non-cancerous human prostate (WPE1-NA22) cell line; an indication of selective cytotoxicity. This finding supports previous report that the presence of *p*-(methylsulfonyl)phenyl groups at the C-3 position of 7,8-Diacetoxy-3-arylcoumarin ring modulates selective cytotoxic activity in certain types of cancer cell lines (26). Furthermore, comparison of the cytotoxic activity of compound 5f with Tamoxifen (TAM) revealed that (i) compound 5f and TAM showed similar cytotoxicity in PC-3 cell line, and (ii) compound 5f showed twofold decrease in cytotoxicity compared to TAM in MDA-MB-231 cell line (Table I).

Cell cycle is a sequence of complex events by which cells grow and divide. Studies have demonstrated that many anti-cancer drugs exert their cytotoxic activity by arresting cells at various cell cycle phases (27, 28). In this present investigation, compound 5f arrested PC-3 cells in G_0/G_1 phase in a concentration-dependent manner with respect to the untreated control cells; indicating apoptotic mode of cell death (Figure

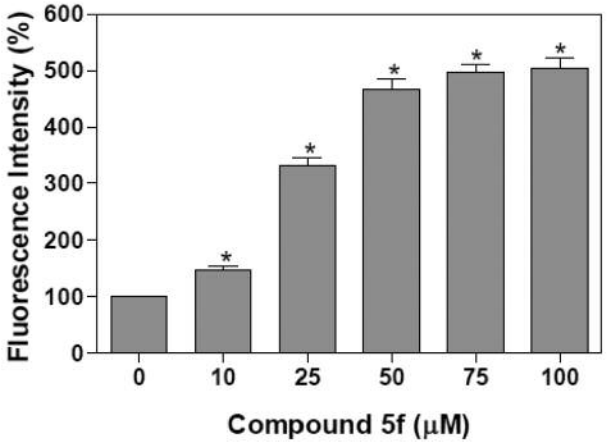


Figure 6. *Effect of compound 5f on reactive oxygen species (ROS) production in PC-3 cells. Data are represented as mean±SD, n=3.*

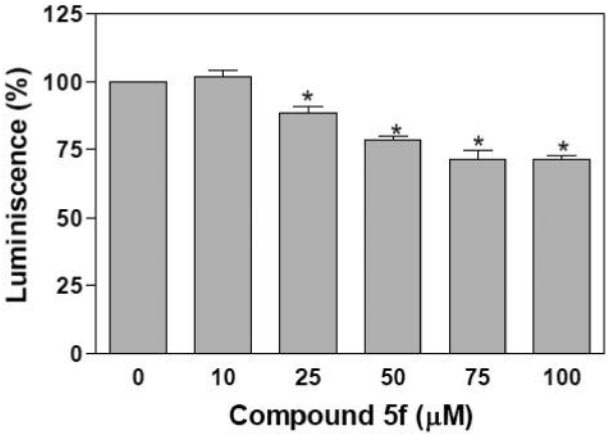


Figure 7. *Effect of compound 5f on GSH level in PC-3 cells. Data are represented as mean±SD, n=3.*

2). Based on this finding, we decided to explore whether compound 5f induced cytotoxic activity *via* apoptosis. Compounds that induce apoptosis are known to kill cancer cells by stimulating apoptotic signaling either through activation of extrinsic death-receptor-mediated or intrinsic mitochondrial-mediated pathways (29). Our results from western blot analysis confirmed that compound 5f induced apoptosis in PC-3 cell line through the mitochondrial (intrinsic) apoptotic signaling pathway that is independent of Cytochrome *c* release based on the up-regulation of Caspases 3/8, Bid and Smac/DIABLO proteins expression (Figure 3). This finding is supported by previous reports indicating that Bid serves as an initiator of Caspase 8 during Fas or TRAIL induced apoptotic signaling, leading to the generation of truncated Bid (tBid) that binds to the mitochondria and releases Smac/DIABLO from the mitochondria. Smac/DIABLO (pro-apoptogenic mitochondrial protein) interacts and neutralizes Inhibitors of apoptosis proteins (IAPs), thus allowing the activation of Caspases leading to apoptosis (30-33). It has also been reported that the interaction between Bcl2 family members, XIAP and Smac/DIABLO, plays a major role in the intrinsic apoptotic pathway (32). Furthermore, activation of Caspase-3, -8, and -9 are the most common processes occurring in the apoptotic signaling events (34, 35). The result from our Caspases measurement showed increase in the cells viability with either Caspase 3 (Z-DEVD-fmk) or Caspase 8 (Z-IETD-fmk) inhibitors pretreatment (Figure 4); indicating the involvement of Caspases 3/8 in compound 5f induced apoptotic cell death of PC-3 cells.

Mitochondria play a key role in the activation of apoptosis and previous reports have shown that a decrease in Rhodamine-123 fluorescence is directly proportional to the loss of MMP (36, 37). Thus, evaluation of MMP changes using Rhodamine-123, a green-fluorescent dye, is an important tool for monitoring the effect of chemicals on mitochondrial function in living cells (38). Our result from the MMP measurement showed a decrease in fluorescence intensity compared to the untreated control cells in PC-3 cells after 24 h treatment with Rhodamine-123 dye; indicating loss of MMP (Figure 5). This finding is consistent with previous studies suggesting that 7,8-Diacetoxy-3-arylcoumarins caused loss of MMP in initiating cell death (25, 26). Furthermore, mitochondria are an important source of ROS production in cells and chemotherapeutic agents that raised ROS level above a safe threshold can eventually lead to cell death *via* apoptotic or other mechanisms (39, 40). The result from our intracellular ROS level measurement in PC-3 cells showed concentration-dependent increase in ROS production compared to the untreated control cells (100%) (Figure 6). This finding is consistent with previous studies suggesting that 7,8-Diacetoxy-3-arylcoumarins elevated the level of ROS production in initiating cell death (25, 26, 41).

Glutathione (GSH, L-γ-glutamyl-cysteinyl-L-glycine) is a natural antioxidant that neutralizes harmful ROS produced in normal cells and its depletion plays a central role in cell death, including apoptotic cell death. An increase in ROS generation is known to induce oxidative stress in response to redox alteration. One way to selectively kill cancer cells without causing significant toxicity is to change the ROS levels using GSH modulation. GSH depletion causes increase susceptibility to oxidative stress and served as a potent activator of apoptosis signaling (42, 43). Our evaluation of whether compound 5f induced apoptotic cell death was the result of excessive ROS production by measuring GSH levels indicates a significant GSH depletion (Figure 7). This result supports oxidative stress since GSH depletion elevates ROS level in the cells and *vice versa* (43).

In conclusion, our studies demonstrated that compound 5f containing the *p*-methylsulfonylphenyl groups at C-3 position exhibits highest cytotoxicity and selectivity against PC-3 cell line *vs*. the non-cancerous WPE1-N22 cell line. The cytotoxic mode of action of compound 5f in PC-3 cells is associated with cell cycle arrest at G_0/G_1 phase, loss in mitochondrial membrane potential (MMP), induction of ROS production, depletion of GSH level and apoptosis through mitochondrial (intrinsic) apoptotic signaling pathway that is independent of Cytochrome *c* release as a result of oxidative stress. The present investigation revealed that *p*-methylsulfonylphenyl group on the 7,8-Diacetoxy-3-arylcoumarin ring is critical in modulating selective cytotoxic activity and induction of apoptosis. The present study provided a new insight into how compound 5f induced cell death in PC-3 cells, that may be helpful in the development of future promising therapeutic agents for prostate cancer treatment.

Conflicts of Interest

The Authors declare that they have no financial or non-financial competing interests.

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