

## 7,8-Dihydroxy-3-arylcoumarin Induces Cell Death Through S-Phase Arrest in MDA-MB-231 Breast Cancer Cells

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**Abstract.** *Background/Aim:* Coumarins remain one of the most versatile classes of compounds for anticancer drug design and discovery. The present study aimed to evaluate the *in vitro* cytotoxic activity of 7,8-Dihydroxy-3-arylcoumarin derivatives (7a-i) in A549, MDA-MB-231 and PC-3 cancer cell lines. *Materials and Methods:* Cell viability, cell-cycle progression and regulatory protein expression were evaluated using crystal violet dye-binding assay, flow cytometry and western blot analysis. *Results:* 7,8-Diacetoxy-3-(4-nitrophenyl)coumarin (7h) showed the highest cytotoxic activity with  $CC_{50}$  of  $7.51 \pm 0.07 \mu M$  in MDA-MB-231 cell line. The mechanism of cytotoxic action indicated that 7h caused significant ( $p < 0.05$ ) MDA-MB-231 cells arrest in the S phase as well as moderate cells arrest in the G<sub>2</sub>/M phase; confirmed by up-regulation of cyclins A/B1, p21 and CDKs 4/6, and down-regulation of cyclin E2 and CDK2 regulatory proteins. *Conclusion:* These results suggest that 7h could serve as a valuable template for the development of novel synthetic compounds for breast cancer treatment.

The parent compound name “Coumarin” comes from “Coumarou”, a vernacular name for the tonka bean (*Dipteryx odorata* Wild). Coumarins (2H-1-benzopyran-2-one) are naturally-occurring oxygen heterocyclic compounds found throughout the plant and can be synthesized chemically (1). Naturally-occurring coumarins (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 (2). Synthetic coumarins (SCs) on the other hand have been the focus of chemical modification of the coumarin skeleton, in order to synthesize novel analogs with improved therapeutic activity and reduced toxicity (3, 4). Coumarins whether NOCs or SCs possess diverse biological activities such as anti-coagulant, anti-cancer, anti-tuberculosis, anti-thrombotic, anti-microbial, anti-inflammatory, anti-asthmatic, anti-viral, anti-platelet, anti-Alzheimer and anti-oxidant (5-8). These diverse pharmacological and biochemical activities are due to their structural diversity, attributed to the nature of substituent and its pattern of substitution on the core coumarin molecule (9-11). Interestingly, coumarins remain one of the most versatile class of compounds for anti-cancer drug design and discovery. They are known to target a number of pathways in cancer such as inhibition of kinases, cell-cycle phases, angiogenesis, heat shock protein (HSP90), telomerase, anti-mitotic activity, carbonic anhydrase, monocarboxylate transporters, aromatase and sulfatase (12).

For the last few years, our group has been deeply interested in the synthesis and biological evaluation of 3-Arylcoumarins as potential anti-cancer agents. Recently, we demonstrated that 7,8-Dihydroxy-3-(4-nitrophenyl)coumarin (7,8-Dihydroxylated Coumarin, DHNPC, Figure 1; 1) showed higher cytotoxic activity than 7,8-Diacetoxy-3-(4-nitrophenyl)coumarin (7,8-Diacetylated Coumarin, DANPC, Figure 1; 2) in HepG2 cell line (13). Additionally, studies have shown that 7,8-Dihydroxycoumarin (DHC, Figure 1; 3), a 7,8-Dihydroxylated coumarin, possess anti-cancer activity in different human cancer cell lines (14-16). Interestingly, coumarins are known to target a number of pathways (such as cell cycle phases) in cancer (17). As part of our ongoing investigation involving 7,8-Dihydroxy-3-arylcoumarins as potential anti-cancer agents, we herein report the evaluation of the *in vitro* cytotoxic activity of 7,8-Dihydroxy-3-arylcoumarins (7a-i, Table I) in human lung (A549), breast (MDA-MB-231) and prostate (PC-3) cancer cell lines in comparison to current drugs, tamoxifen (TAM) and docetaxel (DOC). In addition, mode of action of the most active compound on cell-cycle progression and regulatory protein expressions were also investigated.

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**Key Words:** 7,8-Dihydroxy-3-arylcoumarins, *in vitro* cytotoxicity, cell-cycle arrest, cyclins, cyclin-dependent kinases.

Table I. The  $CC_{50}$  values ( $\mu M$ ) for compounds **7a-i** tested in A549 (Lung), MDA-MB-231 (Breast) and Prostate (PC-3) cancer cell lines after 48 h treatment.

	Compounds	$CC_{50}$ ( $\mu M$ ) mean $\pm$ SD		
		A549	MDA-MB-231	PC-3
<b>7a</b>		26.40 $\mu M \pm 2.31$	56.30 $\mu M \pm 0.35$	56.7 $\mu M \pm 4.11$
<b>7b</b>		>100	66.67 $\mu M \pm 1.14$	>100
<b>7c</b>		20.37 $\mu M \pm 0.23$	23.30 $\mu M \pm 0.67$	61.4 $\mu M \pm 0.72$
<b>7d</b>		13.00 $\mu M \pm 1.37$	68.60 $\mu M \pm 0.62$	39.3 $\mu M \pm 0.58$
<b>7e</b>		49.27 $\mu M \pm 0.20$	22.00 $\mu M \pm 0.25$	37.50 $\mu M \pm 0.39$
<b>7f</b>		21.70 $\mu M \pm 0.17$	85.77 $\mu M \pm 0.37$	46.00 $\mu M \pm 0.15$
<b>7g</b>		26.40 $\mu M \pm 2.31$	56.30 $\mu M \pm 0.35$	56.7 $\mu M \pm 4.11$
<b>7h</b>		9.70 $\mu M \pm 0.27$	7.51 $\mu M \pm 0.07$	20.6 $\mu M \pm 0.55$
<b>7i</b>		56.75 $\mu M \pm 1.25$	22.75 $\mu M \pm 1.05$	22.8 $\mu M \pm 1.05$
	<b>Tamoxifen</b>	32.30 $\mu M \pm 0.71$	16.03 $\mu M \pm 0.83$	32.8 $\mu M \pm 0.45$
	<b>Docetaxel</b>	9.40 $\mu M \pm 0.07$	7.30 $\mu M \pm 0.03$	9.44 $\mu M \pm 0.44$

Data represent the average of triplicate from three experiments. 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. Drugs effects were determined after 48 h treatment.

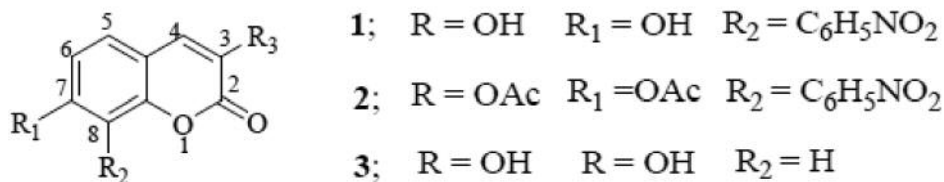


Figure 1. Structures of compounds. 7,8-Dihydroxy-3-(*p*-nitrophenyl)coumarin (DHNPC, 1), 7,8-Diacetoxy-3-(*p*-nitrophenyl)coumarin (DANPC, 2), and 7,8-Dihydroxycoumarin (DHC, 3).

## Materials and Methods

**Chemicals.** F12K and DMEM-F12 media, penicillin-streptomycin anti-biotic solution (100×), fetal bovine serum (FBS), trypsin-EDTA solution (1×), phosphate buffered saline (PBS), 50% glutaraldehyde, crystal violet, IGEAL CA-630, propidium iodide, tamoxifen, docetaxel and RNase were obtained from Sigma-Aldrich company (St. Louis, MO, USA). Total protein cell lysis buffer was obtained from AMRESCO (Solon, OH, USA). The EZ Block protease inhibitor cocktail (BioVision, Milpitas, CA, USA) was obtained from VWR International (Suwanee, GA, USA). p21<sup>Waf1/Cip1</sup>(12D1) rabbit mAb 2947, CDK2 rabbit mAb 2556, cyclin A2 mouse mAb 4639, cyclin B1 rabbit mAb 12231, cyclin E2 Ab 4132, CDK 4 rabbit mAb 12790, and CDK6 mouse mAb 3136 were purchased from Cell Signaling Technology (Danvers, MA, USA).

**Cell line maintenance.** The human lung (A549), breast (MDA-MB-231) and prostate (PC-3) cancer cell lines 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 (A549, PC-3) and DMEM-F12 (MDA-MB-231) media containing 100 units of penicillin/ml, 100 µg of streptomycin/ml, 2 mM L-glutamine and 10% FBS in T-75 cm<sup>2</sup> flasks at 37°C in a 5% CO<sub>2</sub> incubator.

**Treatment and measurement of cell viability.** The cells (MRC-9, MDA-MB-231 or PC-3) were plated at a density of 5×10<sup>4</sup> cells per well in a polystyrene, flat bottom 24-well microtiter plate (Corning Costar, Rochester, NY, USA) in cell type pertaining medium containing 10% FBS and allowed to stabilize overnight in a 5% CO<sub>2</sub> incubator at 37°C. Afterwards, the cells were treated with compounds (7a-i) at different concentrations of (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<sub>2</sub> incubator. All studies were repeated at least twice. At the end of incubation period, the viability was evaluated by crystal violet dye uptake assay and cytotoxic concentration (CC<sub>50</sub>) was calculated according to our previously reported method (6). Glutaraldehyde (400 µl of 0.25%) was added to each well and incubated for 30 min at room temperature (RT) to fix the cells. The glutaraldehyde (0.07% final concentration in the well) in the crystal violet dye staining assay procedure fixed the viable cells after treatment with the compound. 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. To solubilize the dye, 1 ml of 0.05 M sodium phosphate solution (monobasic) in 50% ethyl alcohol was

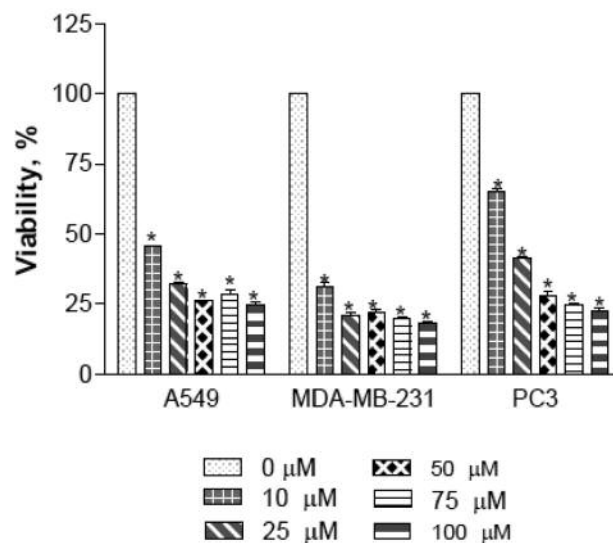


Figure 2. The cytotoxic activity of the most active compound 7h. The cells were treated with compound 7h for 48 h and the viability was measured by crystal violet binding assay.

added to each well 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 control samples was considered as 100% and the treated sample percentages were calculated by comparing the treated samples absorbance with the control.

**Cell cycle analysis.** Cell cycle analysis was carried out according to our previous report (6) by treating 1.3×10<sup>6</sup> MDA-MB-231 cells / T-25 flask in complete medium, in triplicate flasks for each treatment with 7h (0, 5, 10 µM) for 24 h. 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 IGEAL CA- 630 (1 µl/ml). The cells were stained at 4°C overnight and analyzed using a flow cytometer for cell cycle analysis at different phases, at a low flow rate of ~150 cells/sec or less.

**Preparation of samples for western blot.** Approximately 4.4×10<sup>6</sup> MDA-MB-231 cells were plated in T-175 cm<sup>2</sup> flasks in complete DMEM-F12 medium and allowed to stabilize overnight. The cells

were treated with 7h at different concentrations (0, 20 and 40  $\mu$ M) and incubated for 24 h in a 5% CO<sub>2</sub> incubator at 37°C. At the end of incubation, the cells were trypsinized and pelleted by centrifuging at 2,500 rpm for 5 min. The control and treated cell pellets were suspended in 100  $\mu$ l 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 a -80°C freezer till further use.

**Protein estimation.** The protein concentration of cell lysates was quantitatively measured using the Pierce BCA Protein Assay (Life technologies, Grand Island, NY, USA). Different concentrations of albumin standard (BSA) and working reagent were prepared according to the kit's manual. Each standard concentration and 1:10 diluted cell lysate (25  $\mu$ l) were pipetted in triplicate wells in a 96-well plate and 200  $\mu$ l of working reagent was added to each well. The plate was incubated at 37°C for 30 min and then read at 540 nm in a plate reader (Bio-Tek EL800 Plate Reader, Winooski, VT, USA). The protein concentration of cell lysate was determined using the generated standard curve by the KC Junior plate reader software.

**Western blot analysis.** The protein (30  $\mu$ g) from each sample in 1X SDS gel loading buffer was loaded into 15% SDS PAGE, run at 80 V and electro-transferred to a nitrocellulose membrane at 30 V for 16 h. The western blot analysis was performed according to manual instructions by Cell Signaling Technology. 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 (5% Non-fat milk in TBS/T buffer) for 1 h at RT, and incubated with a primary antibody (1:1,000 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 (3 $\times$ ), 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 (3 $\times$ ) with 15 ml of TBS/T. The membrane was then incubated with 10 ml LumiGLO® (Cell Signaling Technology, Danvers, MA, USA) (0.5 ml 20 $\times$  LumiGLO®, 0.5 ml 20 $\times$  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, and the membrane was wrapped in plastic wrap followed by imaging using 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).

**Statistical analysis.** The data were presented as mean $\pm$ standard deviation (SD, n=3). All treated cells data 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 from the respective untreated control were considered statistically significant when  $p < 0.05$ . The viability and CC<sub>50</sub> graphs were plotted in Prism 3.00 software (GraphPad Software, Inc., San Diego, CA, USA). The CC<sub>50</sub> value was deduced from the point of the graph where the live and dead cells lines meet (the dose at which 50% of the cells dies) using the Prism 5 software.

## Results

**Cytotoxic activity of compounds 7a-i in cancer cell lines.** In the present investigation, the *in vitro* cytotoxic activity of 7,8-Dihydroxy-3-arylcoumarins (7a-i) at different concentrations (0, 25, 50, 75 and 100  $\mu$ M) were analyzed in human lung (A549), breast (MDA-MB-231) and prostate (PC-3) cancer cell lines after treatment for 48 h, using crystal violet dye binding assay. The CC<sub>50</sub> values of 7a-i, TAM and DOC are shown in Table I. The *in vitro* cytotoxic activity results indicate that 7h containing *p*-nitrophenyl- group at the Carbon-3 (C-3) position showed the highest cytotoxic activity in A549 (CC<sub>50</sub>=9.70 $\pm$ 0.27  $\mu$ M), MDA-MB-231 (CC<sub>50</sub>=7.51 $\pm$ 0.07  $\mu$ M) and PC-3 (CC<sub>50</sub>=20.60 $\pm$ 0.55  $\mu$ M) cancer cell lines with respect to untreated control cells (Figure 2). Overall, 7h showed the highest cytotoxic activity (CC<sub>50</sub>=7.51  $\mu$ M) in MDA-MB-231 cell line. Comparison of the cytotoxic activity of 7h with TAM and DOC (currently used anti-cancer drugs) revealed that (i) 7h showed higher cytotoxic activity in A549 (three-fold), MDA-MB-231 (two-and-a-half fold) and PC-3 (one-and-a-half fold) cell lines compared to TAM, (ii) 7h and docetaxel showed similar cytotoxic activity in A549 and MDA-MB-231 cell lines, and (iii) 7h showed two-fold decrease in cytotoxic activity compared to DOC in PC-3 cell line (Table I).

**Compound 7h induced cell cycle arrest in S-phase in breast cancer cells.** To explore the molecular target leading to the higher cytotoxic activity of the most active compound 7h in MDA-MB-231 cells, we evaluated the effect of this compound (5, 10  $\mu$ M) on cell cycle progression after 24 h treatment. The control and treated cells were stained with propidium iodide (PI) and the percentage of cells in each cell cycle phase was analyzed using a flow cytometer. As shown in Figure 3, the percentage of cells at S phase increased significantly from 20.2 $\pm$ 0.6% (control) to 25.0 $\pm$ 0.3% (5  $\mu$ M) and 33.1 $\pm$ 1.0% (10  $\mu$ M) in a concentration-dependent manner and moderately at G<sub>2</sub>/M phase from 41.2 $\pm$ 0.7% (control) to 44.2 $\pm$ 0.7% (5  $\mu$ M) with respect to the untreated control cells.

**Compound 7h modulated S phase regulatory proteins in breast cancer cells.** Based on the above finding that 7h modulates S phase cell cycle arrest, we evaluated the expression of cell cycle regulatory proteins (cyclins A, B, and E; CDKs 2, 4, and 6; and CDK inhibitor (p21<sup>Waf1/Cip1</sup>(12D1))) in treated MDA-MB-231 cells (5,10  $\mu$ M) after 24 h treatment. Western blotting analysis results showed that the expression levels of cyclins A2 (154.8 $\pm$ 2.5 at 5  $\mu$ M and 219.6 $\pm$ 6.2 at 10  $\mu$ M; Figure 4A), cyclins B1(135.2 $\pm$ 0.3 at 5  $\mu$ M and 116.7 $\pm$ 0.4 at 10  $\mu$ M; Figure 4B), CDK 4 (129.1 $\pm$ 0.9 at 5  $\mu$ M and 213.9 $\pm$ 1.8 at 10  $\mu$ M; Figure 4E) and CDK 6 (143.9 $\pm$ 0.7 at 5  $\mu$ M and 121.7 $\pm$ 1.0 at 10  $\mu$ M; Figure 4F) proteins as well as CDK inhibitor p21<sup>Waf1/Cip1</sup>(12D1) (139.4 $\pm$ 0.5 at 5  $\mu$ M and

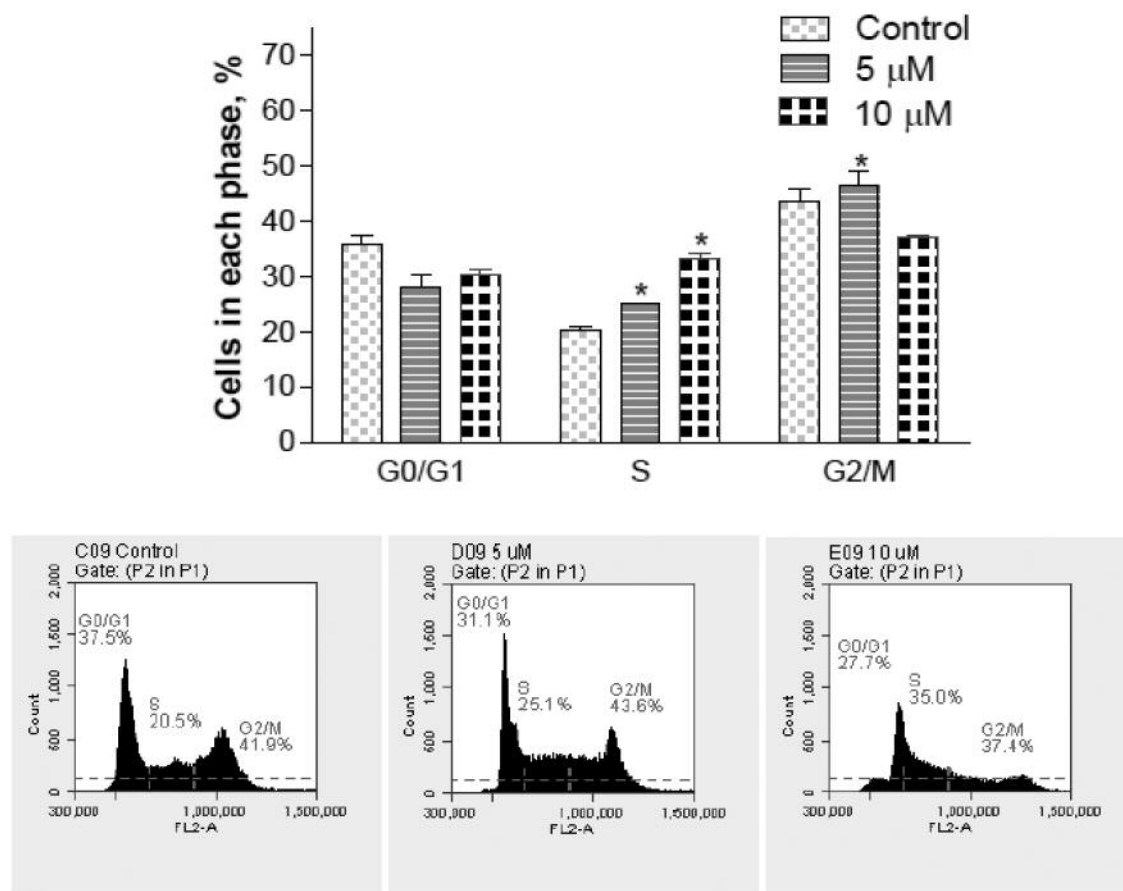


Figure 3. Effect of compound 7h on cell-cycle progression in MDA-MB-231 cells. The cells were treated with compound 7h for 24 h and cells in each phase were analyzed by flow cytometry. Data are represented as mean and SD,  $n=3$ . \*Statistically significant difference from the control ( $p<0.05$ ) using Dunnett's multiple comparison test.

130.2% $\pm$ 0.2 at 10  $\mu$ M; Figure 4G) were up-regulated compared to untreated control cells (100%). Furthermore, the expression levels of cyclin E2 (100.8% $\pm$ 0.5 at 5  $\mu$ M and 88.6% $\pm$ 0.1 at 10  $\mu$ M; Figure 4C) and CDK 2 (86.7% $\pm$ 0.1 at 5  $\mu$ M and 88.2% $\pm$ 0.5 at 10  $\mu$ M; Figure 4D) were down-regulated compared to untreated control cells (100%).

## Discussion

Coumarins are important naturally-occurring compounds used in drug discovery and still remain as one of the most versatile class of compounds against cancer cells. As part of our ongoing investigation involving 7,8-Dihydroxy-3-arylcoumarins as potential cytotoxic agents, we herein report that 7h containing *p*-nitrophenyl- group at the C-3 position showed the highest cytotoxic activity in A549, MDA-MB-231 and PC-3 cancer cell lines with respect to untreated control cells (Table I). Based on structure activity relationship study (SARs), the presence of *p*-nitrophenyl- group (containing NO<sub>2</sub>, strong electron-

withdrawing group) enhanced the cytotoxic activity of 7,8-Dihydroxy-3-arylcoumarins significantly compared to benzo[d][1,3]dioxol-6-yl-; 3, 4, 5-trimethoxy phenyl-; 3, 5-dimethoxyphenyl-; 4-methoxyphenyl-; naphthalenyl-; phenyl-; 4-hydroxyphenyl-; 4-(methyl sulfonyl)phenyl- groups in A549, MDA-MB-231 and PC-3 cancer cell lines (Table I). This finding is consistent with previous finding from our research group indicating that *p*-nitrophenyl- group is a good structural feature for enhancing the cell-specific cytotoxic activity of 7,8-Dihydroxy-3-arylcoumarin in certain types of cancer cell lines (18). Furthermore, comparison of the cytotoxic activity of 7h with TAM or DOC revealed that 7h showed a higher cytotoxic activity in the order of A549>MDA-MB-231>PC-3 cancer cell lines compared to TAM, similar cytotoxic activity in A549 and MDA-MB-231 cell lines and lower cytotoxic activity in PC-3 cell line compared to DOC (Table I).

Coumarins have been reported to exert their cytotoxic activity by arresting cells in different phases (G<sub>0</sub>/G<sub>1</sub> or S or G<sub>2</sub>/M phase) of the cell cycle depending on the cell line (19).

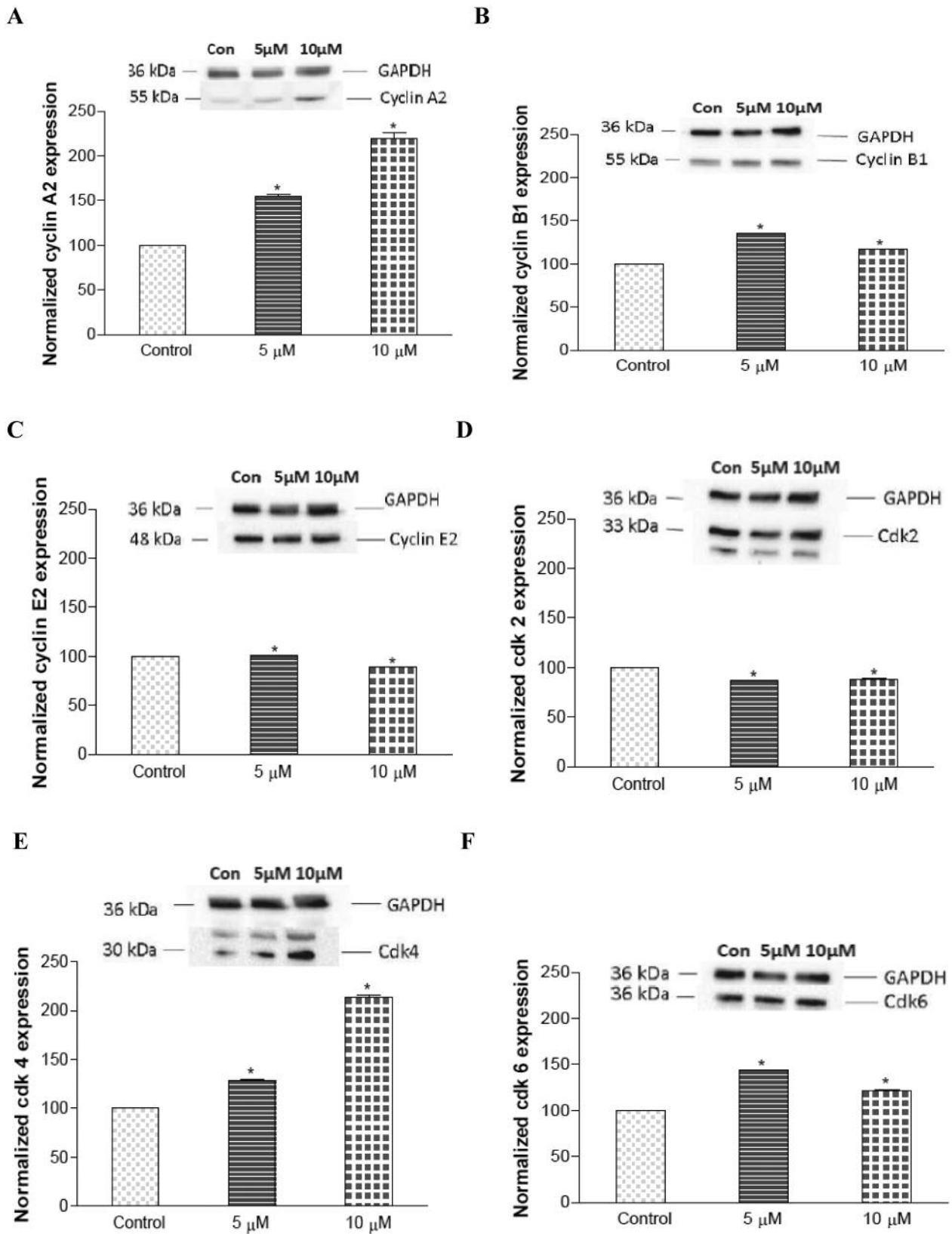


Figure 4. Continued

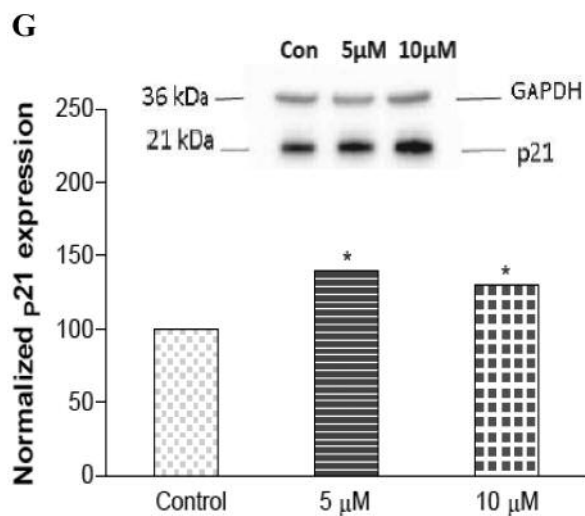


Figure 4. Effect of compound 7h on cell-cycle regulatory proteins. MDA-MB-231 cells were treated with compound 7h for 24 h and expression of cyclins A, B, and E; CDKs 2, 4, and 6; and p21<sup>Waf1/Cip1(12D1)</sup> proteins was measured using western blotting. Data are represented as mean±SD, n=3.

Cell cycle is a sequence of complex events by which cells grow and divide. Therefore, control of cell-cycle progression may be a successful strategy for halting tumor growth since cancer cells show uncontrolled growth (20). In the present investigation, 7h arrested MDA-MB-231 cells in S phase in a concentration-dependent manner with respect to the untreated control cells; indicating that one of the mechanisms by which 7h induced cell death in MDA-MB-231 cells is by inhibition of DNA synthesis (Figure 3). The progression of cells through the different phases of cell cycle is regulated by cyclins and CDK (cyclin dependant kinases) proteins. These cell-cycle regulatory proteins are potential molecular targets for cancer therapy/prevention because their functions are regulated differently in cancer cells and normal cells (21). Cyclin A and CDK2 are known to play an important role in the regulation of DNA synthesis during cell-cycle progression at S phase and are deregulated or up-regulated in several cancers such as breast, liver, and lung (22, 23). Furthermore, previous reports have shown that the 1) synthesis of cyclin A starts in S phase and reaches maximal levels at the S/G<sub>2</sub> transition; 2) cyclin B starts at the end of S phase and reaches maximal levels as the cell enters into phase M; and 3) cyclin E begins in mid-phase G<sub>1</sub>, reaches maximal levels at the G<sub>1</sub>/S transition and is degraded during S phase (24). The S phase cell-cycle arrest has been reported to occur with the loss of Cdk2 activity due to up-regulation of p21<sup>Waf1/Cip1(12D1)</sup>, a well-known inhibitor of Cdk 2 (25-27). CDK4 and CDK6 have been reported to help drive the progression of cells into the S (DNA synthetic) phase of the

cell cycle in mammalian cells (28). Therefore, based on western blotting analysis (Figure 4), it can be speculated that 7h induced cell death in MDA-MB-231 cells by altering expression of cell cycle regulatory proteins, which govern the S/G<sub>2</sub> phase cell-cycle progression.

In conclusion, our studies demonstrated that 7h containing the *p*-nitrophenyl- group at Carbon-3 position showed the highest cytotoxic activity in MDA-MB-231 cell line. The cytotoxic mode of action of 7h in MDA-MB-231 cells was associated with cell cycle arrest at S phase through up-regulation of cyclins A and B1, CDKs 4/6 and p21<sup>Waf1/Cip1(12D1)</sup>, and down-regulation of cyclin E1 and CDK2 regulatory proteins. This compound also showed higher cytotoxic potency than the standard anti-cancer drug, TAM. Thus, the present investigation provided a new insight on how 7h induced cell death in MDA-MB-231 cell line, which may be helpful in the development of future promising therapeutic agents for breast cancer treatment.

## Conflicts of Interest

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

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