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Synthesis and *in vitro* evaluation of 3-(4-nitrophenyl)coumarin Derivatives in Tumor Cell Lines

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Abstract: Coumarins are naturally-occurring compounds that have attracted considerable interest due to their numerous biological activities depending on their pattern of substitution on the coumarin molecule. In this present investigation, we synthesized 3-(4-nitrophenyl)coumarin derivatives (**9a-e**) and evaluated their *in-vitro* cytotoxic effect on human lung (A549), breast (MDA-MB-231) and prostate (PC3) cancer cell lines for 48 h using crystal violet dye binding assay. Cytotoxic effects of the most active compound on normal human lung (MRC-9) and breast (MCF-10A) cell lines, cell cycle analysis using flow cytometry and mitochondrial membrane potential (MMP) using Tetramethyl Rhodamine Methyl Ester (TMRM; rhodamine-123) fluorescent dye were also examined. Among the compounds that were evaluated, **9c** showed cytotoxic effect (active), caused significant cells arrest (p<0.05) in G₀/G₁ and S phases of cell cycle and loss of MMP in A459, MDA-MB-231 and PC3 cell lines. Additionally, the cytotoxic effect of **9c** was compared to reference drugs (Coumarin and Docetaxel) for comparative study. These results further demonstrate that acetoxy group at C-7 and C-8 positions of **9c** are responsible for the observed cytotoxic effect in these cancer cell lines.

1. Introduction

Coumarins are important class of naturally-occurring heterocyclic compounds known as benzo- α -pyrones, a pyrone ring fused with a benzene nucleus (1, 2). Most coumarin compounds occurred as secondary metabolites in green plants, while some are produced by fungi and bacteria (3). They exhibit diverse array of pharmacological and biochemical properties such as anti-coagulant (4), anti-tumor (5, 6), anti-thrombotic (7), anti-viral (8, 9), anti-bacterial (10), anti-microbial (11), anti-inflammatory (12, 13) and anti-oxidant (14, 15) properties. However, the anti-proliferative and anti-tumor activities of various coumarins have been extensively reported, e.g. simplest coumarin (1) and its metabolite, 7-hydroxycoumarin (2) have been reported to inhibit cell growth proliferation in various human cancer cell lines, such as A549 (lung), ACHN (renal), H727 (lung), MCF-7 (breast) and HL-60 (leukemia) (16-190). They have also been reported in clinical trials to demonstrate activity against breast cancer, prostate cancer, malignant melanoma, and metastatic renal cell carcinoma (5, 20, 21). In addition to their biological activities, coumarins are used as additives in food and cosmetics, and as optical brightening agents (22, 23). These properties of coumarins have led to considerable interest in their synthesis and possible applications as therapeutic agents for the treatment of various diseases.

Recent studies have shown that therapeutic applications, including pharmacological and biochemical properties of coumarins depends on the nature of the group present and its pattern of substitution on the core coumarin molecule (24, 25). For examples, acetoxycoumarin such as 7,8-diacetoxy-4-methylcoumarin (DAMC; **3**) exhibits anti-cancer and pro-oxidant activities in human tumor cell lines (26, 27); nitrocoumarins exhibit potent anti-microbial, anti-allergic and anti-cancer properties (528-30); hydroxycoumarins exhibit diverse biological properties such as

anti-coagulants, anti-arthritis, anti-inflammatory, anti-pyretic, anti-bacterial, anti-viral, and anticancer properties (5, 31, 32); and 3-arylcoumarins commonly produced by plants have shown different types of biological properties such as antioxidant, anti-inflammatory, anticancer, anti-HIV and antimicrobial activities (33–36). As part of our on-going investigation on coumarins as anticancer agents, we recently reported that 3-arylcoumarin containing acetoxy group (4) and basic amino side chain (5 and 6) exhibits cytotoxic activity in various cancer cell lines (37, 38). These studies and others not mentioned here strongly support the potential therapeutic applications of coumarin and its derivatives, making them attractive for further evaluation as novel therapeutic agents for cancer treatment.

Recent advances in anti-cancer studies have shown that heterocyclic compound containing *p*-nitrophenyl pharmacophore offers great potential for the development of novel anticancer agents (39, 40). This finding aroused our interest in the presence cytotoxicity studies of coumarins containing *p*-nitrophenyl groups substituted at the 3-position with the aim of obtaining more potent biologically active coumarins. Therefore, we herein report the synthesis of 3-(4nitrophenyl)coumarin derivatives (**9a-e**, Table I); and evaluation of their *in-vitro* cytotoxic effect on A549, MDA-MB-231 and PC3 cancer cell lines. In addition, cytotoxic effect of the most active compound on normal (MRC-9 and MCF-10A) cell lines, cell cycle and MMP were also evaluated. Finally, cytotoxic effect of the most active compound was also compared to Coumarin and Docetaxel drugs.

Fig. 1.

2.0. Materials and Methods

2.1. Chemistry

2.1.1. Materials

Commercial grade solvents and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Alfa Aeser (Ward Hill, MA, USA) and used without further purification. The NMR spectra were recorded on Varian 300 MHz spectrometer (¹H: 300 MHz, ¹³C: 75MHz,). The appropriate deuterated solvents are indicated in the experimental procedure and line positions recorded in (δ) *ppm* from the reference signal. ESI-TOF Mass Spectrometer was recorded on Finnigan LCQ – Quadrupole Ion Trap (Thermo Finnigan, San Jose, CA), and the HPLC pump was an Agilent (HP) 1100 series pump or Applied Biosystems model 400 pump. CHN elemental analysis was performed by combustion using automatic analyzers. To estimate the degree of purity of the active compound (**9c**), HPLC analysis was conducted with Waters Alliance 2996 HPLC system equipped with Wasters 2996 Photodiode array detector. HPLC grade acetonitrile and HPLC water (Merck) was used for the chromatography. Melting points were determined on a Gallenkamp (UK) apparatus.

2.1.2. General Experimental Procedures

To a mixture of 2-hydroxybenzaldehyde (7a) [or 2-hydroxyacetophenone (7b), 2,3,4trihydroxy benzaldehyde (7c), 2-hydroxy-4-methoxybenzophenone (7d) or 2-hydroxybenzophenone (7e)] (7.4 mmol) and 4-nitrophenylacetic acid (8) (8mmol) in acetic anhydride (3mL) was added triethylamine (99%, 1 mL). The reaction mixture was then refluxed overnight at 90°C, allowed to cool to r.t. and 10mL of ether was added with occasional stirring for another 2-3hrs to afford the desired product as a precipitate that was collected using vacuum filtration.

The synthesis and structural characterization of 9a has been previously reported in literature (41-43).

2.1.2.1. 3-(4-nitrophenyl)coumarin (**9a**)

This title compound was prepared according to the above experimental procedure starting from 2-hydroxybenzaldehyde (**7a**) to afford **9a** as a yellow precipitate (1.02g, 52%) with m.p. = 267.2-268.4 °C (Literature reported m.p. = 267-268 °C). ¹H-NMR (300 MHz, CDCl₃) δ : 7.33-7.43 (2H, ArH), 7.59-7.65 (2H, ArH), 7.91-7.96 (3H, ArH) and 8.30-8.33(2H, ArH); HRMS (ESI) (m/z): for C₁₅H₉NO₄ calcd: 268.0604; [M+H]; found: 268.0605.

2.1.2.2. 4-methyl-3-(4-nitrophenyl)coumarin (9b)

This title compound was prepared according to the above experimental procedure starting from 2-hydroxyacetophenone (**7b**) to afford **9b** as a yellow precipitate (1.52g, 77%) with m.p. = 200.6-201.8 °C. ¹H-NMR (300 MHz, CDCl₃) δ : 2.36 (3H, s. CH₃), 7.35-7.41 (2H, ArH), 7.52-7.55 (2H, ArH), 7.58-7.64 (1H, ArH), 7.72-7.45 (1H, ArH) and 8.30-8.33 (2H, ArH). ¹³C-NMR (75MHz, CDCl₃) 16.73 (*CH*₃), 11.7.03, 120.00, 123.63, 124.70, 125.21, 125.35, 131.43, 132.23, 141.35, 147.55, 148.96, 152.74 (Ar-C) and 160.25 (C=O); HRMS (ESI) (m/z): for C₁₆H₁₁NO₄ calcd: 282.0768; [M+H]; found: 282.0767.

2.1.2.3. 7,8-diacetoxy-3-(4-nitrophenyl)coumarin (9c)

This title compound was prepared according to the above experimental procedure starting from 2,3,4-Trihydroxy benzaldehyde (**7c**) to afford **9c** as a yellow precipitate (2.66g, 97%) with m.p. = 224.4-226.1 °C. ¹H-NMR (300 MHz, CDCl₃) δ : 2.37-245 (6H, s, 2 x OCOC*H*₃), 7.17-7.20 (1H, ArH), 7.46-7.49 (1H, ArH), 7.85-7.92 (3H, ArH) and 8.28-8.30 (2H, ArH). ¹³C-NMR (75MHz, CDCl₃) 20.34 and 20.68 (2 x OCOCH₃), 117.88, 119.62, 123.70, 125.49, 125.83, 129.45, 140.56, 146.00, 145.85, 146.86, 147.87, 158.39 (Ar-C), and 167.33 and 167.72 (C=O);

HRMS (ESI) (m/z): for C₁₉H₁₃NO₈ calcd: 384.0714; [M+H]; found: 384.0716. Anal. Calcd for C₁₉H₁₃NO₈: C, 59.54; H, 3.42; N, 3.65. Found: C, 59.70; H, 3.41; N, 3.69. Purity (HPLC): 99.17%.

2.1.2.4. 7-methoxy-3-(4-nitrophenyl)-4-phenylcoumarin (9d)

This title compound was prepared according to the above experimental procedure starting from 2-Hydroxy-4-Methoxybenzophenone (**7d**) to afford **9d** as a yellow precipitate (1.45g, 52%) with m.p. = 214.9-216.6 °C. ¹H-NMR (300 MHz, CDCl₃) δ : 3.91 (3H, s. OC*H*₃),), 6.78-6.82 (1H, ArH), 6.94-6.95 (1H, ArH), 7.01-7.17 (3H, ArH), 7.27-7.36 (5H, ArH) and 8.01-8.06 (2H, ArH). ¹³C-NMR (75MHz, CDCl₃) 55.91 (OCH₃), 100.69, 112.78, 113.50, 121.41, 132.91, 128.66, 128.99, 129.16, 131.90, 133.93, 141.32, 146.72, 153.28, 155.21, 160.86 (Ar-C) and 163.22 (C=O); HRMS (ESI) (m/z): for C₂₂H₁₅NO₅ calcd: 374.1023; [M+H]; found: 374.1035.

2.1.2.5. 3-(4-nitrophenyl)-4-phenyl-coumarin (9e)

This title compound was prepared according to the above experimental procedure starting from 2-hydroxybenzophenone (**7e**) to afford **9e** as a yellow precipitate (1.43g, 56%) with m.p. = 236.1-239.0 °C. ¹H-NMR (300 MHz, CDCl₃) δ : 7.11-7.14 (2H, ArH), 7.24-7.28 (2H, ArH), 7.31-7.37 (5H, ArH), 7.45-7.48 (1H, ArH), 7.58-7.63 (1H, ArH) and 8.04-8.07(2H, ArH). ¹³C-NMR (75MHz, CDCl₃) 116.97, 119.99, 122.97, 124.56, 124.80, 128.10, 128.72, 129.10, 129.18, 131.77, 132.41, 133.60, 140.97, 146.92, 153.08, 153.38 (Ar-C) and 160.49 (C=O); HRMS (ESI) (m/z): for C₂₁H₁₃NO₄ calcd: 344.0917; [M+H]; found: 344.0927).

2.2. Biology

2.2.1. Chemicals

F12 K medium, RPMI medium, penicillin-streptomycin anti-biotic solution (100x), fetal bovine serum (FBS), Trypsin-EDTA solution (1x), phosphate buffer (PBS), 50% glutaraldehyde, crystal violet, propidium iodide, Rhodamine-123, IGPAL CA-630, Coumarin, Docetaxel and RNase were obtained from Sigma-Aldrich Company (St. Louis, MO, USA). The potassium phosphate, EDTA, D-glucose, ethanol were obtained from Thomas Scientific Company (Swedesboro, NJ, USA).

2.2.2. Cell line maintenance

The cell lines (A549, MRC-9, MDA-MB-231, MCF-10A and PC3) 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, PC3) or MEM (MRC-9) or RPMI (MDA-MB-231) or DMEM-F12 (MCF-10A) medium containing 100 units of penicillin/ml, 100 μ g of streptomycin/ml, 2 mM L-glutamine and 10% FBS in T-75 cm² flasks at 37°C in a 5% CO₂ incubator.

2.2.3. Treatment of cells

The cells (A549, MRC-9, MDA-MB-231, MCF-10A or PC3) were plated at a density of 5 x 10^4 cells per well in polystyrene, flat bottom 24-well microtiter plates (Corning Costar, Rochester, NY, USA) in complete medium containing 10% FBS and allowed to stabilize overnight in a CO₂ incubator at 37°C. Next, the cells were treated with compounds (**9a-e**) at different concentrations (0, 10, 25, 50, 75 and 100 μ M) in a final volume of 1 ml per well in triplicate wells for each treatment for 48 h at 37°C in a 5% CO₂ incubator. All studies were repeated at least twice.

2.2.4. Evaluation of cell viability

At the end of incubation period, the cell viability was evaluated by dye uptake assay according to previous reported method by Badisa *et al.* (44). Glutaraldehyde (400 μ l of 0.25%) was added to each well and incubated for 30 min at room temperature 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 the treatment with compounds. 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 μ l 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 added to each well and the plates were read at 540 nm in a plate reader (Bio-Tek EL800 Plate Reader). The mean absorbance value of control was considered as 100% and the treated sample percentages were calculated by comparing the treated samples absorbance with the mean absorbance of control.

2.2.5. Cell cycle analysis

The cell cycle analysis was evaluated using Accuri flow cytometer (BD Biosciences, San Jose, CA). Cells (A549, MDA-MB-231 and PC3) at a density of 0.65 x 10^6 cells per T-25 cm² flask were plated and incubated overnight. The cells were then treated with 0, 10 and 20 μ M of the active compound in triplicate flasks and incubated for 48 h in a 5% CO₂ incubator at 37°C. At the end of incubation, the cells were trypsinized and centrifuged at 2,500 rpm for 10 min at room temperature. The pellet was suspended in 1 ml of PBS and transferred to eppendorff tube. The cells were pelleted by centrifugation at 2,500 rpm for 5 min. Then the cells were stained with 500 μ l of Vindelov's reagent containing 0.01mg/ml of ribonunuclease A and 0.075mg/ml of

propidium iodide and 1 µl/ml of IGPAL CA-630 in phosphate buffered saline for overnight at 4°C in dark. The distribution of cells in each phase was analyzed with the Accuri flow cytometer (Becton Dickinson, San Jose, CA). In each sample, a total of 50,000 events were analyzed separately. The Accuri C6 software was used for acquisition and analysis of the data and the percentage of cells in each phase.

2.2.6. Measurement of MMP

The loss of MMP was evaluated using rhodamine-123 fluorescent dye in A549, MDA-MB-231 and PC3 cells. At the end of incubation, cells were fixed with 400 μ l of 0.25% aqueous glutaraldehyde containing rhodamine-123 to yield a final concentration of 1 μ M for 30 min at room temperature. The supernatant was discarded, and the plates were washed with tap water and air dried in the hood. Finally, 500 μ l of 0.1% Triton X 100 in dPBS was added per well and incubated at 37^oC for 1 h. The plates were read with the excitation filter set at 485 nm and the emission filter at 538 nm in TECAN microplate Fluorometer.

2.2.7. Statistical analysis

The viability values were presented as mean \pm standard deviation (n=3). All treated cells data were presented as percentage values in comparison to the untreated control (100%). The viability and CC₅₀ graphs were plotted in Prizm 3.00 software (GraphPad Software, Inc., San Diego, CA, USA). The CC₅₀ value was calculated from the CC₅₀ graph where the two lines meet (the dose at which 50% of the cells dies) using Prizm 5 software.

3.0 RESULT

3.1 Synthesis of 3-(4-nitrophenyl)coumarin derivatives (9a-e)

In this present investigation, we reported the synthesis of 3-(4-nitrophenyl)coumarin derivatives (9a-e) via modified reported based-catalyzed Perkin condensation reaction (45-47) (Scheme 1). This process involved refluxing a mixture of benzaldehyde (acetophenone or benzophenone) and 4-nitrophenylacetic acid in the presence of triethylamine in acetic anhydride overnight at 90°C to afford the desired compounds as solid between 52-97% yields. The synthesized compounds were characterized using proton (¹H) and carbon (¹³C) NMR, HRMS and CHN elemental analyses as indicated in the experimental section. The HPLC analysis was also conducted to estimate the degree of purity of the active compound (9c) and result indicated 99.17% purity. MAR

Scheme 1.

Table 1.

3.2. **Cytotoxicity Studies**

The synthesized compounds (9a-e) were evaluated for their *in-vitro* cytotoxic effect using crystal violet dye-staining assay. The cytotoxic concentration values (CC_{50} , the concentration of tested drug where 50% dead cells is observed in the treated sample compared to the untreated control) for **9a-e**, Coumarin and Docetaxel were calculated according to the Ipsen' method (48) and are given in Table 1. Result indicated that **9**c showed cytotoxic effect in A549 (CC₅₀ = 12.2) μ M), MDA-MD-231 (CC₅₀ = 27.6 μ M) and PC3 (CC₅₀ =18.2 μ M) cancer cell lines, while **9a**, **9b**, **9d** and **9e** didn't show cytotoxic effect ($CC_{50} > 100 \mu M$; inactive). The compound **9c** was most cytotoxic to A549 cells, causing dose-dependent cell death in comparison to the untreated control cells (Fig. 2). Comparison of the cytotoxic effect of 9c with Coumarin and Docetaxel revealed

that (1) Coumarin was not active in the three cancer cell lines ($CC_{50} > 100$) and (2) **9c** showed comparable cytotoxicity to Docetaxel on A549 cell line and lesser cytotoxicity compared to Docetaxel on both MDA-MD-231 and PC3 cell lines (Table 1). Additionally, **9c** showed similar cytotoxic effect on both cancer (A549 and MDA-MB-231) and normal (MRC-9 and MCF-10A) cell lines (Table 1).

Fig. 2.

3.3. Cell cycle Analysis

Cells (A549, MDA-MB-231 and PC3) were treated with **9c** for 48 h, followed by staining with propidium iodide. The percentage of cells in G₁, G₂/M and S phases of the cell cycle was analyzed using a flow cytometer. Result indicated that treatment of A549, MDA-MB-231 and PC3 cells with **9c** caused cell cycle arrest (p<0.05) in concentration dependent manner (Fig. 3A-C). Furthermore, it was observed that **9c** arrest (i) A549 cells in G₀/G₁ phase at 10 µM concentration (2.17%) (Fig. 3A), (ii) MDA-MB-231 cells in G₀/G₁ phase at 30 µM concentration (2.90%), and S phase at both 15 µM concentration (4.72 %) and 30 µM concentration (2.23%) (Fig. 3B) and (iii) PC3 cells in G₀/G₁ phase at both 10 µM concentration (3.04%) and 20 µM concentration (2.84%) with respect to the untreated control cells (Fig. 3C).

Fig. 3.

3.4. Measurement of MMP

Cells (A549, MDA-MB-231 and PC3) were treated with **9c** for 48 h and then fixed with rhodamine-123 dye. Results indicated that **9c** caused loss of MMP in the following order; A549 (lung) > PC 3 (prostate) >MDA-MB-231 (breast) cells (Fig. 4a). The percentage of MMP in

comparison to the untreated control cells (100%) in (i) A549 cells at 10 μ M (74.2%±3.86), 25 μ M (38.7%±0.99), 50 μ M (35.8%±1.21), 75 μ M (34.9%±1.45) and 100 μ M (32.2%±2.82); (ii) MDA 231 cells at 10 μ M (90.8%±2.56), 25 μ M (82.4%±1.87), 50 μ M (81.9%±2.52), 75 μ M (80.7%±2.772) and 100 μM (72.4%±2.47) and (iii) PC3 cells at 10 μM (91.1%±4.22), 25 μM $(70.9\% \pm 3.07)$, 50 µM (59.6% ±0.66), 75 µM (55.2% ±4.29) and 100 µM (55.0% ±0.66); indicating loss of MMP respectively. Additionally, cells exposed to 9c treatment at shorter period of time (4, 6 and 24 h) also showed dose and time dependent loss of MMP in all three cancer cell lines as ANU indicated in Figure 4b.

Fig. 4.

4.0. DISCUSSION

Interest in the synthesis and biological evaluation of coumarins has increased over the years due to their diverse pharmaceutical activities. As part of our ongoing investigation involving coumarins aims at evaluating the role of substituent effect in modulating cytotoxicity, we synthesized series of 3-(4-nitrophenyl)coumarin derivatives (9a-e, Table 1) and evaluated their cytotoxic effect on A549, MDA-MB-231 and PC3 cancer cell lines. The in-vitro cytotoxicity results indicate that 9c bearing acetoxy group at C-7 and C-8 positions on the benzenoid ring of coumarin molecule showed significant cytotoxic effect with respect to untreated cells, while absence of this group as indicated in 9a, 9b, 9d and 9e resulted in loss of cytotoxic activity (Table 1). This finding is supported by previous reports indicating that the presence of acetoxy group at C-7 and C-8 positions of the coumarin molecule (3) enhanced drug activity such as anti-cancer, antioxidant and radicals scavenging properties (26, 27, 49). Interestingly, Coumarin was relatively non-toxic in all tested cancer cell lines; indicating that it's

cytotoxic effect was metabolism and dose-time dependent (50, 51). Additionally, compound 9cshowed similar cytotoxicity on A549, and two-fold (PC3) and fourth-fold (MDA-MB-231) decreased in cytotoxicity compared to Docetaxel. Docetaxel (formerly called taxotere) belongs to a new class of cytotoxic agents known as taxanes and is used in the treatment of breast cancer, lung cancer, prostate cancer, stomach cancer, and head/neck cancer (52). Comparison of 9a and 9c based on structural relationship (SAR) activity indicates that presence of *p*-nitrophenyl group at the C-3 position did not make any contribution to the observed cytotoxic effect of 9c; further supporting the beneficial effect of acetoxy group at C-7 and C-8 positions on the coumarin molecule in modulating cytotoxicity. The order of 9c cytotoxic effect based on CC_{50} values is A549 > PC3 > MDA-MD 231 cells. However, the non-selectivity of 9c in normal MRC-9 (CC₅₀) = 17.1 μ M±1.07) and MCF-10A (CC₅₀ = 9.6 μ M±1.41) cell lines is of utmost concern, since one of the important criteria in the development of therapeutic drugs for cancer treatment is to have few or no side-effects on normal cells of patients undergoing chemotherapy. A possible reason for the lack of 9c selective cytotoxicity could be due that the two selected non-malignant lines are female-origin cells (53).

One effective strategy for controlling tumor growth is the inhibition of cell cycle progression in cancer cells (54). Previous studies have demonstrated that toxic effect of compound on different phases of the cell cycle can activate or inhibit of cell growth and proliferation of cancer cells (55, 56). In the present investigation, it was observed that **9c** caused cell arrest of (i) A549, MDA-MB-231 and PC3 cells in G_0/G_1 phase, indicating apoptotic mode of cell death and (ii) MDA-MB 231 cells in S phase, indicating inhibition of DNA synthesis (Fig. 3). This finding is consistent with previous investigations indicating that coumarins caused G_0/G_1 and S phases cell arrest in different cancer cell lines (57, 58).

Rhodamine-123, a green-fluorescent dye, has been used to measure MMP changes in cells (59). This dye is selectively taken up by mitochondria and the amount taken up by the cells is directly proportional to its membrane potential (59). Thus, evaluation of MMP changes in living cells is of critical important in accessing the effect of chemicals on mitochondria function (60). The present investigation indicates loss of MMP in the following order: MDA-MB-231 > PC3 >A549 cell lines after 48 h treatment with **9c** (Fig. 4a). Furthermore, **9c** caused dose-time dependent loss of MMP in A549 cells during the early stages of the treatment; suggesting that it might be using intrinsic apoptotic pathway to inflict cell death (Fig. 4b) (61). Interestingly, the observed correlation between cytotoxic effect and loss of MMP in these cancer cell lines (Fig. 1 and 4) indicates that the elicited differential cytotoxicity of **9c** may be associated with the loss of MMP.

5.0. CONCLUSION

A series of 3-(4-nitrophenyl)coumarin derivatives (**9a-e**) were synthesized, characterized and evaluated for their *in vitro* cytotoxic effect on A549, MDA-MB-231 and PC3 cancer cell lines. Compound **9c** bearing acetoxy group at C-7 and C-8 positions showed cytotoxic effect compared to other synthesized analogs (**9a**, **9b**, **9d** and **9e**). The cytotoxic effect of **9c** is associated with cell arrest in G_0/G_1 and S phases of the cell cycle, and loss of MMP. Additionally, the elicited differential cytotoxic effect and loss of MMP. This present investigation further correlates the importance of acetoxy group at C-7 and C-8 positions on the coumarin molecule with respect to modulating cytotoxic effect and thus indicates that **9c** could serve as an

attractive leads in the future development of new cytotoxic compounds that are more potent and selective.

Declaration of interest statement

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

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Figure Captions

Scheme 1. Reagents and Conditions: (i) AC₂O, NEt₃, refluxed overnight at 90°C

Fig. 1. Structures of (1) Coumarin, (2) 7-hydroxycoumarin. (3) 7,8-diacetoxy-4-methyl coumarin (DAMC), (4) 4-(7-(diethylamino)-4-methyl-2-oxo-2H-chromen-3-yl)phenyl acetate (5) 3-(4-(2-(dimethylamino)ethoxy)phenyl)-7-methoxy-4-phenylcoumarin (6) 3-(4-(2-(pyrrodin-1-yl)-ethoxy)phenyl)-7-methoxy-4-phenylcoumarin

Fig. 2. Effect of compound **9c** on A549, MDA-MB-231 and PC3 cell viability. Data are represented as the mean and SD, n=6.

Fig. 3: Effect of compound **9c** on (**A**) A549, (**B**) MDA-MB-231 and (**C**) PC3 cell cycle. Data are represented as mean and SD, n=3. *Statistically significant difference from the control (p<0.05) using Dunnett's multiple comparison test.

Fig. 4. Effect of compound **9c** on mitochondrial membrane potential in (A) A549, MDA-MB-231 and PC3 cell lines for 48 h and (B) A549 cell lines for 4, 6, 24 h. Data are represented as mean and SD, n=3.*Statistically significant difference from the control (p<0.05) using Dunnett's multiple comparison test.



Scheme 1.





Fig. 3.

A.



Β.



C.



Fig. 4.



Table 1. The CC_{50} values (μM for compounds 9a-e and reference drugs (Coumarin and Docetaxel) tested on lung (A549), breast (MDA-MD-231) and prostate (PC3) cancer cell lines for 48 h.

| | CC ₅₀ (μM) mean ± SD | | | | |
|-----------------|---------------------------------|-------------|-----------|------------|-----------|
| Compounds | A549 | MRC-9 | MDA-MB-23 | MCF-10A | PC-3 |
| NO ₂ | | | | 0 | |
| 9a 000 | >100.0 | ND | >100.0 | ND | >100.0 |
| NO ₂ | >100.0 | ND | >100.0 | ND | >100.0 |
| 9b 000 | | | J. | | |
| | 12.2±1.85 | 17.1µM±1.07 | 27.6±3.39 | 9.6μM±1.41 | 18.2±0.15 |
| | | A | | | |
| NO ₂ | >100.0 | ND | >100.0 | ND | >100.0 |
| 9d 0 0 0 | | | | | |
| NO ₂ | >100.0 | ND | >100.0 | ND | >100.0 |
| 9e 4040 | | | | | |
| Docetaxel | 9.40±0.07 | ND | 7.30±0.03 | ND | 9.44±0.44 |
| Coumarin | >100 | ND | >100 | ND | >100 |
| | | | | | |

¹Data represent the average of two values (n = 6). ²The cytotoxic concentration (CC₅₀) value was determined from the graph where the live and dead cells line graphs meet in the Graph pad Prism. ³ Drugs effects were determined for 48 h exposure

⁴ ND: Not Determined

Graphical Abstract:



Highlights

- ➤ 3-(4-nitrophenyl)coumarin derivatives (9a-e) were synthesized
- 9a-e were evaluated for *in-vitro* cytotoxic effect in cancer cell lines
- 9c bearing acetoxy group at C-7 and C-8 positions exhibited cytotoxic effect
- > 9c caused G_0/G_1 and S phases cell arrest and loss of MMP