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7,8-Diacetoxy-3-(4-methylsulfonylphenyl)-4-phenylcoumarin Induces ROS-dependent Cell Death in the A549 Human Lung Cancer Cell Line

MUSILIYU A. MUSA[1] and QUDUS KOLAWOLE[2]

[1]*Department of Chemistry, Florida A&M University, Tallahassee, FL, U.S.A.;*
[2]*Department of Biological Sciences, Florida A&M University, Tallahassee, FL, U.S.A.*

Abstract. *Background/Aim: Coumarins comprise of a very large class of naturally occurring compounds with growing interest in their synthesis and possible applications in the treatment of various diseases. We herein report the in-vitro cytotoxic activity of 3,4-Diarylcoumarins (4a-i) in A549 (lung) and PC-3 (prostate) cancer cell lines. Materials and Methods: The cytotoxic activity was evaluated using crystal violet dye-binding. The most active compound effect on the cell-cycle phases, mitochondrial membrane potential (MMP), reactive oxygen species (ROS) production and apoptosis were also evaluated. Results: Among the synthesized compounds that were evaluated, 7,8-Diacetoxy-3-(4-(methylsulfonyl)phenyl)-4-phenylcoumrin (4f) showed highest cytotoxicity (CC_{50}=13.5%±0.15μM) in A549 cancer cell line. The mechanism of its cytotoxic action indicated significant cell arrest in G_1/G_0, S and G2 phases of the cell cycle, loss of mitochondrial membrane potential (MMP), increase in reactive oxygen species (ROS) production and induction of apoptotic cell death. The cell viability result of pretreated A549 cells with antioxidant N-acetylcysteine (NAC), followed by compound 4f treatment confirmed ROS-dependent cell death. Conclusion: The presence of 3-4-methylsulfonyl and 7,8-diacetoxy groups on 3,4-Diarylcoumarin is critical in modulating higher cytotoxic activity and could serve as a valuable template for the development of novel synthetic compounds as potential anticancer agents for lung cancer treatment.*

Correspondence to: Dr. Musiliyu A Musa, Department of Chemistry, Florida A & M University, 1530 S. Martin Luther King Jr. Blvd, 219 Jones Hall, Tallahassee, FL 32307, U.S.A. Tel: +1 8505993509 and +1 8505993638, Fax: +1 8505612388, e-mail: musiliyu.musa@famu.edu/musiliyu@gmail.com

Key Words: 7, 8-Diacetoxy-3,4-diarylcoumarin, cell cycle, *in vitro* cytotoxicity, mitochondrial membrane potential, MMP, reactive oxygen species, ROS, apoptosis.

Coumarins (benzopyran-2-ones) are comprised of a wide group of naturally-occurring heterocyclic compounds consisting of fused benzene and α-pyrone rings (1, 2). The name "Coumarin" comes from "Coumarou," a vernacular name for the Tonka Bean (Dipteryx odorata wild) that was first isolated as a natural product in 1820 and synthesized in 1868 *via* the Perkin reaction (3, 4). Currently, coumarins are classified as synthetic (SCs) and naturally occurring coumarins (NOCs). NOCs represent photochemical compounds found in higher plants, fungi and microorganisms functioning as growth regulators, controllers of respiration, bacteriostats, fungistats, as well as prophylactics against infection (5). On the other hand, SCs are widely used as optical brighteners (*e.g.* 7-Diethylamino-4-methylcoumarin), dispersed fluorescent laser dyes, perfumes, cosmetics, food additives, pharmaceuticals, fragrances and for imparting pleasant odors to industrial products (6). Coumarins, whether SCs or NOCs, have been the subject of extensive studies because of their useful and diverse biological activities, such as photochemotherapy, anti-coagulant, anti-cancer, anti-thrombotic, anti-inflammatory, anti-HIV, anti-bacterial, anti-coagulant. anti-microbial, anti-inflammatory, anti-influenza, anti-tuberculosis, anti-asthmatic, anti-hyperlipidemic, anti-platelet, anti-Alzheimer, anti-oxidant, anti-allergic and monoamine oxidase (MAO) inhibitory activities (1, 7-10).

One of the most widely reported activity of coumarins is their anticancer effects, where they are known to target a number of pathways (*e.g.*, inhibition of kinases, cell cycle phases *etc.*) (11-12). Coumarins exhibit diverse arrays of pharmacological and biochemical activities because of their structural diversity, attributed to the nature of substituent and its pattern of substitution on the core coumarin molecule (10, 13-14). For example, arylcoumarins (*e.g.*, 3,4-Diarylcoumarins) have been found to possess significant anti-oxidant, anti-inflammatory, anti-cancer, anti-HIV, anti-microbial, anti-fertility, and monoamine oxidase activities (15-18). For the last

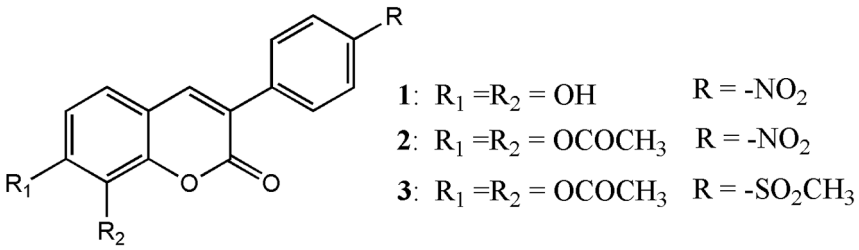


Figure 1. *Structures of compounds. 7,8-Dihydroxy-3-(p-nitrophenyl)coumarin (DHNPC, 1), 7,8-Diacetoxy-3-(p-nitrophenyl)coumarin (DANPC, 2), and 7,8-Diacetoxy-3-(4-methylsulfonyl phenyl)coumarin (DAMSPC, 3).*

few years, our group has been deeply interested in the synthesis and biological evaluation of arylcoumarins as anti-cancer agents. Recently, we demonstrated that arylcoumarins (1-3, Figure 1) enhanced cytotoxic activity in various cancer cell lines (19-21). As part of our ongoing investigation on these molecules, we herein report the evaluation of the *in vitro* cytotoxic activity of 3, 4-Diarylcoumarin (4a-i, Table I) in A549 (lung) and PC-3 (prostate) cancer cell lines in comparison to the current anti-cancer drug Docetaxel (DOC). Furthermore, the cytotoxic mode of action of the most active compound was also investigated.

Materials and Methods

Chemicals. F12K (A549 and PC-3) media, penicillin-streptomycin anti-biotic solution (100×), fetal bovine serum (FBS), trypsin-EDTA solution (1×), phosphate buffered saline (PBS), 50% glutaraldehyde, crystal violet, IGEPAL CA-630, propidium iodide, 2',7'-Dichlorofluorescin diacetate (DCFDA), Tetramethyl Rhodamine Methyl Ester (Rhodamine 123), Acridine orange (AO), Ethidium bromide (EB), Docetaxel, N-acetylcysteine (NAC) and RNase were obtained from Sigma Aldrich (St. Louis, MO, USA).

Cell culture and cell viability assay. Human cell lines (A549 and PC-3) were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured as per the guidelines supplied using the crystal violet dye uptake assay according to our previously reported method (19-21). Cell viability assay was performed in F12K medium, and the cytotoxic concentration (CC_{50}) was determined after 48 h of treatment. Additionally, the involvement of ROS was determined by measuring the cell viability in pretreated A549 cells with antioxidant NAC (1 mM) for 1 h, followed by compound 4f for 48 h.

Cell cycle analysis. Cell cycle analysis was carried out according to our previously reported method by treating 1.3×10^6 A549 cells per T-25 flask in complete medium with compound 4f (0, 15, 30 μM) for 48 h, stained with propidium iodide and analyzed using a C6 Accuri flow cytometer (Accuri Cytometers, Ann Arbor, MI, USA) (22).

Measurement of MMP and ROS production. MMP and ROS measurement were carried out according to our previously reported method by treating 2×10^4 cells/well in a 24-well microtiter plate in complete medium with compound 4f (0-100 μM) in a final volume of 1 ml per well in triplicate wells for 30 min using rhodamine-123 (MMP) or 6 h using DCFDA dye (ROS) (22).

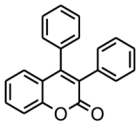
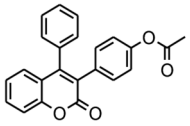
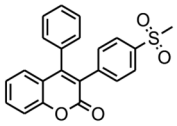
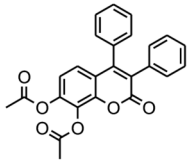
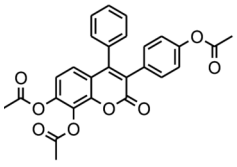
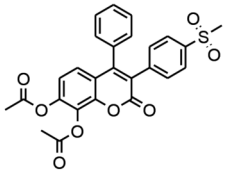
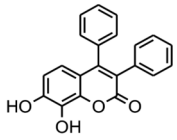
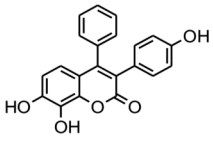
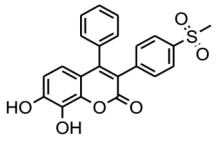
Acridine orange / ethidium bromide (AO/EtBr) staining. AO/EtBr fluorescent staining was carried out according to previously reported method by Kasibhatla *et al*. (23) by treating 1×10^6 A549 cells/ml with different concentrations (0, 5, 10, 15, 25, and 50 μM) of compound 4f for 24 h and followed by the addition of 10 μl of fluorescent dyes containing AO and EtBr. The cells were then visualized immediately under a fluorescence microscope (Nikon, Inc. Japan) with excitation at 488 nm and emission at 550 nm at 200× magnification.

Statistical analysis. Data are presented as mean±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 GraphPad Prism v. 5.00 (GraphPad Software, Inc., San Diego, CA, USA). Differences from the respective untreated control were considered statistically significant when $p<0.05$.

Results

Cytotoxic effect of compounds 4a-h in cancer cell lines. The cytotoxic activity of 7,8-Diacetoxy-3,4-diarylcoumarins (4a-i) and DOC (standard anticancer drugs) was evaluated by a simple and reproducible crystal violet dye-staining assay at different concentrations (0, 25, 50, 75 and 100 μM) in lung (A549) and prostate (PC-3) cancer cell lines. The CC_{50} values are summarized in Table I and indicated that compounds (4d-i) showed cytotoxic activity in PC-3 (CC_{50}=30.6±0.90μM to 86.5±1.36 μM) and A549 (CC_{50}=13.7±0.15μM to 83.5±1.82μM) cell lines with respect to untreated control cells (100%). In summary, compound 4f is the most active in A549 cell line, while compound 4g is the most active in PC-3 cell line (Table I). Overall, compound 4f is the most active compound based on its cytotoxic activity in A549 cell line. However, comparison of the cytotoxic activity of compound 4f with Docetaxel revealed that the later showed approximate twofold decrease in potency in A549 cell line (Table I).

Table I. *The CC-50 values (μM) for compounds (4a-i) tested in PC-3 (prostate) and A549 (lung) cancer cell lines after 48 hours of treatment.*

	Compounds	CC_{50} (μM) mean±SD	
		PC-3	A549
4a		>100	>100
4b		>100	>100
4c		>100	>100
4d		69.5±0.623	83.6±1.71
4e		86.5±1.36	44.3±2.83
4f		47.05±0.35	13.7±0.15
4g		30.6±0.90	79.6±0.98
4h		47.7±0.48	83.5±1.82
4i		36.9±0.27	38.9±1.31
Docetaxel		9.44±0.44	9.40±0.07

Data represent the average of triplicate concentrations. The cytotoxic concentration (CC50) value was determined from the graph where the live and dead cells line graphs meet in GraphPad Prism. Drugs effects were determined after 48 hours of exposure.

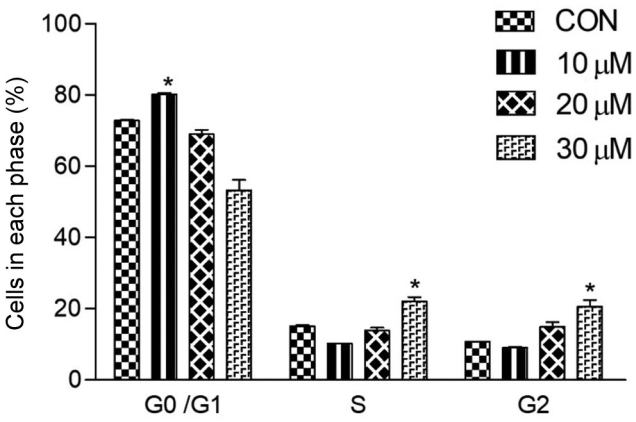


Figure 2. *The effect of compound 4f on the cell cycle (G_1/G_0, S and G2) phases in A549 cell line at concentrations of 10, 15 and 30 μM, respectively. Data are represented as mean and SD, n=3. #Statistically significant difference compared to control ($p<0.05$) using Dunnett's multiple comparison test.*

Compound 4f affects cell cycle progression. The effect of higher cytotoxic activity of compound 4f in A549 (10, 20 and 30 μM) cells on cell cycle progression was evaluated. The percentage of cells in the cell cycle phases was analyzed and compound 4f was shown significantly to induce cell death in A549 cells by arresting cell cycle progression in a concentration-dependent manner: G_0/G_1 (10.08%±0.38 at 10 μM; $p<0.05$), S (45.89%±1.27 at 30 μM; $p<0.001$) and G_2/M (92.83%±1.82 at 30 μM; $p<0.001$)) phases with respect to the untreated control cells (Figure 2).

Compound 4f decreases MMP and ROS production. The effect of compound 4f in A549 (0, 10, 25, 50, 75 and 100 μM) cells on mitochondrial function was then evaluated. Results indicated that the percentage of MMP decreased in concentration-dependent manner (significant, $p<0.01$): 10 μM (87.1%±1.18), 25 μM (81.0%±2.16), 50 μM (68.1%±0.98), 75 μM (62.3%±1.65) and 100 μM (50.8%±0.97) with respect to the untreated control cells (100%) (Figure 3A). Secondly, the intracellular ROS levels indicated a concentration-dependent increase in fluorescence intensity (significant, $p<0.01$): 50 μM (141.6%±6.45), 75 μM (163.5%±3.89), and 100 μM (182.3%±8.33) with respect to untreated control cells (100%) (Figure 3B). Finally, the cell viability result of the pretreated A549 cells with NAC, followed by compound 4f treatment indicated increase in the cell viability (significant, $p<0.01$) at 10 μM (70.1%±1.02) with respect to the compound 4f-treated cells without NAC (Figure 3C).

Acridine orange / ethidium bromide (AO/EtBr) staining of compound 4f. The effect of compound 4f (0, 10, 15 and 25μM)

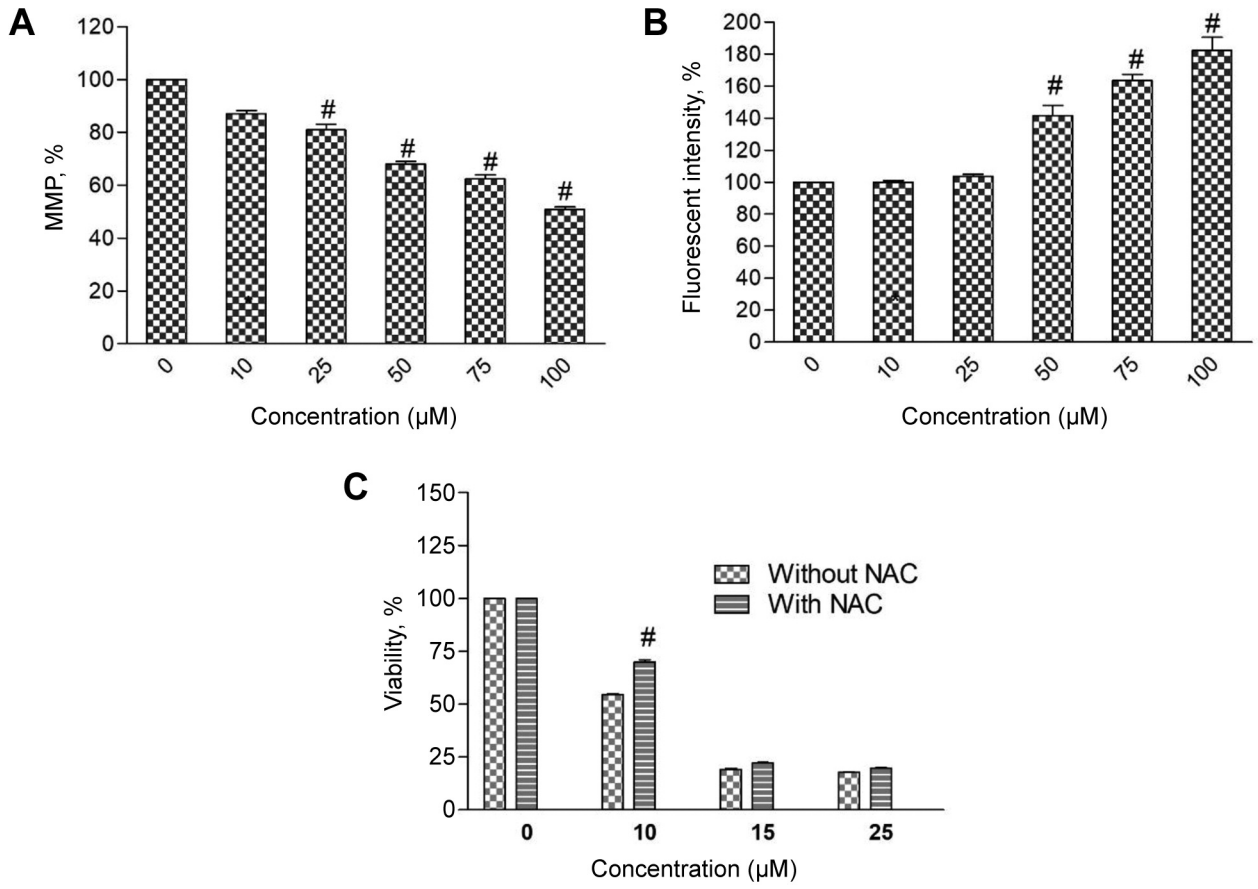


Figure 3. *Effect of compound 4f on (A) mitochondrial membrane potential (MMP), (B) reactive oxygen species (ROS) production, and (C) cell viability in the presence of antioxidant NAC in A549 cells. Data are represented as mean and SD, n=3. #Statistically significant difference compared to control (p<0.05) using Dunnett's multiple comparison test. NAC, N-acetylcysteine; SD, standard deviation.*

in A549 cells on apoptosis and cell viability using the AO/EB staining was evaluated. The results indicated that compound 4f-treated cells showed morphological changes including bright-green and orange nuclei, nucleus condensation, fragmentation of chromatin in the nucleus with cell shrinkage in a dose-dependent manner in comparison to the untreated control cells (Figure 4).

Discussion

Coumarins are important naturally-occurring compounds used in drug discovery that have attracted considerable interest over the years due to their diverse pharmaceutical activities. As part of our ongoing investigation involving 3,4-Diarylcoumarins as potential cytotoxic agents, we herein report that compound 4f containing 7,8-diacetoxy- and 3-(*p*-(methylsulfonyl)- groups showed higher cytotoxic activity in A549 cell line, while the compound 4g containing 7,8-dihydroxy group showed higher cytotoxic activity in PC-3 cell line with respect to the untreated control cells (100%) (Table I). The structural activity relationship study (SARs) indicates that the presence of 7,8-diacetoxy (or 7,8-dihydroxy-) groups enhanced drug cytotoxic activity in both cancer cell lines, while the absence of this group results in no cytotoxic activity (CC_{50}>100). This finding agrees with previous investigations showing that the presence of either 7,8-diacetoxy or 7,8-dihydroxy groups on the core arylcoumarin ring enhanced drug cytotoxic activity in certain types of cancer cell lines (19, 21-22, 24). Overall, the cytotoxic activity is most profound with compound 4f in the A549 cell line (Table I).

Coumarins are known to induce apoptotic cell death by arresting cells at different phases of the cell cycle progression (G_0/G_1-, S- and G_2/M) (21-22, 25). Flow cytometric analysis of cell cycle has been used to measure the apoptotic changes in cells by staining them with propidium iodide (DNA) dyes (26). In the present study, it was observed that compound 4f-induced cell death in A549 cells by arresting cell cycle progression in a concentration dependent manner (p<0.05): it induced apoptosis at lower concentrations (G1/G0 phase) and inhibited DNA replication at higher concentrations (S-

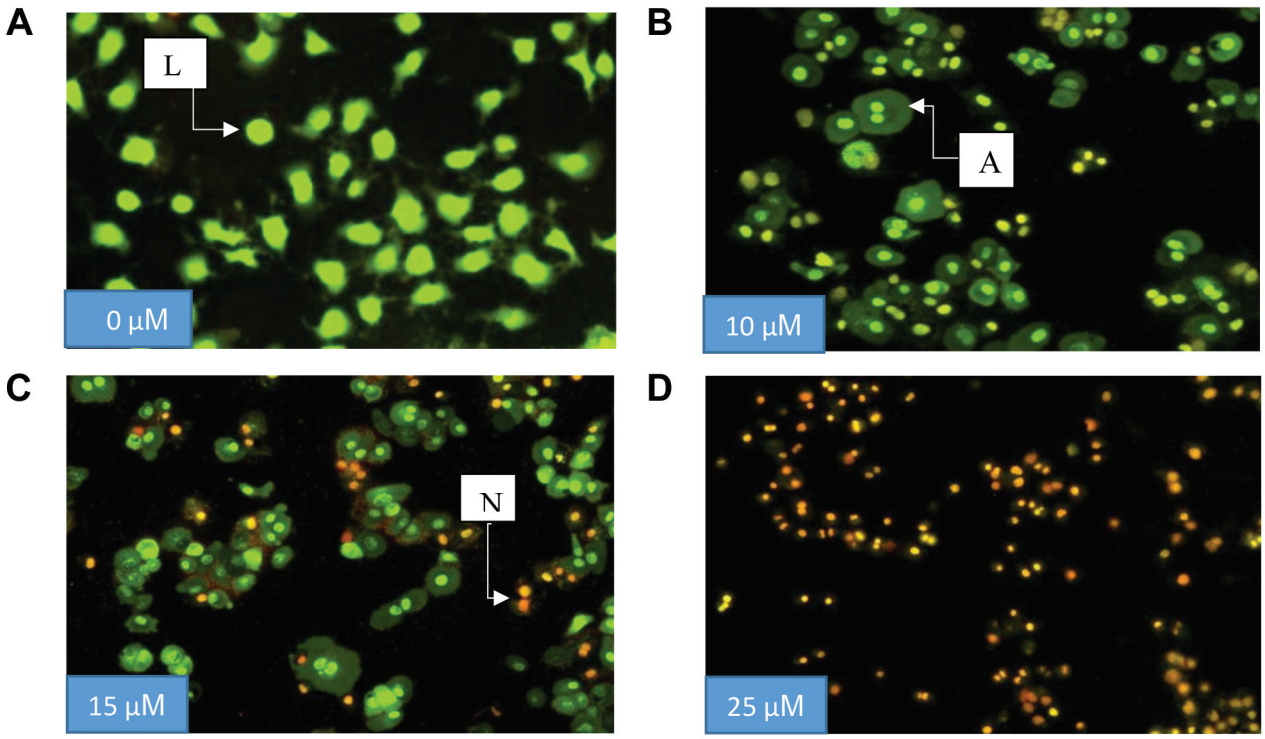


Figure 4. *AO/EtBr staining of A549 cells treated with compound 4f at 10, 15 and 25 µM concentrations. (A) White arrows next to "L" point to live cells stained uniformly (normal green) and represent control cells without treatment with the compound. (B) White arrows next to "A" indicate apoptotic cells and represent cells stained bright green with "dots" of condensed or fragmented chromatin. (C) White arrows next to "N" indicate necrotic cells and represent cells stained with orange color, with cell shrinkage, because of the entry of ethidium bromide into these cells.*

and G_2/M phases) (Figure 2). Based on the above findings, we evaluated whether the mitochondria play a key role in the activation of apoptosis in compound 4f-induced cytotoxic activity. The result showed a dose-dependent decrease in fluorescence intensity with respect to the untreated control cells in A549 cells, indicating that the elicited cytotoxic activity is associated with the disruption of MMP (Figure 3A). This finding is consistent with previous studies suggesting that 7,8-Diacetylated arylcoumarins cause loss of MMP (19, 27). Mitochondria are an important source of ROS production in cells and chemotherapeutic agents that raise ROS level above a safe threshold can induce DNA damage, cell cycle arrest and apoptosis (22). The result of intracellular ROS level measurements in A549 cells showed a concentration-dependent increase in ROS production with respect to the untreated control cells (Figure 3B), thus resulting in oxidative stress. This finding is consistent with previous studies suggesting that 7,8-Diacetylated arylcoumarins elevate the level of ROS production in cancer cell lines (19, 27-29). Furthermore, viability results of the pretreated A549 cells with NAC, followed by compound 4f treatment did show significant changes in cell viability with respect to the with cells treated without NAC (Figure 3C), clearly demonstrating that compound 4f-induced cell death is dependent on ROS production in A549 cells. Lastly, the efficacy of anticancer drugs is measured by their ability to detect cancer cells and selectively promote their apoptosis as visualized by changes in morphological features and extensive DNA fragmentation (23, 30-32). Dual AO/EB fluorescent staining can be used to identify apoptosis-associated changes of cell membranes during the process of apoptosis. The results from this study showed that compound 4f-induced apoptosis/necrosis in A549 cancer cells in dose dependent manner as evident by the observed increase in apoptotic cells at lower concentrations (10 and 15 µM; Figure 4B and C) and necrosis at higher concentrations (15 and 25µM; Figure 4C and D). This finding is consistent with our previous studies suggesting that 3,4-Diarylcoumarins induced cell death in the A549 cell line *via* apoptosis mechanisms (33).

Conclusion

In conclusion, the present study demonstrated that compound 4f exhibited higher cytotoxicity in A549 cells compared to the other synthesized analogs. Its cytotoxic mode of action

is associated with cell cycle arrest at different phases, loss in MMP and ROS-dependent cell death. The present investigation revealed that the presence of 7,8-diacetoxy and 3-*p*-(methylsulfonyl) groups on the 3,4-Diarylcoumarin ring is critical in modulating higher cytotoxic activity.

Conflicts of Interest

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

Authors' Contributions

Musiliyu A. Musa: Designed and conducted the cytotoxicity studies of 3,4-Diarylcoumarins, including the write-up and revision of the manuscript. Qudus Kolawole: Designed and performed the assay, analyzed the data and wrote the section on AO/EtBr staining. Both Authors approved the final version of the manuscript.

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