

Novel triazole analogs of apigenin-7-methyl ether exhibit potent antitumor activity against ovarian carcinoma cells via the induction of mitochondrial-mediated apoptosis

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Abstract. Ovarian cancer is one of the main causes of cancer-associated mortality across the world. Currently, ovarian cancer is mainly treated with chemotherapy. However, ovarian cancer is detected at advanced stages and chemotherapy has numerous side effects. In addition, the results of current chemotherapy on the treatment of ovarian cancer are less than satisfactory. Therefore, there is an urgent need to develop novel and more viable chemotherapeutic agents that can be used to treat ovarian cancer. The present study was designed to synthesize a series of novel triazole analogs of the bioactive apigenin-7-methyl ether to evaluate its anticancer activity against three human ovarian cancer cell lines. A total of eight novel triazole derivatives were synthesized and screened for their anticancer activity. Of all the derivatives, a derivative named 3d exhibited significant and dose-dependent anticancer activity against the SKOV3 ovarian cancer cell line. The IC₅₀ of 3d was found to be 10 μ M against the SKOV3 cancer cell line. It was also observed that 3d induced apoptosis in SKOV3 cancer cells through the accretion of reactive oxygen species and reduction in mitochondrial membrane potential. The molecule also modulated the expression of B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X protein. Taken together, these results showed that the apigenin-7-methyl ether novel derivative 3d may prove an important lead molecule for the treatment of ovarian cancer.

Introduction

Ovarian cancer remains one of the main causes of cancer-associated mortality. Ovarian cancer is mainly treated by chemotherapy and/or by surgical interventions (1,2). Although, the initial responses to chemotherapy are encouraging, the tumors often reoccur. Additionally, only limited anticancer agents are available for the treatment of ovarian cancer (3,4). Naturally occurring compounds have gained considerable attention for the prevention of various types of cancer (5-7). Flavonoids consist of a large group of polyphenolics having a benzo- γ -pyrone skeleton and are widely distributed in the plant kingdom (8). These are also frequently found in fruits, grains, green tea and other dietary supplements (9,10). Numerous biological activities have been reported for flavonoids, including antioxidant, antitumor, anti-inflammatory, antiallergenic and hepatoprotective activities (11,12). It has been found that a flavonoid rich diet reduces the risk of chronic diseases, particularly cancer, including prostate and breast cancer, indicating their potential role as anticancer agents (13,14). Flavonoids, including flavopiridol, epigallocatechin gallate and quercetin, have emerged as potent anticancer drug candidates and a number of these have already entered clinical trials (15).

Apigenin-7-methyl ether is a naturally occurring flavonoid known to possess several pharmacological properties, including anti-inflammatory, antioxidant, and cytotoxic properties (16). Among these, its anticancer effect has been reported against various human cancer cells (16). These findings suggest that apigenin is an ideal bioactive scaffold for the synthesis of a series of analogues and examination of their structure-activity associations, and justify its further investigation. In this context, the present study targeted apigenin-7-methyl ether to synthesize 1,2,3-triazole analogs. Heterocyclic moieties, including 1,2,3-triazoles, are frequently occurring structural motifs in various pharmaceuticals, and are reported to exhibit diverse biological activities, including anti-human immunodeficiency virus (17), antimicrobial (18) and anticancer effects (19,20). The addition of such heterocyclic groups can influence the effectiveness, polarity and aqueous solubility of the parent compound (21,22). Furthermore, triazoles are stable against

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acidic and basic hydrolysis, which is advantageous in resisting metabolic degradation (23). These also have a high dipole moment to facilitate hydrogen bond formation and dipole-dipole interactions while interacting with membrane proteins (24,25).

In view of the preceding discussion, the present study introduced a triazole moiety at the 4'-OH position of apigenin-7-methyl ether through a linker to synthesize desired derivatives using Hugen's 1,3 dipolar cyclo-addition approach. All the synthesized triazolyl hybrids were evaluated against the SKOV3 ovarian cancer cell line. It was demonstrated that these analogs have the potential to induce apoptosis in human ovarian cancer cell lines. The results facilitate the identification of a lead compound capable of inhibiting colony formation in SKOV3 cells and inducing apoptosis via loss of mitochondrial membrane potential (MMP).

Materials and methods

Chemistry. In the present study, the reagents and solvents were purchased from Sigma Aldrich; Merck Millipore (Darmstadt, Germany). TLC (0.25 mm silica gel 60 F254; Merck Millipore) plates were used to monitor the reaction progress. Compound purification was performed by column chromatography using silica gel 60-120 mesh. Bruker DPX 410 and DPX 540 NMR instruments were used to record ¹H NMR and ¹³C NMR spectra, TMS as the internal standard and CDCl₃ as the solvent. The chemical shifts are expressed in δ ppm and coupling constants in Hertz.

Preparation of propargyl apigenin-7-methylether (compound 2). In a typical procedure, the apigenin-7-methyl ether (1 mmol), propargyl bromide (1.1 mmol) and K₂CO₃ (1.5 mmol) were added to a round-bottom flask containing methanol (10 ml). The reaction mixture was then vigorously stirred on a magnetic stirrer at 80°C until the initial material had completely disappeared, which was monitored by TLC. Following completion, the reaction mixture was partitioned with EtOAc and water three times. The collected organic layers were concentrated in a vacuum and purified by column chromatography using silica gel (60-120 mesh) and EtOAc: hexane as eluting solvents to produce compound 2 at 98% yield.

General procedure for the synthesis of triazolyl derivatives (3a-g). Compound 3 (1 eq) and respective organic azides (1.1 eq) were added to a round bottom flask containing 15 ml of 1:1 water: ethanol mixture, to which 10 mol% each of sodium ascorbate and CuSO₄·5H₂O was added. The reaction mixture was stirred on a magnetic stirrer at room temperature until its completion. The crude reaction mixture was then partitioned using aqueous ethylacetate. The collected ethylacetate layer was dried over anhydrous magnesium sulphate and subjected to column chromatography using EtOAc: hexane as eluting solvents to produce the pure desired products (3a-g) in quantitative yields.

5-hydroxy-2-(4-((1-(2-(hydroxymethyl)benzyl)-1H-1,2,3-triazol-4-yl)methoxy)phenyl)-7-methoxy-4H-chromen-4-one (3a). The details of product 3a were as follows: White crystalline solid, yield: 93%; ¹H NMR (400 MHz, CDCl₃) δ 7.55 (d, J=6.3 Hz, 1H), 7.49 (d, J=7.5 Hz, 2H), 7.37 (s, 1H), 7.23-7.17 (m, 3H), 7.01

(d, J=7.4 Hz, 2H), 6.33 (s, 1H), 6.19 (d, J=1.5 Hz, 1H), 6.13 (d, J=1.4 Hz, 1H), 5.16 (s, 2H), 4.76 (s, 2H), 3.81 (s, 3H). ¹³C NMR (125 MHz,) δ 182.28, 166.02, 163.74, 161.60, 159.47, 140.09, 137.73, 136.17, 132.36, 129.19, 127.52, 127.24, 126.97, 125.85, 125.31, 115.31, 105.68, 104.77, 98.01, 93.64, 63.36, 57.74, 56.03.

5-hydroxy-2-(4-((1-(4-hydroxybenzyl)-1H-1,2,3-triazol-4-yl)methoxy)phenyl)-7-methoxy-4H-chromen-4-one (3b). The details of product 3b were as follows: White solid, yield: 89%; ¹H NMR (400 MHz, CDCl₃) δ 7.50 (d, J=7.5 Hz, 2H), 7.42-7.31 (m, 3H), 7.02 (d, J=7.6 Hz, 2H), 6.82 (d, J=7.5 Hz, 2H), 6.30 (s, 1H), 6.21 (d, J=1.4 Hz, 1H), 6.13 (d, J=1.4 Hz, 1H), 5.23 (s, 1H), 3.81 (s, 3H); ¹³C NMR (125 MHz, CDCl₃) δ 182.28, 166.02, 163.74, 161.60, 161.27, 159.47, 136.00, 133.10, 128.4, 128.06, 127.24, 125.3, 117.03, 115.3, 105.68, 104.77, 98.01, 93.64, 57.74, 56.03.

5-hydroxy-7-methoxy-2-(4-((1-(4-methoxybenzyl)-1H-1,2,3-triazol-4-yl)methoxy)phenyl)-4H-chromen-4-one (3c). The details of product 3c were as follows: White solid, yield: 89%; ¹H NMR (400 MHz, CDCl₃) δ 7.63 (d, J=7.5 Hz, 2H), 7.41-7.31 (m, 3H), 7.02 (d, J=7.5 Hz, 2H), 6.81 (d, J=7.5 Hz, 2H), 6.27 (s, 1H), 6.19 (d, J=1.4 Hz, 1H), 6.09 (d, J=1.4 Hz, 1H), 5.27 (s, 1H), 3.81 (s, 3H), 3.80 (s, 3H).

2-(4-((1-(2-fluorobenzyl)-1H-1,2,3-triazol-4-yl)methoxy)phenyl)-5-hydroxy-7-methoxy-4H-chromen-4-one (3d). The details of product 3d were as follows: White solid, yield: 93%; ¹H NMR (400 MHz, CDCl₃) δ 7.56 (d, J=7.5 Hz, 2H), 7.48 (m, 1H), 7.39 (s, 1H), 7.15 (m, 1H), 7.10 (m, 1H), 7.07-6.97 (m, 3H), 6.52 (s, 2H), 6.26 (d, J=1.4 Hz, 1H), 6.11 (d, J=1.6 Hz, 1H), 5.18 (s, 2H), 3.81 (s, 3H).

3-((4-((5-hydroxy-7-methoxy-4-oxo-4H-chromen-2-yl)phenoxy)methyl)-1H-1,2,3-triazol-1-yl)methyl)benzonitrile (3e). The details of product 3e were as follows: Yellowish solid, yield: 93%; ¹H NMR (400 MHz, CDCl₃) δ 7.98 (m, 1H), 7.92 (d, J=1.5 Hz, 1H), 7.51 (d, J=7.5 Hz, 2H), 7.42-7.32 (m, 3H), 7.03 (d, J=7.5 Hz, 2H), 6.32 (s, 1H), 6.21 (d, J=1.4 Hz, 1H), 6.14 (d, J=1.4 Hz, 1H), 5.22 (s, 2H), 3.79 (s, 3H).

2-(4-((1-butyl-1H-1,2,3-triazol-4-yl)methoxy)phenyl)-5-hydroxy-7-methoxy-4H-chromen-4-one (3f). The details of product 3f were as follows: Yellow amorphous powder, yield: 93%; ¹H NMR (400 MHz, CDCl₃) δ 7.52 (d, J=7.5 Hz, 2H), 7.11 (s, 1H), 7.03 (d, J=7.5 Hz, 2H), 6.30 (s, 1H), 6.25 (d, J=1.4 Hz, 1H), 6.21 (d, J=1.4 Hz, 1H), 5.22 (s, 2H), 3.82 (s, 3H), 2.93 (m, 2H), 1.26-1.37 (m, 4H), 0.91 (t, 3H, J=7.2 Hz).

2-(4-((1-pentyl-1H-1,2,3-triazol-4-yl)methoxy)phenyl)-5-hydroxy-7-methoxy-4H-chromen-4-one (3g). The details of product 3g were as follows: Yellow amorphous powder, yield: 93%; ¹H NMR (400 MHz, CDCl₃) δ 7.50 (d, J=7.4 Hz, 2H), 7.09 (s, 1H), 7.03 (d, J=7.4 Hz, 2H), 6.31 (s, 1H), 6.27 (d, J=1.4 Hz, 1H), 6.22 (d, J=1.4 Hz, 1H), 5.22 (s, 2H), 3.82 (s, 3H), 2.95 (m, 2H), 1.29-1.38 (m, 6H), 0.92 (t, 3H, J=7.2 Hz).

Antiproliferative assay. The antiproliferation effect of the novel triazole analogs of apigenin-7-methyl ether against three human ovarian cancer cell lines, OVCAR-3, Caov-3,

and SKOV3, (Type Culture Collection of Chinese Academy of Sciences, Shanghai, China) was investigated with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were cultured at the density of 1×10^6 cells/well in 96-well plates for a time period of 12 h at 37°C. The cells were then subsequently treated with 0-200 μ M doses of the apigenin-7-methyl ether derivatives for 24 h at 37°C. Following this, 20 μ l of MTT solution was added to each well. Prior to the addition of 500 μ l of DMSO, the medium was completely removed. For solubilizing the MTT formazan crystals, dimethylsulfoxide (500 μ l) was added. The absorbance at 570 nm was measured using an ELISA plate reader. As the derivative 3d was found to be most active, only this molecule was used for further experimentation.

Colony formation assay. To investigate the effect of the 3d derivative on the colony formation potential of SKOV3 cells, the cells were collected at the exponential growth phase and then counted using a hemocytometer. The plating of the cells was performed at 200 cells/well, and the plates were then incubated at 37°C for 48 h to permit the cells to adhere. This was followed by the addition of various concentrations (0, 5, 10, and 20 μ M) of 3d. Following treatment with 3d, the cells plates were incubated for 6 days at 37°C. Following 6 days of incubation, the cells were washed with PBS and fixed with methanol. Subsequently, the cells were treated with crystal violet for 30 min at room temperature and then counted under a light microscope (magnification, x200).

Detection of apoptosis. The SKOV3 cells were seeded at the density of 1×10^6 cells/well in 6-well plates and then treated with 0, 10, 20 and 40 μ M 3d for the time period of 24 h at 37°C. This was immediately followed by 4',6-diamidino-2-phenylindole (DAPI) staining at 25°C for 5 min. The cell samples were then examined and images were captured with a fluorescence microscope (magnification, x200). To estimate the apoptotic cell populations, the SKOV3 cells were seeded at a density of 1×10^6 cells/well in 6-well plates and treated with varied concentrations (0, 5, 10, and 20 μ M) of 3d for 24 h at 37°C. The cells were then collected and washed with PBS. The cells were then incubated with Annexin V/FITC and PI for 15 min and the apoptotic cell populations were estimated by flow cytometry (BD Biosciences, San Jose, CA, USA). The estimated percentage of cells in each phase of the cell cycle was quantified using WinMDI software v2.0 (Informer Technologies, Inc., Los Angeles, CA, USA).

Determination of ROS and MMP. The SKOV3 cells were seeded at a density of 2×10^5 cells/well in a 6-well plate and incubated at 37°C for 24 h and treated with 0, 5, 10, and 20 μ M of 3d for 24 h at 37°C in 5% CO₂ and 95% air. Subsequently, the cells from all samples were collected, washed twice PBS and resuspended in 500 μ l of DCFH-DA (10 μ M) for ROS estimation and DiOC₆ (1 μ mol/l) for MMP at 37°C in a dark room for 30 min. The samples were then examined immediately using a flow cytometer and BD FACSuite software v1.0 (BD Biosciences, San Jose, CA, USA).

Western blot analysis. Protein expression was determined by western blot analysis. Briefly, the cells were lysed in a

lysis buffer (20 mM HEPES, 350 mM NaCl, 20% glycerol, 1% Nonidet P 40, 1 mM MgCl₂, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF, 2 mM protease inhibitor cocktail and 10% phosphatase inhibitor cocktail). The proteins present in the cell extracts were quantified using a BCA assay and proteins (50 μ g/lane) from each sample were resolved by SDS-PAGE on a 10% gel. This was followed by transference onto a nitrocellulose membrane. The membrane was then treated with non-fat milk (5%) in PBS, and then incubated with a suitable primary antibody: B-cell lymphoma 2-associated X protein (Bax; cat. no. sc-6236) and Bcl-2 (cat. no. sc-509) purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA) overnight at 4°C (dilution 1:1,000), followed by incubation with horseradish peroxidase-conjugated (cat. no. 9003-99-0) and anti-rabbit secondary antibody (cat. no. sc-2372) (dilution 1:1,000) for 1 h at room temperature. The western blots were then observed in an ECL western blot analysis system (GE Healthcare Life Sciences, Chalfont, UK).

Statistical analysis. The experiments were repeated three times and results are presented as the mean \pm standard deviation. The significance was determined, compared with the untreated control, using one way analysis of variance and Tukey's test with GraphPad prism 7 software (GraphPad Software, Inc., La Jolla, CA, USA). P<0.01 was considered to indicate a statistically significant difference.

Results

Synthesis of novel triazole analogs of apigenin-7-methyl ether. In the present study, apigenin-7-methylether (compound 1) was isolated from the ethanolic extract of leaves of *Aquilaria sinensis*. The isolated natural product (compound 1) was subjected to propargylation using propargyl bromide in presence of base K₂CO₃ to give compound 2. Compound 2 was then reacted with different substituted organic azides under click chemistry conditions (Fig. 1) to produce desired 1,2,3-triazole products in quantitative yields. In the ¹H NMR, products were easily identified by a characteristic singlet for H-5 in the 1,2,3-triazole moiety, which appeared as singlet downfield (~7.5 ppm) with other aromatic protons. All the prepared triazolyl analogs were characterized by ¹H NMR, ¹³CNMR and MS spectroscopic analysis.

Anticancer effects of synthesized derivatives on ovarian cancer cell lines. To investigate the antiproliferative role of synthesized compounds on three human ovarian cancer cell lines (OVCAR-3, Caov-3, and SKOV3), the cells were treated with different concentrations of the synthesized compounds and the IC₅₀ was determined for all compounds (Table I). Compound 3d exhibited a potent antiproliferative effect against SKOV3 cells in a dose-dependent manner with an IC₅₀ of 10 μ M (Fig. 2). In the formazan crystal assay, it was revealed that administering 3d to cells reduced the number of formazan crystals in a concentration-dependent manner (Fig. 3). As 3d exhibited highest activity against the SKOV3 cells, this cell line was used for further experimentation.

Compound 3d induces apoptosis in SKOV3 ovarian cancer cells. Following treatment with the different concentrations

Table I. IC₅₀ values of novel triazole analogs of apigenin-7-methyl against ovarian cancer cell lines, determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

Derivative	SKOV3 (μM)	OVCAR-3 (μM)	Caov-3 (μM)
1	29	30	30
2	20	25	20
3a	18	20	20
3b	17	20	20
3c	25	30	25
3d	10	15	20
3e	40	40	40
3f	40	30	25
3g	40	40	30

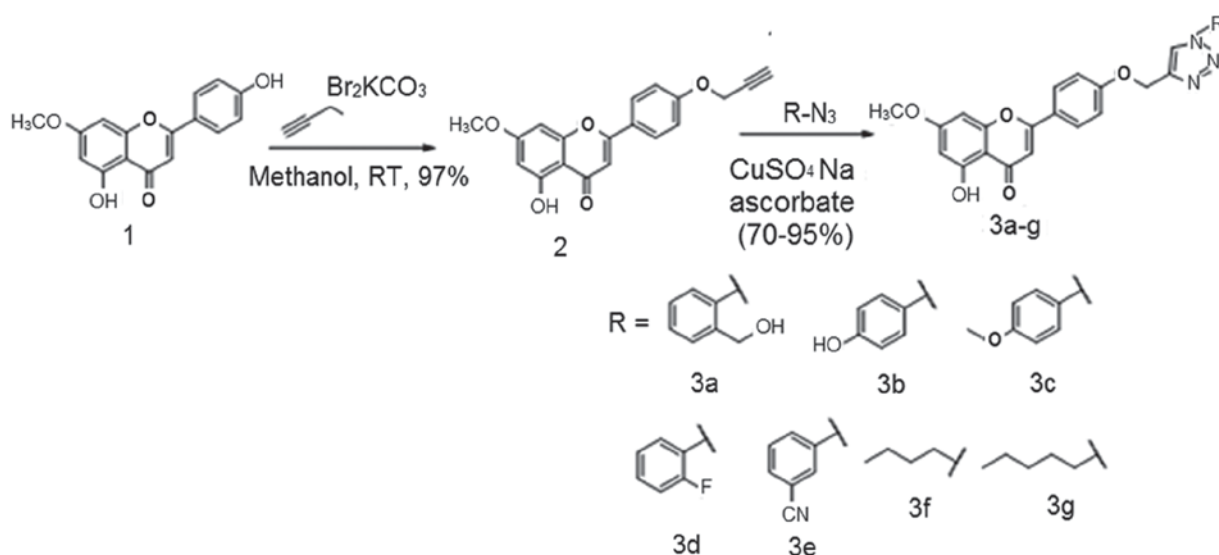


Figure 1. Schematic diagram showing different steps for the synthesis of novel triazole analogs of apigenin-7-methyl ether.

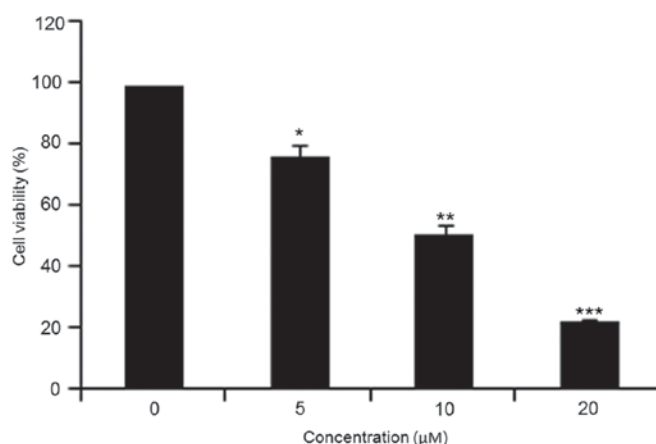


Figure 2. Effect compound 3d on the viability of SKOV3 cells. The experiments were repeated three times and data are presented as the mean \pm standard deviation. (* $P < 0.01$, ** $P < 0.001$ and *** $P < 0.0001$, vs. untreated control).

of compound 3d, apoptosis was detected by DAPI staining. The results indicated that compound 3d caused apoptosis

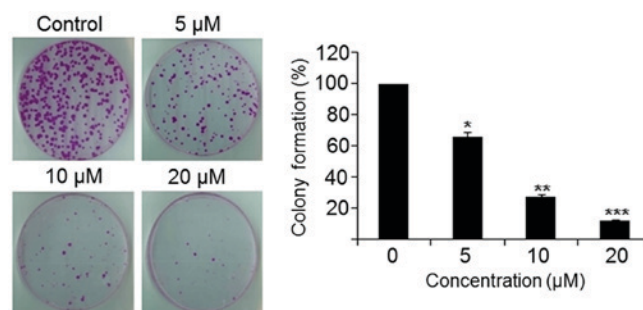


Figure 3. Effect of indicated doses of compound 3d on the colony formation potential of SKOV3 cells. The experiments were repeated three times and data are presented as mean \pm standard deviation (* $P < 0.01$, ** $P < 0.001$ and *** $P < 0.0001$, vs. untreated control).

in a concentration-dependent manner, as evident from the increased density of white-colored nuclei (Fig. 4). The apoptotic cell populations were further estimated by annexin V/PI staining and it was observed that the apoptotic cell populations increased from 0.05% in the control to 39.62% at 20 μ M

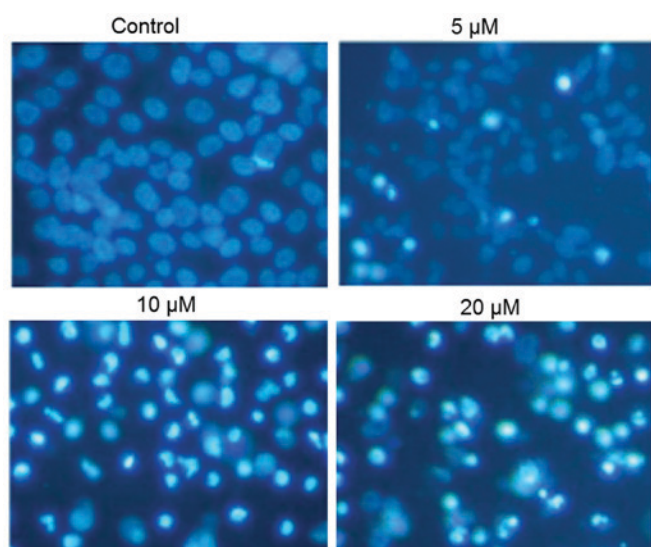


Figure 4. Compound induces apoptosis of SKOV3 cancer cells. Apoptosis was evident from the DAPI staining (magnification, x200). The experiments were repeated three times.

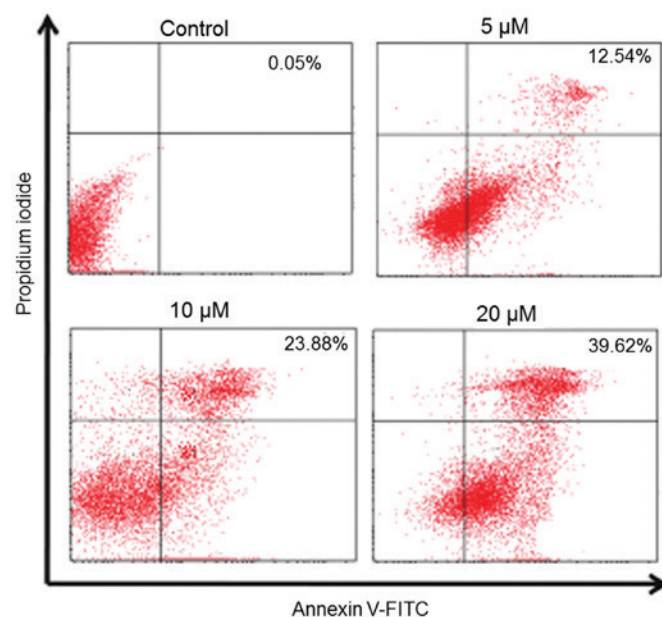


Figure 5. Estimation of apoptotic cell populations at the indicated doses of compound 3d. The experiments were repeated three times.

concentrations of 3d (Fig. 5). In addition, this was associated with the increase in the expression of Bax and a decrease in the expression of Bcl-2 (Fig. 6).

Compound 3d triggers ROS activation in SKOV3 ovarian cancer cells. The potential of 3d to induce apoptosis, as observed through DAPI staining, indicated that 3d may trigger the production of intracellular ROS. Therefore, the present study estimated the ROS level at different concentrations of 3d for 48 h. The results showed that the intracellular ROS levels of the treated cells increased up to 255%, compared with the untreated cells (Fig. 7). This result suggested that compound 3d is an effective molecule for stimulating the generation of ROS in SKOV3 cells.

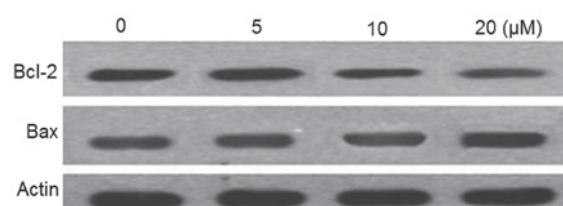


Figure 6. Effect of indicated doses of 3d on Bax and Bcl-2 proteins. Protein expression of Bax and Bcl-2 are shown in the western blots. The experiments were repeated three times. Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated x protein.

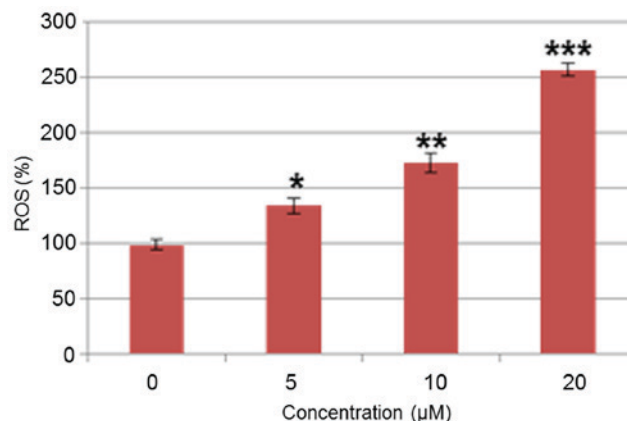


Figure 7. Effect of indicated doses of compound 3d on ROS production. The experiments were repeated three times and data are presented as mean \pm standard deviation (* P <0.01, ** P <0.001 and *** P <0.0001, vs. untreated control). ROS, reactive oxygen species.

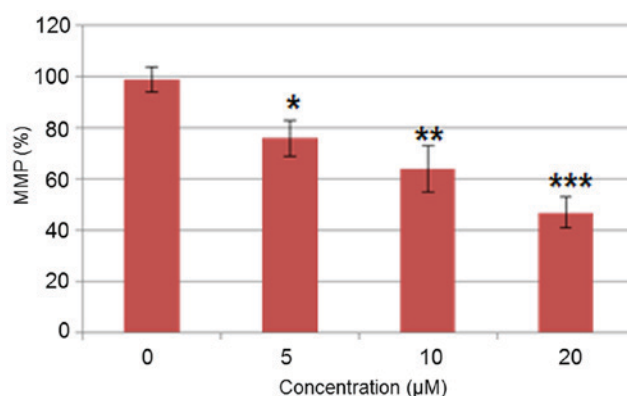


Figure 8. Effect of indicated doses compound 3d on MMP. The experiments were repeated three times and results are presented as the mean \pm standard deviation (* P <0.01, ** P <0.001 and *** P <0.0001, vs. untreated control). MMP, mitochondrial membrane potential.

Compound 3d reduces MMP. The generation of ROS causes mitochondrial mutilation and disrupts the outer mitochondrial potential, ultimately leading to the discharge of death-promoting proteins (16). Therefore, the present study investigated whether compound 3d decreased the MMP in the SKOV3 cells administrated with various doses (0-20 μM). The compound 3d-administrated SKOV3 cells showed a considerable decrease in MMP in a dose-dependent manner. The MMP decreased up to 63% at 20 μM of compound 3d, compared with that in the untreated control (Fig. 8).

Discussion

Of types of gynecological cancer, ovarian cancer is one of the main causes of cancer-associated mortality around the world. Despite preliminary responses to chemotherapy, the tumors consistently relapse (1,2). Apeginin-7-methyl ether is a naturally occurring flavonoid reported to possess several biological activities, including antioxidant, anti-inflammatory and antitumor activities (15). Among these, its anticancer effect has been reported against various human cancer cells. These findings suggest that apigenin is an ideal bioactive scaffold for the synthesis of a series of analogues and examination of their structure-activity associations, and justifies its further investigation. In this context, the present study targeted apeginin-7-methyl ether to synthesize 1,2,3-triazole analogs. All derivatives exhibited potential growth inhibitory effects on the three ovarian cancer cell lines, as evident from the proliferation assay, however 3d exhibited the most potent activity against the SKOV3 cancer cell line. As it has been shown previously, several anticancer drugs trigger antiproliferative effects through the induction of apoptosis (26,27). For example, the anticancer drugs cisplatin, taxol and 5-fluorouracil (28-34) have been shown to activate apoptotic pathways and cause DNA damage (35). To assess whether compound 3d triggers apoptosis in SKOV3 cells, the treated cells were subjected to DAPI staining. The results revealed that compound 3d induced apoptotic damage in a concentration-dependent manner. In addition to this, it was observed that the compound 3d-treated cells showed that ROS promoted a reduction in MMP (33). Therefore, these results indicated that compound 3d may trigger apoptosis by the accretion of intracellular ROS and lessening of MMP. These results are well supported by earlier studies wherein a number of anticancer drugs have been shown to cause cancer cell death partly by the generation of high levels of ROS (35). In addition, the role of mitochondria in ROS is key (36). For example, capsaicin disrupts MMP and modulates oxidative stress, resulting in apoptosis of pancreatic cancer cells (37). Therefore, the inhibitory effect of compound 3d on ovarian cancer cells may prove beneficial in the treatment and management of ovarian cancer.

In conclusion, a small series of apeginin-7-methylether derived 1,2,3-triazole hybrids were synthesized using an alkyne azide cyclo-addition reaction. All the prepared triazolyl analogs were evaluated against the SKOV3 human ovarian cancer cell line, however, the biological data revealed that compound 3d exhibited the lowest IC₅₀ and exerted its anticancer activity through the induction of apoptosis through ROS-mediated alterations in MMP. The present study confirmed that potential anticancer agents can be synthesized from flavonoids.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YQ, ZD, YY and DM performed all the experiments. FR, HY and AC collected the materials and provided instrumental suggestion for the present study. AC designed the study.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors confirm that they have no competing interests.

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