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Differential Mechanism of Action of 3,4',7-O-trimethylquercetin in Three Types of Ovarian Cancer Cells

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Abstract. *Background/Aim: 3,4',7-O-trimethylquercetin (34'7TMQ), a derivative of quercetin, inhibited ovarian cancer cell migration and invasion without affecting proliferation. In this study, the apoptotic effect of 34'7TMQ on three cancer cell lines (CRL-1978, CRL-11731, SK-OV-3) was evaluated. Materials and Methods: Expression of pro-apoptotic proteins such as Bax/Bcl-2 ratio, p38 MAP kinase, and caspase-9 were measured by western blot analysis. Annexin-V staining was performed to visualize apoptotic signaling. Results: Caspase-9 was up-regulated in all three cell lines. Bax/Bcl-2 ratio was up-regulated in CRL-1978 and SK-OV-3 but down-regulated in CRL-11731. The p38 MAPK was down-regulated in CRL-1978, up-regulated in SK-OV-3, and had differential expression in CRL-11731. Annexin V staining indicated that 34'7TMQ at 6.25 μM induced apoptotic signaling in the CRL-1978 ovarian cancer cell line. Conclusion: 34'7TMQ induced apoptosis in three types of cancer cell lines but it appears to have a different mechanism of action in each cell line.*

Ovarian cancer is the most malignant gynecological cancer. To better understand the pathogenesis of this cancer type, experts have been studying the ovarian-cancer associated genes that are fundamental for the initiation and metastasis of ovarian cancer (1). It is necessary to be able to distinguish between benign and malignant ovarian tumours because stage IV ovarian cancer is lethal, and the chance of survival is approximately 15% (2). Ovarian cancer is the fourth leading cause of cancer mortality in the United States, accounting for the death of 14,000 women annually (2, 3). The statistical 5-year survival rate is approximately 39% and survival rates are approximately 90% if the cancers are detected in either stage I or II. Researchers have identified some risk factors for ovarian cancer such as family history of breast/ovarian cancer, increasing age, high fat diet, and usage of fertility drugs (4). Mutation of the Breast Cancer Resistance Genes (*BRCA*) 1 and 2 are also considered high-risk factor for the development of ovarian cancer (5). Significant progress has been made to improve diagnostic methods, discovering new biomarkers, and introducing phytochemicals to treat different forms of cancer (6). A novel anti-cancer treatment which is under investigation is a derivative of quercetin known as 3,4',7-O-trimethylquercetin (34'7TMQ) (7). A study by Yamauchi *et al.* showed this compound suppressed the invasive and migratory capacity of ovarian cancer cells without statistically affecting their proliferation (8). Further studies are being performed to elucidate the differential effects of this compound on different types of ovarian cancer cells (8). Quercetin's anti-oxidant properties, in addition to the ability to modulate different signal transduction pathways, are shown to prevent and impede numerous inflammatory reactions (9, 10). Epigenetic pathways that are essential for cancer progression are also potential targets of quercetin. Quercetin increases the cytosolic Ca^{2+} levels leading to activation of pro-apoptotic proteins such as caspase-3, -8, and -9 (11). Quercetin dihydrate is known to target the cell cycle of tumor cells and prevent further growth of the tumor. This compound modulates cell cycle components such as cyclin-dependent kinases and topoisomerase II to block the cell cycle at the G_2/M or at the G_1/S transition (11, 12). Quercetin has also shown to down-regulate the

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Key Words: Ovarian cancer, apoptosis, Annexin-V, methylquercetin.

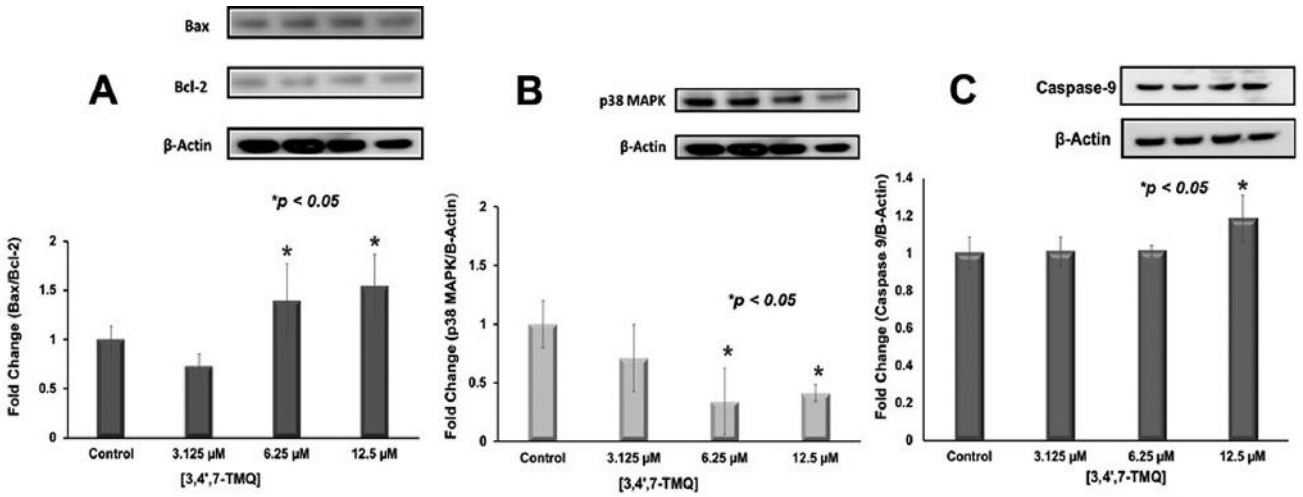

Figure 1. *Western Blot analysis of Bax/Bcl-2 (A), p38 MAPK (B), and caspase-9 (C) expression in CRL-1978 cell line. Data are expressed as means±SEM; n=3; *p≤0.05 compared to respective control values.*

HGF/c-Met pathway which is identified to induce tumor invasion, migration, and metastasis (12). Cao *et al*. found that melanoma cells pretreated with quercetin reduced the phosphorylation of c-Met in the HGF-stimulated cells compared to the cells that were not pretreated with quercetin. Real-time PCR data displayed the decrease in the mRNA expression of HGF in melanoma cells that were pretreated with quercetin (9).

One of the common approaches to understand the mechanism of action of a compound is to investigate whether the compound causes natural cell death in cancer cells. Cancer cells lack contact inhibition and therefore are able to proliferate in an uncontrollable manner in absence of apoptosis. This study, investigates the expression of a number of pro-apoptotic proteins in response to 34'7TMQ. Bax serves as an apoptosis regulator in humans and accelerates programmed cell death by binding to and antagonizing the apoptosis repressor protein Bcl-2. Overexpression of Bax accelerates cell death induced by cytokine deprivation in an interleukin-3 (IL-3) dependent cell line. Human Bcl-2 is an anti-apoptotic protein that promotes cell survival through protein–protein interactions. Bcl-2 can also interfere with the activation of cytochrome c/Apaf-1 pathway leading to stabilization of the mitochondrial membrane and inhibiting apoptosis. p38 MAPK plays a significant role in cell adhesion, differentiation and apoptosis; uses Mg^{2+} as a cofactor to catalyze the ATP-dependent phosphorylation of pro-apoptotic target proteins. Caspase-9 is responsible for apoptosis execution by binding Apaf-1 leading to activation of a protease which then cleaves and activates caspase-3.

Materials and Methods

General experimental procedures. Human ovarian cancer cells were purchased from Cell Biolabs (San Diego, CA, USA). Antibodies against Bax (N-20), Bcl-2 (N-19), p38α/β (H-147), β-actin (C4), horseradish peroxidase (HRP)-conjugated anti-rabbit IgG donkey antibody (sc-2313) and HRP-conjugated anti-mouse IgG goat antibody (sc-2318) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Antibody against Caspase-9 (E-23) was purchased from abcam (Cambridge, UK). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Gaithersburg, MD, USA). Penicillin-streptomycin-amphotericin Cocktail (LS-1085) was purchased from LifeLine (Frederick, MD, USA). Cell lysis buffer was purchased from Cell Signaling Tech. (Beverly, MA, USA).

Cell culture. Three ovarian cancer cell lines, CRL-1978, CRL-11731 and SK-OV-3 (American Type Culture Collection, Manassas, VA, USA), were grown in DMEM supplemented with 10% FBS and penicillin-streptomycin-amphotericin B cocktail. Cells were cultured at 37˚C in a humidified atmosphere of 5% CO_2.

Western blot assay. Ovarian cancer cells treated with 34'7TMQ at 0 μM, 3.125 μM, 6.25 μM and 12.5 μM for 48 h were lysed with cell lysis buffer containing a protease inhibitor cocktail at 0˚C for 10 min. Protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit (#23227; Fisher Scientific, Waltham, MA, USA) and a bovine serum albumin solution as a standard. Cell lysates were loaded at 10 μg of protein per lane and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on Invitrogen™ Novex™ NuPAGE™ 4-12% Bis-Tris gel (Fisher Scientific, Waltham, MA, USA). Proteins were subsequently transferred onto a nitrocellulose membrane (Bio Rad, Hercules, CA, USA) using a wet transfer

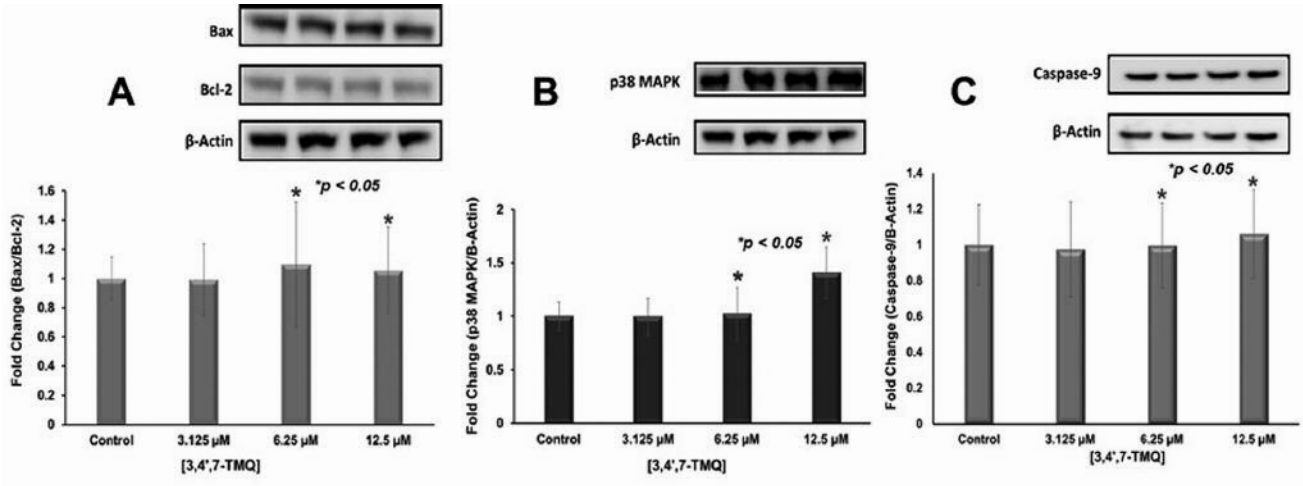

Figure 2. *Western Blot analysis of Bax/Bcl-2 (A), p38 MAPK (B), and caspase-9 (C) expression in SK-OV-3 cell line. Data are expressed as means±SEM; n=3; *p≤0.05 compared to respective control values.*

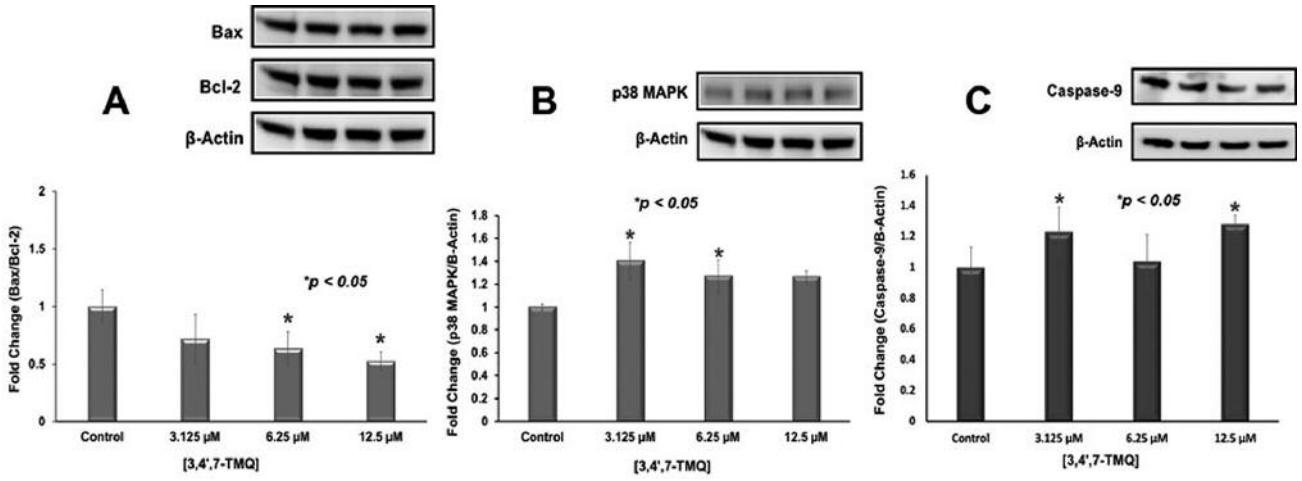

Figure 3. *Western Blot analysis of Bax/Bcl-2 (A), p38 MAPK (B), and caspase-9 (C) expression in CRL-11731 cell line. Data are expressed as means±SEM; n=3; *p≤0.05 compared to respective control values.*

system for 2 h. The membrane was blocked with 5% dry milk in tris-buffered saline Tween20 (TBST) at 4˚C for 2 h and then incubated with dilutions of mouse monoclonal anti-β-actin (1:10,000), rabbit polyclonal anti-Bax (1:100), rabbit polyclonal anti-Bcl-2 (1:200), mouse monoclonal anti- p38α/β (1:200) or rabbit monoclonal anti-Caspase-9 (1:1,000) antibodies. Following overnight incubation at 4˚C, the membranes were washed and incubated with 1: 2,000 diluted HRP-conjugated secondary antibodies for 2 h. Following addition of the SuperSignal® substrate (Fisher Scientific), protein density was visualized using enhanced chemiluminescence detection system (LAS-4000; Fujifilm, Tokyo, Japan) and quantified by Image J Software® (https://imagj.nih.gov/ij/).

Annexin-V staining. CRL-1978 cells were seeded on coverslips in a 6-well plate and allowed to adhere overnight. The cells were then stimulated with 0 μM and 6.25 μM of 34'7TMQ for 48 h. Afterwards the cells were incubated with biotinylated Annexin-V (Roche Applied Science, Penzberg, Germany) and then Cy3-labeled streptavidin (GE Healthcare, Chicago, IL, USA), the coverslips were mounted on microscope slides with the nuclei marker 4', 6' diamidino-2-phenylindole (DAPI) (Invitrogen, Carlsbad, CA, USA). To measure the apoptotic signal, cells were then observed on an Olympus FluoView FV 300 confocal laser-scanning microscope. Due to time constraints and scheduling conflicts, immunofluorescence was performed on only one cell line for two treatment concentrations (0 μM and 6.25 μM).

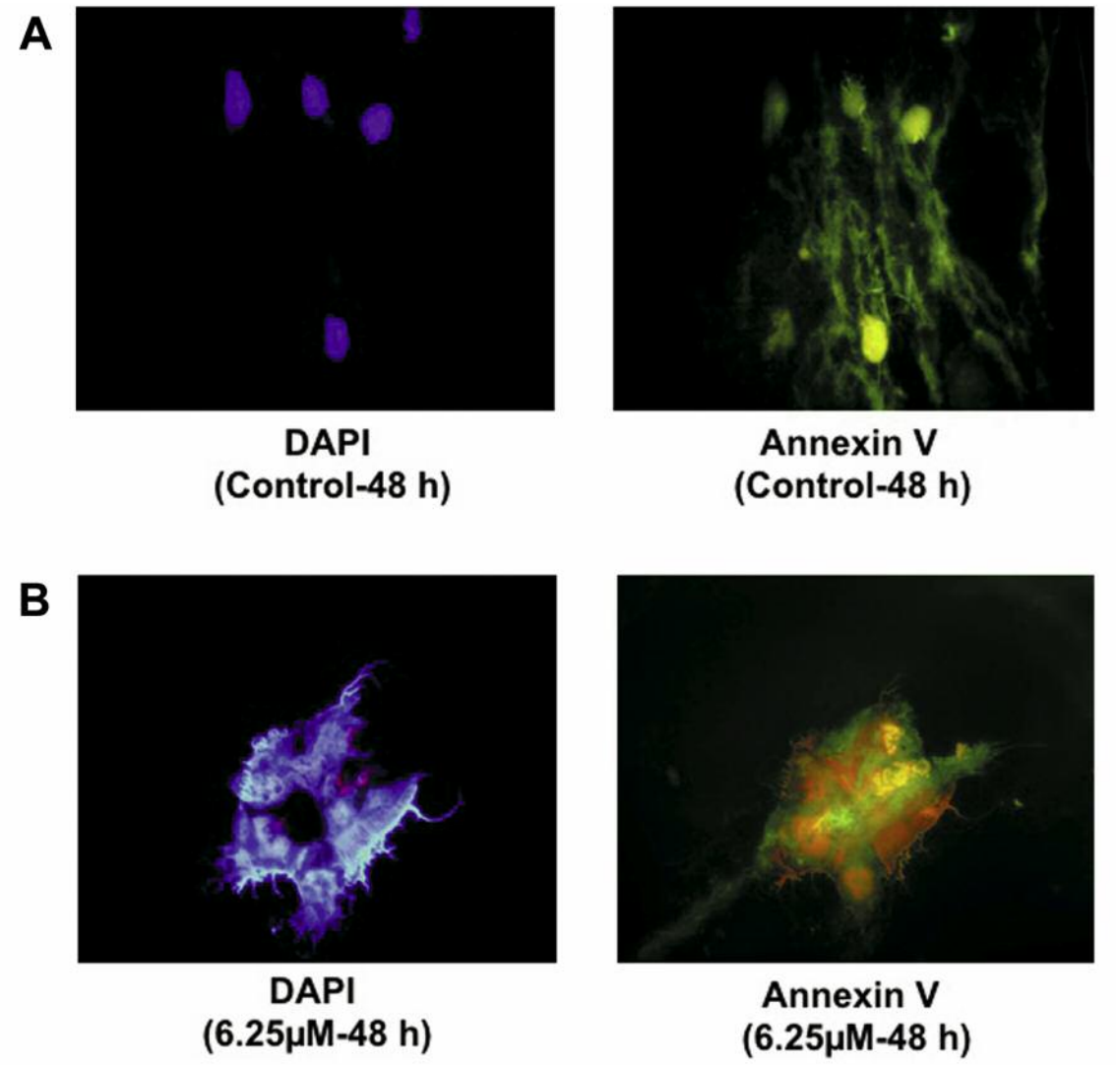

Figure 4. *Evaluation of apoptotic signaling using Annexin-V staining. DAPI and Annexin V staining images for the control sample (A). DAPI and Annexin V staining images for the 6.25 μM treatment (B).*

Statistical analysis. All data are expressed as means±SEM values. Statistical significance of differences was evaluated using the Student's *t*-test. Data from 3417TMQ-treated cells in *in vitro* experiments were compared with those from basal DMSO-treated controls using the Student's *t*-test with repeated measures as 3417TMQ dosages varied within each experiment. Experiments were repeated three times to produce the replicates and data were expressed as means±SD. A *p*-value of less than 0.05 was considered significant.

Results

Western blot assays were performed to investigate the possible mechanism of action of 34'7TMQ. Expression of pro-apoptotic proteins were studied to see if 34'7TMQ induced apoptosis in the three ovarian cancer cell lines (CRL-1978, SK-OV-3, and CRL-11731). According to Figure 1, the expression of Bax/Bcl-2 (A) and caspase-9 (C) increased whereas expression p38 MAPK (B) decreased in CRL-1978 cell line. The up-regulation of Bax/Bcl-2 and caspase-9 in tandem with down-regulation of p38 MAPK demonstrates 34'7TMQ induces apoptosis in CRL-1978 cells in a dose dependent manner. In the SK-OV-3 cell line (Figure 2), the elevated expression of Bax/Bcl-2 (A) and caspase-9 (B) suggests pro-apoptotic signaling however, in contrast, p38 MAPK (C) expression was up-regulated. This suggests the possibility of a differential mechanism of action of 34'7TMQ. CRL-11731 cell line (Figure 3) exhibits differential behavior in the expression of all three apoptotic

proteins in response to 34'7TMQ. Up-regulation of caspase-9 (C) causes apoptosis in the cell line, however, the down-regulation of the Bax/Bcl-2 (A) indicates cell survival. The expression of p38 MAPK (B) varies as it increases with the first two doses of 34'7TMQ (3.125 μM and 6.25 μM), but then decreases at 12.5 μM 34'7TMQ. These results indicate the differential induction of apoptotic signaling in CRL-1978, CRL-11731, and SK-OV-3 cell lines in response to 34'7TMQ.

Annexin V/FITC staining was performed to detect the presence of phosphatidylserine (PS) residues on the surface of the cell (13). This is an early event in apoptosis and can be used to qualitatively and quantitatively detect and measure apoptosis. PS is normally present in the plasma membrane and cannot be detected by Annexin V staining unless the molecule somehow surfaces to the membrane due to the stimulation of programmed cell death (13). In the control sample (Figure 4A), intact nuclei in both the DAPI and FITC filter was observed. The shape of the nuclei appeared normal and no overlapping of stains were detected. When the cells were treated with 6.25 μM 34'7TMQ (Figure 4B), the nuclei appeared distorted and an overlapping of stains (orange) was observed. This demonstrates that 34'7TMQ induces apoptosis in CRL-1978 ovarian cancer cells and validates the results from the western blot analysis.

Discussion

Ovarian cancer has been recognized as a heterogenous disease due to the presence of various histological subtypes with unique clinicopathological and molecular features. The single layer of epithelial cells that lines the surface of the ovary is known to be progenitor cells for ovarian cancer (3). Understanding the etiology of cancer cells and their response to different chemicals has been a challenge for all researchers. Studies are being constantly performed to understand the effect of different novel compounds on proliferation and migration of cells (14, 15). McDowell *et al*. examined the effect of a novel compound, cinobufotalin (CINO), on an ovarian cancer cell line *in vitro* and found that it decreased the proliferation, migration, and invasion of the cancer cells (16). It was also found that CINO was able to hinder the progression of cytotrophoblast cells and ovarian cancer cells by inducing cell cycle arrest and apoptosis (16, 17). Studies like these opened an avenue to discover and explore the benefit of other natural compounds such as Quercetin. Quercetin is classified as a flavonoid and one of approximately 4,000 polyphenolic metabolites that are abundant in different fruits, vegetables, aromatic plants, medical herbs, tea, and red wine. Flavonoids are a diverse group of benzo-y-pyrone derivatives that share a common carbon skeleton of diphenylpropanes (11). Quercetin has been tested in different types of cancer cells and has demonstrated inhibitory effects, preventing cancer cells from proliferating, invading, and greatly reducing their mobility both *in vitro* and *in vivo*. Quercetin derivatives were artificially formulated in the laboratory to test their effectiveness against cancer cells (7, 18). The derivatives were first used to treat melanoma cells given that significant success was achieved in preventing melanoma metastasis with quercetin pretreatment. The quercetin derivative, 34'7TMQ exhibited anti-migratory effect with the lowest cytotoxicity against the melanoma cells (18). The first step in creating this compound was to attach the methyl group to the C-3 hydroxyl group of quercetin (18). Previous studies demonstrated that this compound inhibited melanoma cell migration with minimum cytotoxicity, therefore, Yamauchi *et al*. tested this compound on three different ovarian cancer cell lines: SK-OV-3, CRL-11731, CRL-1978. According to the results, 34'7TMQ inhibited migration and invasion in all three ovarian cancer cells without affecting proliferation. These results suggest that 34'7TMQ has the potential to inhibit metastasis in ovarian cancer cells with minimum cytotoxicity therefore minimizing the risk of affecting non-cancerous cells (8). The presence of 3-methoxyl group in positions C-2, C-3, and C-5 of the quercetin molecule demonstrated significant ability of the derivatives to inhibit migration. In addition to the 3-methoxyl group, 4'-methoxyl group further stimulates the anti-migratory effect of the compound (7).

Our results indicate a differential mechanism of action of 34'7TMQ in inducing apoptosis on the three types of ovarian cancer cells. This provides a possible explanation of the findings of Yamauchi *et al*. that the cancer cell invasion and migration is affected by apoptosis. CRL-1973 responded in the most expected manner where Bax/Bcl-2 and caspase-9 expression increased and p38 MAPK expression decreased indicating the occurrence of an apoptotic event in response to 34'7TMQ. This is further validated by the Annexin-v staining where distortion of the nuclei and overlapping of green and blue staining was observed in the nuclei suggesting apoptotic signaling. Caspase-9 expression was up-regulated in all three ovarian cancer cell lines in response to 34'7TMQ suggesting the cleavage of downstream caspase proteins such as caspase-3, caspase-6, and caspase-7 initiating the caspase cascade and cell death program (19, 20). The balance between pro- and anti-apoptotic proteins can provide valuable insights into the susceptibility of a cell to apoptosis (21, 22). Bax expression is known to promote the intrinsic apoptotic pathway triggered by mitochondrial dysfunction whereas, Bcl-2 blocks programmed cell death and inhibits the activity of Bax (21). Bax/Bcl-2 ratio increased in the CRL-1978 and SK-OV-3 cell lines indicating the promotion of cell death because of the mitochondrial outer membrane becoming more permeable in response to cellular stress

(22). In CRL-11731, we saw a decrease in Bax/Bcl-2 ratio which suggests that apoptosis does not occur though the intrinsic pathway. According to several studies, p38 MAPK is involved in cell-cycle regulation and the growth inhibitory signaling cascade of contact inhibition (23). Increase in p38 MAPK expression is linked to elevated activity of the transcription factor NF-κB. Dysregulated NF-κB activity can lead to uncontrolled cell proliferation due to loss of contact inhibition facilitating cancer cell metastasis (23, 24). The p38 MAPK expression was decreased in CRL-1978 which suggests that the activity of NF-κB is controlled and therefore the cells have not lost completely contact inhibition. The down-regulation of p38 MAPK in response to 34'7TMQ further suggests cell cycle arrest at the G_1/S transition of undifferentiated cells resulting in cell death. On the contrary, a decrease in p38 MAPK in the SK-OV-3 cell line was not observed which suggests that this cell line is not being monitored at G_1/S transition and has possibly lost contact inhibition (24). Expression of p38 MAPK in CRL-11731 requires further investigation as it appears to fluctuate at different concentrations of 34'7TMQ.

Modern anti-cancer research aims to develop a chemotherapeutic agent that possess the minimal cytotoxic effect to humans while maximizing the damage to cancer cells (25). The quercetin derivative, 34'7TMQ has proven to be a potential anti-cancer agent that is able to inhibit migration and invasion in different types of cancer such as melanoma, prostate, and ovarian (8, 9, 18). In the future, mitochondrial membrane potential (MMP) assay and fluorescence-activated cell sorting (FACS) assays should be performed to further elucidate the mechanism of action of this compound as well as to further validate the hypothesis that 34'7TMQ induces apoptotic signaling in cancer cells and has a differential mechanism of action on different ovarian cancer cell lines.

Conflicts of Interest

None of the Authors have any conflict of interest to disclose.

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