Effect of quercetin on doxorubicin cytotoxicity in sensitive and resistant human MCF7 breast cancer cell lines

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Abstract. Chemoresistance is the major cause of cancer recurrence, relapse and eventual death. Doxorubicin resistance is one such challenge in breast cancer. The use of quercetin, an antioxidant, in combination with doxorubicin has been investigated for offering protection to normal cells from the toxic side effects of doxorubicin in addition to modulation of its resistance. The present study aimed to investigate the effects of quercetin in prevention of a doxorubicin-chemoresistant phenotype in both doxorubicin-sensitive and -resistant human MCF-7 breast cancer cell lines. A doxorubicin-resistant MCF-7 cell line was established. The development of resistant cells was closely monitored for changes in morphological features. Sensitivity to doxorubicin and the doxorubicin/quercetin combination was assessed using the tetrazolium assay. To determine the mechanism by which quercetin sensitizes the doxorubicin MCF-7-resistant cell line to doxorubicin, gene expression alterations in breast cancer-related genes were examined using the reverse transcription-quantitative PCR (RT-qPCR) array technology. Resistant MCF cells were successfully developed and the inhibitory concentration (IC_{50}) value of doxorubicin increased from 0.133 to 4 μM (wild-type to resistant). The effects of the quercetin/doxorubicin combination exhibited different effects on wild-type vs. resistant cells. The IC_{50} of doxorubicin was reduced in wild cells, whereas resistant cells showed an increase in cell viability at lower concentrations and a potentiation of the effects of doxorubicin only at higher concentrations. Annexin V/propidium iodide staining demonstrated that quercetin drives cells into late apoptosis and necrosis, but in resistant cells, necrosis predominates. RT-qPCR results revealed that quercetin led to a reversal in doxorubicin effects via up- and downregulation of important genes such as SNAI2, PLAU and CSF1 genes. Downregulation of cell migration genes, SNAI2 (-31.23-fold) and plasminogen activator, urokinase (PLAU; -30.62-fold), and the apoptotic pathway gene, colony stimulating factor 1 (CSF1; -17.25-fold) were the most important querticin-associated events. Other gene alterations were also observed involving cell cycle arrest and DNA repair pathways. The results of the present study indicated that quercetin could lead to a reversal of doxorubicin resistance in breast cancer cells via downregulation of the expression of important genes, such as SNAI2, PLAU and CSF1. Such findings may represent a potential strategy for reversing breast cancer cell-related chemoresistance.

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Introduction

Chemotherapy usually leads to an improvement in tumor cell death and produces a decreases in tumor mass; however, in some patients this therapy fails due to recurrence or even death (1). The response to chemotherapy varies greatly from individual to individual. Some patients can achieve a complete response with just one or two cycles, while others require eight or more cycles (2,3).

Although progress in the treatment of breast cancer has been made, chemoresistance still remains an obstacle to the effective management of all breast cancer types (4). In general, two types of drug resistance exist: i) Intrinsic, which exists before treatment; and ii) acquired, which is generated after chemotherapy. Chemoresistance is the major cause of breast cancer recurrence, relapse and mortality (5). It is caused by different mechanisms, such as drug efflux pumps, dysregulation of apoptosis and cancer stem cells (6) and genetic mutations and/or epigenetic changes (5). The two main mechanisms of chemoresistance are drug efflux and DNA repair, both of which epigenetically regulated (7).

Doxorubicin is one of the most effective chemotherapeutic drugs used for treatment of breast cancer (7-9). It is a naturally occurring anthracycline antibiotic (10). One major cause of treatment failure with doxorubicin is the development of drug resistance and even tumor growth (11) that leads to poor prognosis and survival (12-14). Doxorubicin resistance is still an unresolved issue in treatment of patients with breast cancer despite investigations into several doxorubicin resistance mechanisms (10). Unfortunately, doxorubicin has acute

adverse effects that occur within 2 to 3 days of administration and include nausea, vomiting, neutropenia, alopecia and/or arrhythmias (15). Moreover, doxorubicin leads to an increase in the risk of clinical cardiotoxicity by 5.43-fold and the risk of heart attack by 4.94-fold when compared with other non-anthracycline regiments (16). The emergence of drug resistance and life-threatening cardiac injury after doxorubicin treatment has led to limitations for successful cancer treatment (17). Overcoming doxorubicin resistance and minimizing its toxic cardiac and kidney effects would represent a major improvement in the effective management of breast cancer (18).

Quercetin is a polyphenolic compound that is found in abundance in a number of fruits, vegetables and plants and exerts various biological effects, such as antioxidant, antiviral, anticancer, cell cycle modulatory and anti-angiogenesis effects (19). It has been reported that quercetin is effective in inhibiting cancer cell growth via the induction of apoptosis (20,21). In addition, quercetin has been reported to upregulate the expression of estrogen receptors α and β.

The overall outcome on cell fate is reflected by inhibition of cell proliferation, cell cycle arrest in the G_1 phase and reduction in cell migratory potential due to actin cytoskeleton disorganization (22). To produce an improvement in antitumor efficacy and a reduction in chemotherapeutic chemoresistance, the natural flavonoid quercetin has been found to have antitumor potential and synergistic effects when combined with doxorubicin (23). The safety of quercetin makes it an attractive candidate for producing a reduction in cardiotoxicity attributed to anticancer drugs, such as doxorubicin (24). The effects of addition of quercetin to doxorubicin has been previously reported, and multiple mechanisms accounting for such effects have been suggested (24-26). Its use in combination with doxorubicin in resistant cell lines and the exploration of possible mechanisms of enhancing doxorubicin toxicity toward cancer cells has yet to be explored.

The aim of the present study was to investigate the effects of quercetin treatment in prevention of a doxorubicin-chemoresistant phenotype in both doxorubicin-sensitive and -resistant human MCF-7 breast cancer cell lines.

Materials and methods

Cell culture growth conditions. A wild-type MCF-7 cell line. MCF-7 breast cancer cell line was originally obtained from the American Cell Culture Collection (cat. no. HTB-22). The starting passage numbers was five. Cells were grown as an attached monolayer culture in the commercially defined RPMI 1640 medium (Euroclone SpA), supplemented with 10-20% (v/v) heat-inactivated fetal bovine serum (FBS; Euroclone SpA), 1% 2 mM L-glutamine (Euroclone SpA), 1% 100X penicillin-streptomycin concentration (Euroclone SpA) and 0.2% 10 mg/ml gentamicin concentration (Euroclone SpA). The cells were grown in 75 or 25 cm^2 filter-cap culture flasks (SPL life Sciences). The cells were then incubated at 37˚C in a 90% humidified atmosphere of 5% CO_2 and maintained in a tissue culture incubator (BINDER GmbH).

Establishment of doxorubicin resistant sub-line. Doxorubicin 2 mg/ml (Fresenius SE & Co.) was used to induce resistance. A freshly prepared stock solution consisted of 100 μM and was stored at 4˚C. The drug was further diluted with cell culture medium to a concentration of 6.65 nM and added to the cell media.

First, 6.65 nM of doxorubicin was added to the MCF-7 cells, and when the cells reached appropriate confluency at a certain concentration, they were passaged and doxorubicin concentration was increased by 0.05 nM over the previous one. This process took ~3 months as growth of the cells usually has a lag period to allow them to adapt to the higher concentration. To exclude the effects associated with long term culture of MCF-7 wild type cells, the wild-type cells were cultured under identical conditions and maintained in culture for the same period as the resistant cells but without doxorubicin addition.

Cell viability and proliferation assays for the treatment of MCF-7 with doxorubicin and quercetin. For wild-type cell lines, 5,000 MCF-7 cells from the appropriate cell line were seeded per well in a 96-well plate. After 24 h, six different concentrations of doxorubicin (100, 10, 1.0, 0.1, 0.01 or 0.001 μM) were added to the final volume of 200 μl per well of culture RPMI 1640 medium. For the resistant cell lines, 7,000 MCF-7 cells from the appropriate cell line were seeded per well in a 96-well plate. After 24 h, complete growth media was used for doxorubicin dilution, and using a serial dilution technique, 16 different concentrations of doxorubicin were prepared starting at 500 and decreasing each subsequent concentration by 50% until a concentration of 0.015 μM was reached and added to the final volume of 200 μl per well of culture media. A stock solution of quercetin (Sigma-Aldrich; Merck KGaA) was prepared [50 nM in dimethylsulfoxide (DMSO); Euroclone SpA]. Complete growth media was used for quercetin dilution and nine different concentrations of quercetin were prepared (100, 50, 25, 12.5, 6.25, 3.125, 1.652, 0.826 and 0.413 μM). For the combination (both for wild and resistant cell lines), the same concentrations of doxorubicin were mixed with 20 μM of quercetin for MCF-7 cell line.

The antiproliferative effects of doxorubicin on MCF7/WT and MCF7/D53.2 cells were analyzed using the tetrazolium MTT dye (Sigma-Aldrich; Merck KGaA). This test is based on the reduction of MTT, a yellow tetrazole, to a purple formazan, a process that occurs in the mitochondria of viable cells. A total of 10 ml of MTT solution were prepared by weighing 50 mg of MTT dye dissolved in 10 ml of phosphate-buffered saline (PBS) 1X (Euroclone SpA).

After 72 h of incubation, media were aspirated from the cells containing the drugs and replaced with fresh media (100 μl/well) and incubated for 30 min after which 10-13 μl of the MTT dye solution was added to each well. The plates were returned to the incubator at 37˚C for 4 h after which 100 μl of DMSO (Euroclone SpA) was added to each well. Optical density (OD) at 570 nm wavelength was recorded using a 96-well plate reader (Biotek instruments, Inc.) after 20 min of shaking on the plate shaker (cat. no. 130,000; Boekel Scientific). The percentage of living cells (% living) was calculated by dividing the absorbance of viable cells per well over the mean absorbance of the viable cells in control wells. The mean percentage per concentration was then calculated as mean ± standard deviation (SD). Measurements were performed in triplicate.

Results of the MTT cell proliferation assay were evaluated using GraphPad Prism 8 software (GraphPad Software, Inc.). The inhibitory concentration (IC_{50}), which is the drug concentration at which 50% of cells are viable, was calculated from the logarithmic trend line of the cytotoxicity graphs for each drug alone and in combination. The degree of resistance was estimated in terms of resistance index (R), which is calculated according to the equation: R=IC_{50} resistant cells/IC_{50} sensitive cells.

Phase contrast microscopy. Flasks for control wild-type MCF-7 cells and for each concentration was reached for resistant MCF-7 cells were observed under EVOS XL core imaging system (Thermo Fisher Scientific, Inc.) at magnifications of 4, 10 and 20X and images were captured.

RNA isolation. Next, 75 cm^2 flasks were seeded with $7x10^6$ MCF-7 cells. Treatment was started with 5 μM doxorubicin or the combination of 5 μM doxorubicin with 20 μM quercetin. One flask was allotted as control. RNA was extracted from each individual cell pellet using RNeasy® Plus Mini kit (Qiagen GmbH) following manufacturer's instructions.

Then RNA concentration and purity were measured using a nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). Furthermore, the quality of RNA was visualized under ultraviolet light after performing gel electrophoresis.

cDNA synthesis. The first strand of cDNA was prepared using the RT2 Profiler PCR Array kit (Qiagen GmbH). According to the manufacturer's instructions, aliquots containing 1 μg of total RNA were used from each sample.

Gene expression profiling. Pathway-focused gene expression profiling was performed using a 96-well human cancer drug resistance and metabolism PCR array, RT2 Profiler PCR array (PAHS-131ZD-12), Human Cancer Drug Resistance & Metabolism PCR Array (Qiagen, Inc.). In this array, 84 wells contained all components required for the PCR reaction in addition to a primer for a single gene in each well. A total of five housekeeping genes (ACTB, B2M, GAPDH, HPRT1 and RPLP0) were used as a control for normalization. Notably, Qiagen provide the array with their design primers and they do not provide the sequence of such primers. These particular genes are involved in tumor classification, signal transduction, DNA repair and other commonly affected pathways such as the cell cycle, angiogenesis, apoptosis adhesion and proteolysis. A diluted cDNA, equivalent to 1 μg RNA for each plate, was mixed with the RT2 SYBR® green master mix (Qiagen, Inc.) according to the manufacturer's instructions, and loaded into the 96-well array. Reverse transcription-quantitative (RT-qPCR) reaction was performed using the iCycler (Bio-Rad Laboratories, Inc.) machine by heating the plate to 95˚C for 10 min followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min.

The cycle threshold (Cq) values for each sample were given by the iCycler. The threshold value was manually set on 0.01 as recommended by the PCR array user manual. The analysis was performed automatically according to the SA Biosciences company (Qiagen, Inc.) web portal (https://geneglobe.qiagen.com/jo/analyze), and expression levels were expressed as a fold-increase or decrease (27). The data were normalized across the plates to the following housekeeping genes were used: i) Actin β; ii) β-2-microglobulin, glyceraldehyde-3-phosphate dehydrogenase; iii) hypoxanthine phosphoribosyltransferase 1; and iv) ribosomal protein large, P0.

Gene functional annotation. The Gene Ontology (GO; https://david.ncifcrf.gov/tools.jsp) and pathway database for genes whose expression levels changed by 2-fold was investigated using the web-based application DAVID (http://david.abcc.ncifcrf.gov), which provides for ontology and pathway mapping, annotation, and visualization of results. Results appear in tables that describe the most affected function and the pathway based on genes changes.

Apoptosis analysis. Apoptosis events induced by anticancer drug treatment in MCF-7 wild-type and resistant cell lines treated with doxorubicin alone or in combination with quercetin in addition to no treatment were investigated. The mechanism of cell death was determined by Annexin V/propidium iodide (PI) staining using flow cytometry. MCF-7 wild-type and resistant cells were seeded into six-well plates and incubated overnight at 37˚C. Wild-type MCF-7 cells were exposed to 1.3 μM doxorubicin alone or to a combination of 1.3 μM doxorubicin combined with 20 μM of quercetin. Resistant cells were exposed to 4 μM doxorubicin or to a combination of 4 μM doxorubicin combined with 20 μM of quercetin. For both cell types, control cells remained untreated. After incubation for 72 h, media were removed, and cell cultures were washed two times with PBS. Cells were collected into flow tubes using Accutase solution (800 μl on each well at room temperature for 10 min). After this step, an Annexin V/PI apoptosis kit was used to stain the cell pellets following the kit's instructions. The apoptosis assay was carried out using the annexin v assay (Molecular Probes; Thermo Fisher Scientific, Inc.), and cell death (either apoptosis or necrosis) was analyzed by FACS Diva7, a fluorescein-activated sorter (FACS) Canto II version 3 (BD Biosciences). Flow experiments was performed using single color staining.

The results from the apoptosis assay groups were tested for significance using a two-way analysis of variance (ANOVA) followed by a Šidák multiple comparison test to identify the statistical significance using GraphPad Prism. Data was presented as mean ± standard deviation and the experiments were repeated three times. $P<0.05$ was considered to indicate a statistically significant difference. Fig. 1 presents the study flowchart.

Results

Determination of IC_{50} value of doxorubicin alone and combination in wild-type and resistant MCF-7 breast cancer cell lines. The wild MCF-7 cell viability was determined using the MTT assay as is presented in Fig. 2. The IC_{50} values for doxorubicin only and doxorubicin with 10 μM quercetin combination were 0.133 and 0.114 μM, respectively. Treatment with doxorubicin with 10 μM quercetin at 0.01, 0.001 and 0.0001 μM only yielded significant inhibition of the growth of MCF-7 cells when compared with the growth of cells treated with higher doxorubicin concentrations. Overall, these findings indicated

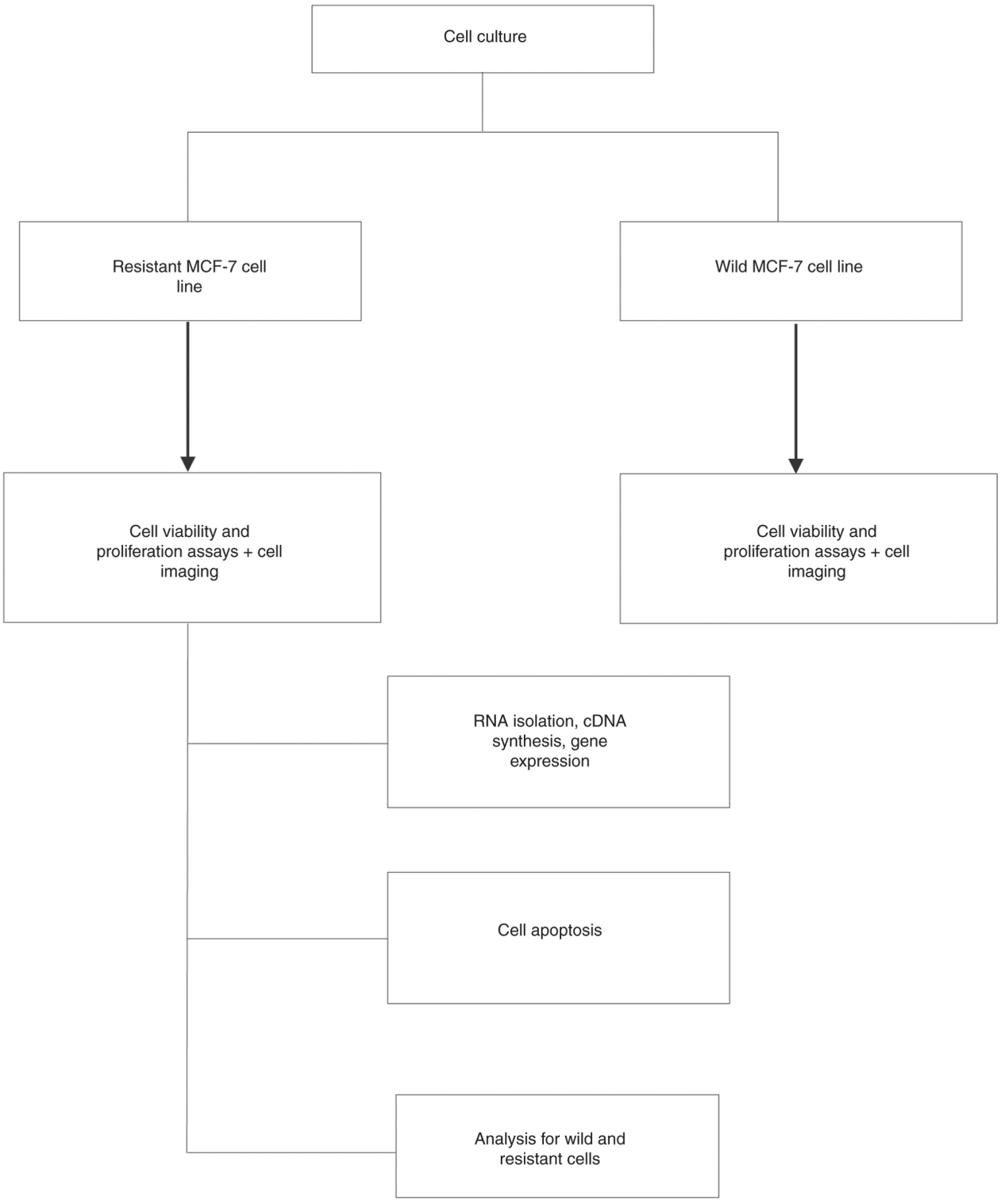

Figure 1. Study flowchart.

that quercetin had a potentiation effect toward doxorubicin against the MCF7 breast cancer cells.

Development and characterization of the MCF-7 resistant cells. The doxorubicin-resistant MCF-7 cell line was established and examined at two different doxorubicin concentrations: i) A final concentration of 26.6 nM; and ii) a final concentration of 53.2 nM.

Morphological features. Fig. 3 presents phase contrast images of wild MCF-7 cells, which present epithelial-like morphology of small, spindle-shaped cells with a single nucleus. Cells grow in colonies, tightly packed and uniform in size. While confocal microscopy images of DAPI-staining are presented in Fig. 4 for wild untreated and 1.3 μM doxorubicin treated cells. It represent how the doxorubicin accumulates within the nuclease of the cells in the treated cells.

Sensitivity to doxorubicin. As presented in Fig. 5 the IC_{50} values of doxorubicin for resistant MCF-7 cell line in addition

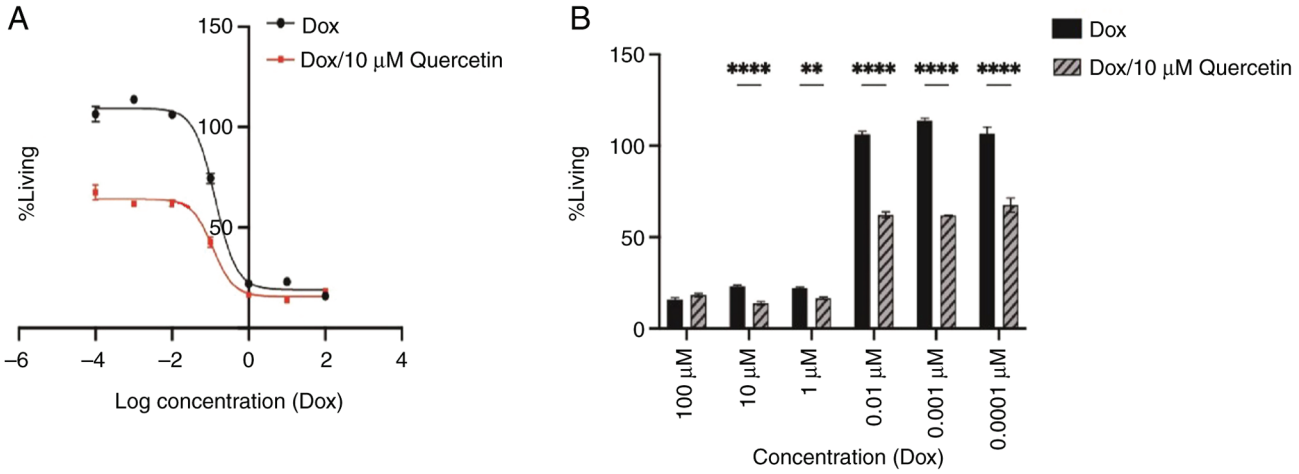

Figure 2. Antiproliferative activity of DOX and DOX/quercetin combination against wild MCF-7. The data represent the viable percentile at each concentration of doxorubicin and DOX/quercetin. (A) DOX and 10 μM quercetin combination; (B) DOX/quercetin combination. Results present the mean ± standard deviation of at least 3 replicates. **P<0.01 and ****P<0.0001. DOX, doxorubicin.

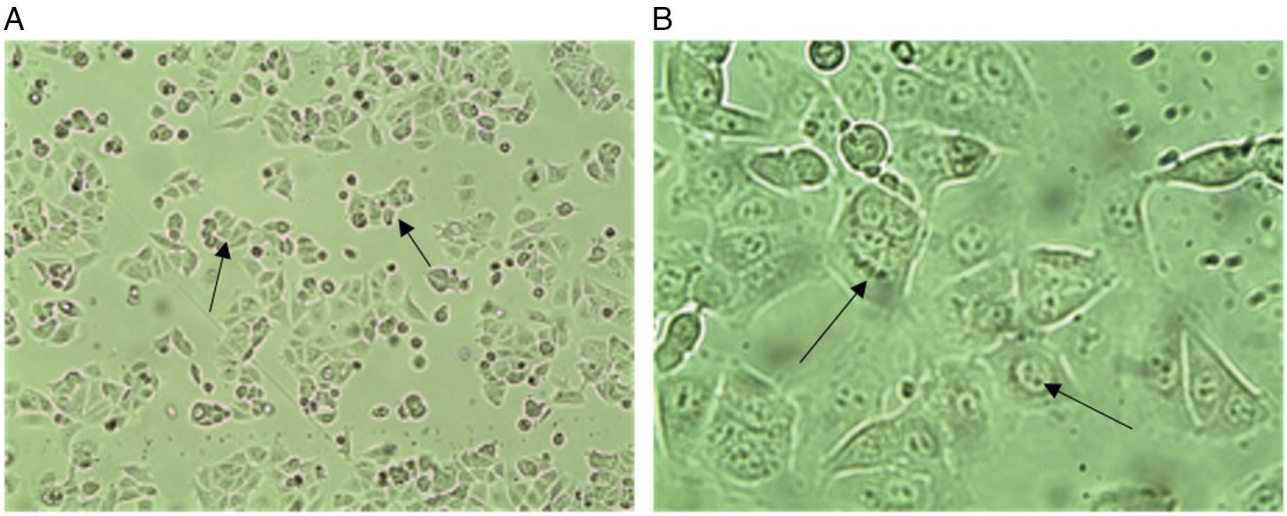

Figure 3. Phase-contrast images of the wild-type MCF-7 cell line. The arrows point to cells that have an epithelial-like morphology; small, spindle-shaped cells with single nucleus (A) at a magnification of 10X (B) and at a magnification of 20X.

to the resistance index were calculated and are shown in Table I.

Although the IC_{50} of the doxorubicin and 20 μM quercetin combination was higher compared with doxorubicin alone in the MCF-7/DOX 26.6 nM group, it was observed that quercetin enhanced anticancer activity when added to higher doxorubicin concentrations as is presented in Fig. 5. The IC_{50} value of the MCF-7/DOX 53.2 nM group was 4.0, μM, which is 30-fold higher compared with the sensitive parent cell line. Treatment with quercetin in the MCF-7/DOX 53.2 nM group led to a significant decrease in the doxorubicin IC_{50} value and resistance fold value from 30- to 9-fold. These findings revealed that quercetin had significant doxorubicin re-sensitizing effects.

Reversal of doxorubicin resistance with verapamil was performed to further investigate the inhibition of P-glycoprotein (P-gp) by verapamil as a standard drug after which proliferation of the cells was compared to the resistant MCF-7 cells with MCF-7 cells in combination (doxorubicin with 10 μM verapamil) as presented in Fig. 6. This figure demonstrates how the verapamil forces doxorubicin outside the cells and therefore reduces the concentration that can reach the nucleus.

Morphological features. In comparison with parent wild type cells, the resistant MCF-7 cells appeared to be larger, irregular and had a satellite-like shape with multiple nuclei and multiple vesicles and stronger adhesion to the underlying surface (Fig. 7).

Confocal imaging in Fig. 8 was used to investigate intracellular doxorubicin accumulation in resistant MCF-7/DOX 53.2 nM cells. The cells were incubated with 400 μM of doxorubicin for 4 h, it was also viewed at 0 h as a control. This demonstrated that control resistant MCF-7/DOX 26.6 nM cells show a low level of fluorescence with a narrow doxorubicin intracellular distribution, while in 400 μM treated doxorubicin group the free doxorubicin surrounded the nucleus. These figures collectively indicated that the main resistant mechanism exemplified in effluxing doxorubicin

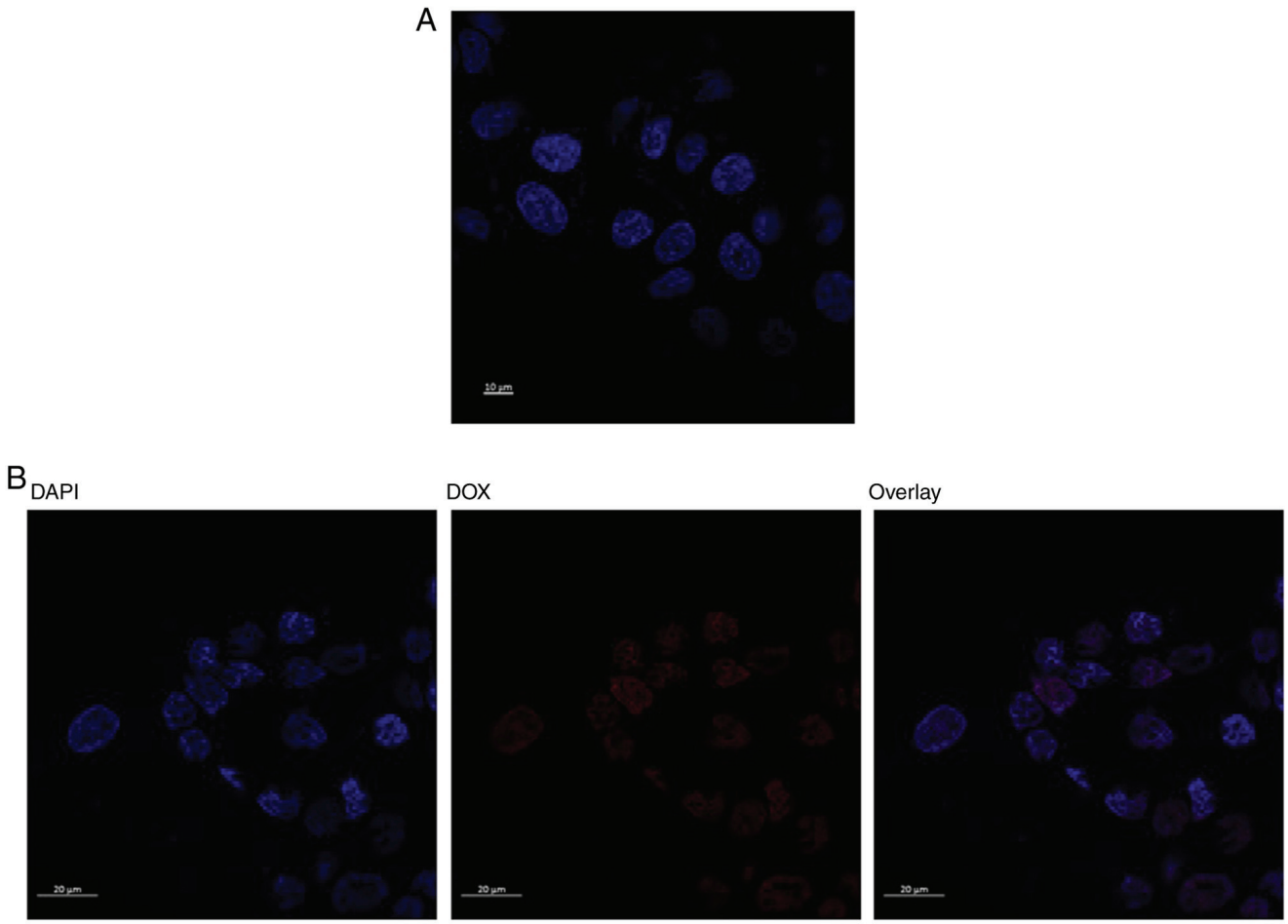

Figure 4. Confocal images of wild-type MCF-7 cells. (A) Control wild-type MCF-7 cell line. (B) 1.3 μM DOX treated wild-type MCF-7 cell line, cell nuclei (blue) stained by DAPI. free DOX diffuses through the cytoplasm. Scale bar, 10 μm. DOX, doxorubicin.

toward the cytoplasm and to engulf them in special type of vesicles.

Apoptosis analysis. To determine whether the effects of quercetin on wild-type and resistant MCF-7 cancer cell proliferation were related to apoptosis, flow cytometry using Annexin V/PI staining was performed. Wild-type MCF-7 cells were treated with 1.3 μM doxorubicin only or a combination of 1.3 μM doxorubicin and 20 μM quercetin, and resistant MCF-7 were treated with 4 μM doxorubicin or a combination of 4 μM doxorubicin and 20 μM quercetin for 72 h.

The cells were sorted according to annexin V and PI status into early apoptotic Q4, late apoptotic Q2, necrotic Q1 and viable cells Q3, and results are presented in Figs. 9 and 10. In wild-type MCF-7 cells as shown in Fig. 9, the late apoptosis rate increased from 7.1% in control cells to 20.6 and 26.3% after treatment corresponding to doxorubicin or the doxorubicin and quercetin combination, respectively. Wild-type MCF-7 cells treated with 1.3/20 μM Dox/quercetin combination had the highest apoptosis rates. Similarly, the percentage of necrotic cells increased from 6.3% in control cells to 20.8 and 25.1% after treatment corresponding to doxorubicin only or the doxorubicin and quercetin combination.

In resistant MCF-7 cells (Fig. 10), the effects of quercetin on the induction of cell apoptosis in breast cancer cells was also measured. It was found that the late apoptosis rate changed from 2.3% in control cells to 1% or 7.25% after treatment corresponding to doxorubicin only or to the doxorubicin and quercetin combination, respectively. By contrast, the percentage of necrotic cells changed from 1.6% in control cells to 0.95% or 25.95% after treatment corresponding to doxorubicin only or the doxorubicin and quercetin combination, respectively. Collectively, the apoptosis assay findings indicated that the mechanism of killing for doxorubicin differs in the presence of quercetin, especially in the resistant MCF-7 cells.

Gene expression changes in the resistant MCF-7 cell line. A gene expression profile analysis was performed using the PCR array and a standard 2-fold change in expression was used as the cut-off. The effects of 5 μM of doxorubicin on the expression of the breast cancer genes were tested against MCF/DOX, such treatment was called Track (1). While the combination of 5 μM dox and 20 μM quercetin were named Track (2). Analysis of track (1) revealed that 52 genes out of the 84 genes were modulated, 41 of these were upregulated, and 11 genes were downregulated (Tables SI and SII).

In the Track 2 analysis, it was revealed that in 46 genes out of the total 84 genes that were examined, 35 were upregulated and 11 genes were downregulated (Tables SIII and SIV). The

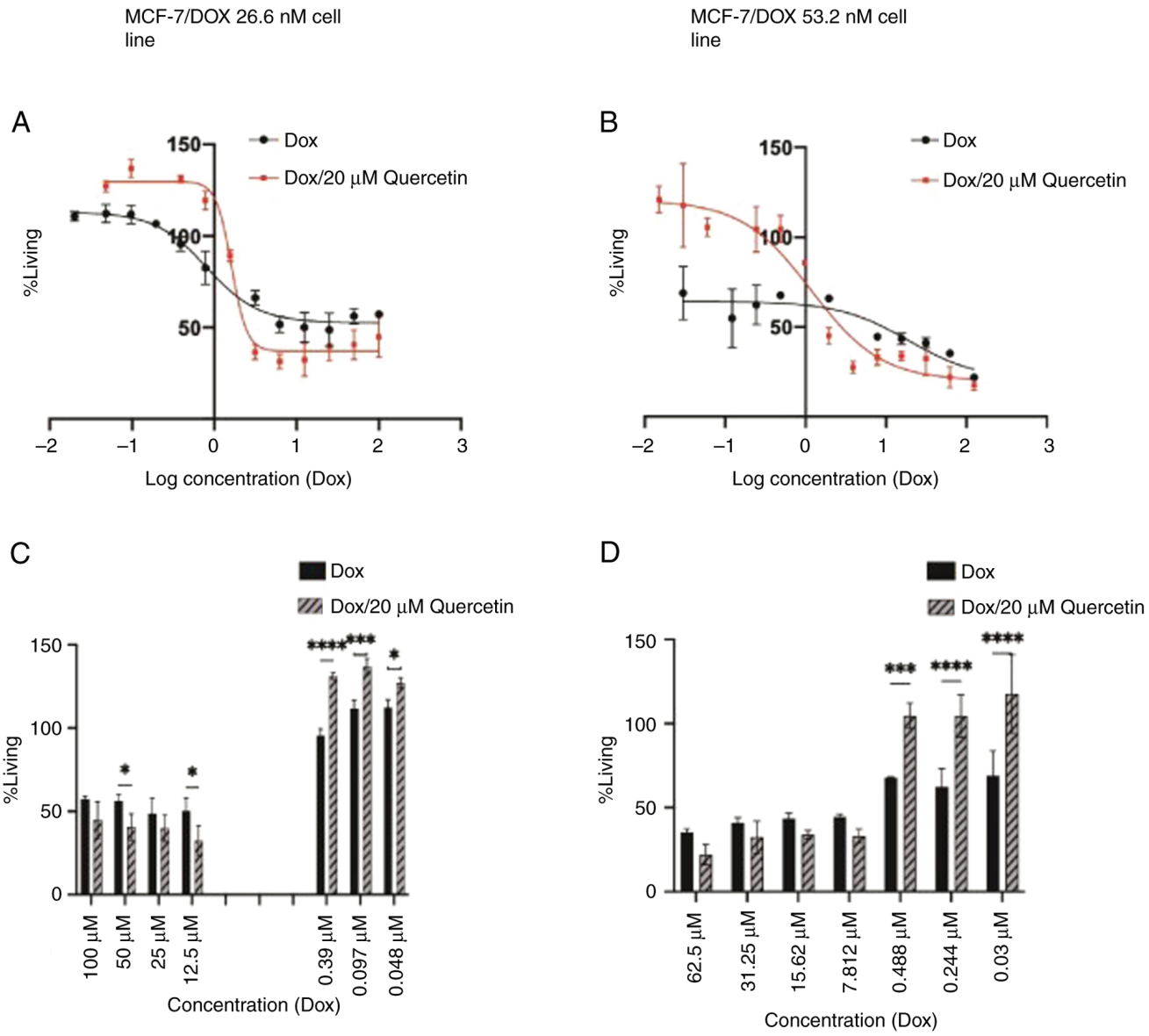

Figure 5. Antiproliferative activity of doxorubicin and Dox/quercetin combination against resistant MCF-7 cells. (A and B) Doxorubicin and 20 μM quercetin combination. (C and D) Doxorubicin/quercetin combination and differences. Results present the mean ± standard deviation of at least 3 replicates. *P<0.05, ***P<0.001, ****P<0.0001. Dox, doxorubicin.

difference between the fold regulation between Tracks (1) and (2) was calculated as shown in Tables SV and SVI using a standard of 5-fold change as the cut-off.

Gene ontology and pathways analysis. Gene Ontology (GO) categories for both up- and downregulated genes for Track (1) are shown in Tables II and III, respectively. The data indicated that the selection for doxorubicin resistance led to changes in gene expression, mainly in upregulated genes, and consisted of 'protein binding', 'negative regulation of cell proliferation', 'positive regulation of transcription from RNA polymerase II promoter', 'negative regulation of apoptotic process' and 'positive regulation of cell migration'. In downregulated genes, it was found that 'positive regulation of transcription from RNA polymerase II promoter', 'positive regulation of transcription, DNA-templated' and 'positive regulation of apoptotic process' were affected. The most important pathways in which the up- and the downregulated genes participated are presented in Tables IV and V, respectively. In the downregulated side, 'pathways in cancer', 'microRNAs in cancer', 'p53 signaling pathway', 'cell cycle' and 'signaling pathway' are the main pathways and 'pathways in cancer' is the only pathway in the downregulated side.

In Track (2) the genes that were upregulated 2-fold were functionally clustered into main categories consisting of 'negative regulation of cell proliferation', 'negative regulation of apoptotic process', 'cell cycle arrest' and 'regulation of cell cycle', as shown in Table VI. In downregulated genes, it was found that 'positive regulation of cell migration', 'positive regulation of apoptotic process', 'negative regulation of transcription from RNA polymerase II promoter' and negative regulation of cell proliferation' were affected, as shown in Table VII. The main pathways in up- and the downregulated genes are presented in Tables VIII and IX, respectively. In

Table I. IC_{50} and R for wild and resistant MCF-7 cell lines.

Cell line	DOX only, μM	R	DOX/20 μM quercetin, μM
MCF-7 wild	0.133		0.114
MCF-7/DOX 26.6 nM	0.800	6.01	1.620
MCF-7/DOX 53.2 nM	4.000	30.0	1.290

DOX, Doxorubicin; R, resistance index.

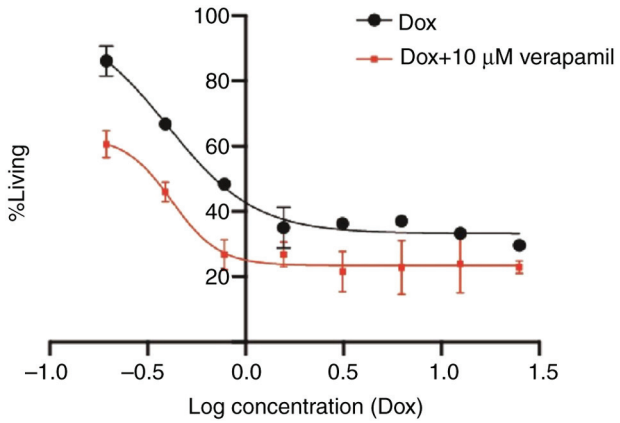

Figure 6. Logarithmic curves for the response of resistant MCF-7 cells to resistant MCF-7 in combination of doxorubicin/10 μM verapamil. The effects of inhibition of P-glycoprotein and partial reversal of doxorubicin resistance. Results present the mean ± standard deviation of at least three wells and the experiment was repeated using 2 different passages. Dox, doxorubicin.

the upregulated side, those pathways are 'pathways in cancer, 'microRNAs in cancer', 'p53 signaling pathway' and 'cell cycle'. In the downregulated side, they are 'microRNAs in cancer' and 'pathways in cancer'.

In terms of the difference between Tracks (1) and (2) genes that were upregulated ≥5-fold, the main functional groups included those involved in 'negative regulation of apoptotic process', 'positive regulation of cell proliferation', 'positive regulation of transcription, DNA-templated', 'cellular response to tumor necrosis factor' and 'DNA replication' (Table X). In downregulated genes, these groups included 'positive regulation of cell migration', 'positive regulation of apoptotic process', 'negative regulation of cell proliferation' and 'transcription, DNA-templated' (Table XI). The main pathways in up- and downregulated genes are presented in Tables XII and XIII, respectively. 'Pathways in cancer', 'p53 signaling pathway', 'signaling pathway' and 'cell cycle' are the main pathway on the upregulated side and 'microRNAs in cancer' and 'pathways in cancer' on the downregulated side.

Notably, after quercetin treatment the colony stimulating factor 1 (CSF1) gene, an apoptotic gene, decreased from 35.26- to 17.58-fold. In addition, as shown in Table II, another alteration in the apoptotic pathway that occurred in the present study after quercetin addition was upregulation of the ATM gene by 4.02-fold as shown in Table VI.

In the DNA repair pathway, BRCA1 and BRCA2 gene expression levels were downregulated in (MCF-7/DOX 53.2 nM) cells by -9.45- and -3.94-fold compared with -2.65 and -4.04-fold in cells treated with quercetin (Table III).

Another valuable observations in the present study was the upregulation of PLAU expression gene in (MCF-7/DOX 53.2 nM) cells by 44.32-fold and downregulation by 13.7-fold after combination treatment as shown in Table II. Moreover, SNAI2 expression decreased by 30.06-fold to -1.17-fold after combination treatment as shown in Table II.

Discussion

The present study was designed to investigate the effects of quercetin in reversing the doxorubicin-chemoresistant phenotype in a MCF-7 breast cancer cell line. First, the doxorubicin-resistant MCF-7 cell line was established using a series of stages of increasing doxorubicin concentrations. The development of resistant cells was closely monitored. Sensitivity to doxorubicin and the doxorubicin with quercetin combination was assessed using the MTT assay. The concentration ranges of quercetin were proven not to be cytotoxic for the cells under investigation. Annexin V/PI staining demonstrated that the presence of quercetin drives cells into late apoptosis and necrosis in agreement with the present findings.

It has been suggested that the reversal property of quercetin is due to its anti-oxidant effects and its capability to disrupt mitochondrial membrane potential, leading to the release of cytochrome C, which is a pro-caspase protein (16). Li *et al* (28) demonstrated that quercetin has little effect on cell proliferation at concentrations <0.7 μM; however, when combined with doxorubicin, the combination leads to significant inhibition of cell proliferation and invasion and suppression of the expression of HIF-1α and P-gp. In 2012, Wang *et al* (29) also found that co-treatment with quercetin (20 mM) and doxorubicin (1 mM) leads to significant potentiation of the antitumor effects of doxorubicin in human liver cancer cells. Shu *et al* (30) reported that the combination treatment of doxorubicin and quercetin leads to a significant promotion of doxorubicin-induced apoptosis in resistant prostate cancer cells. Another study by Huang *et al* (31) showed that quercetin can inhibit oral cancer cell proliferation through induction of apoptosis and death in tongue squamous cell carcinoma cells.

Doxorubicin accumulation patterns or accumulation defects have been described earlier by AbuHammad and Zihlif as reflecting resistance to doxorubicin (11). It is assumed that doxorubicin resistance in several P-glycoprotein-positive non-small cell lung cancer and breast cancer multidrug resistant cell lines can be explained by a summation of accumulation defect and alterations in the efficacy of the drug once present in the cell (32). Furthermore, in our previous study that describes the gene expression changes in MCF-7 cells, a significant overexpression of p-gp gene was reported (11). The accumulation of doxorubicin in the vesicles outside the nucleus has been reported by our group previously (11). Resistant MCF-7 cells were treated with a combination of doxorubicin and verapamil. Verapamil is a calcium channel inhibitor, and it has been shown to efficiently suppress the function of P-gp, leading to the reverse of drug resistance and eventual increase

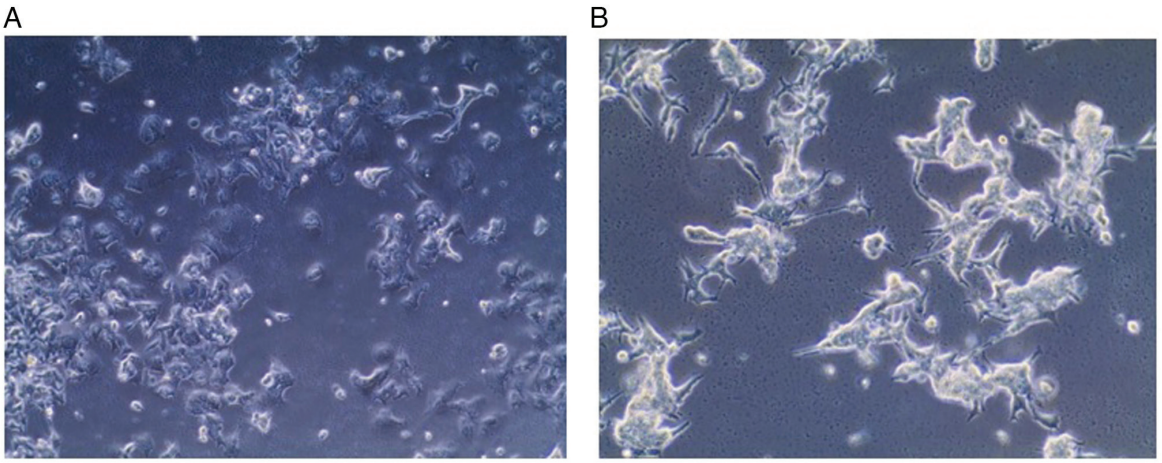

Figure 7. Phase-contrast photography of resistant MCF-7 cell line. Cells appear large, irregular, rounded and have multiple vesicles, and images are presented (A) at a magnification of 10X and (B) at a magnification of 20X.

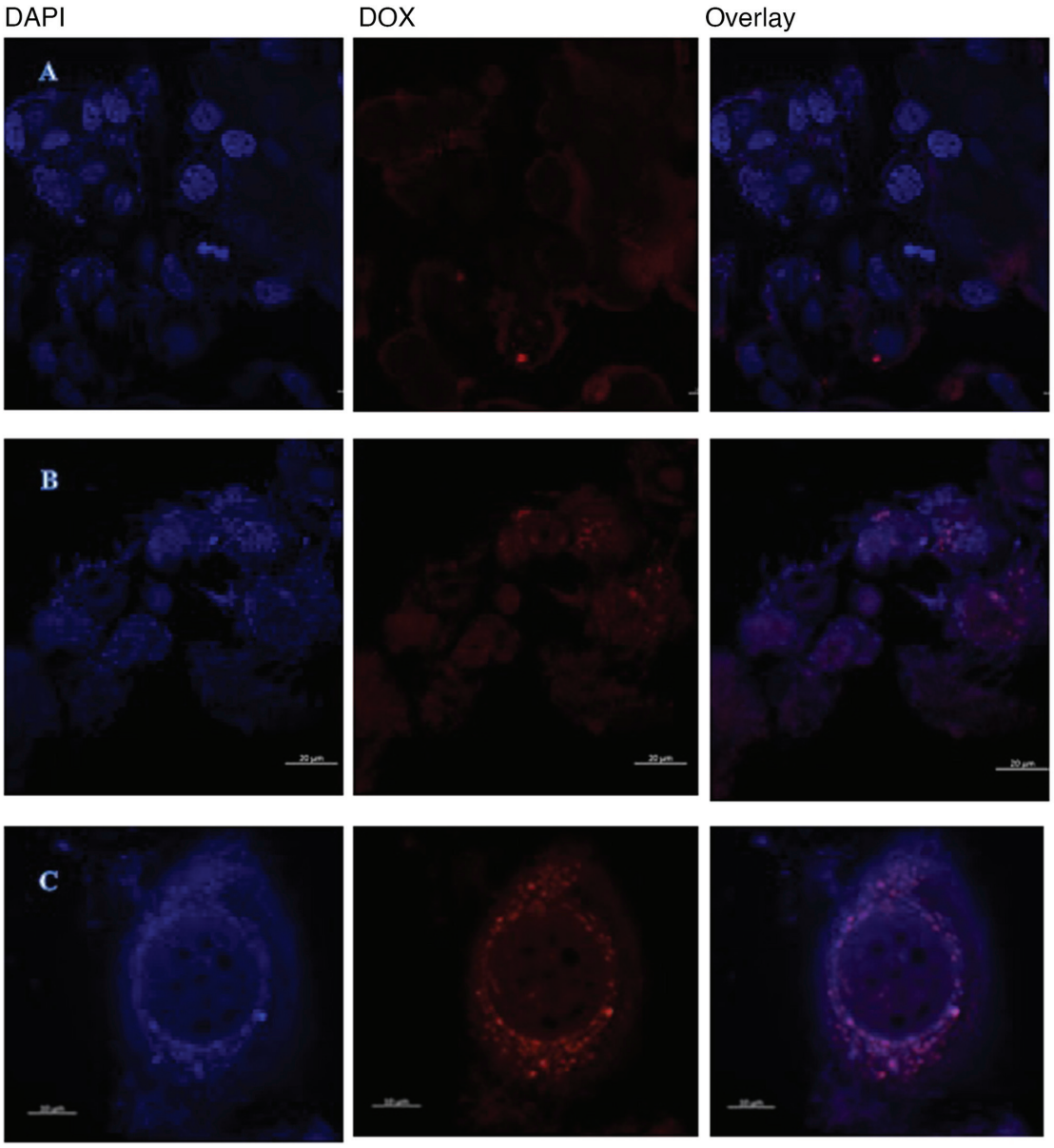

Figure 8. Confocal images of resistant MCF-7. (A) Confocal imaging of control resistant MCF-7/DOX 26.6 nM cell line. (B) Confocal imaging of treated resistant MCF-7 cell line, the free DOX surrounding the nucleus and also presented at (C) a higher magnification of image B. Magnification, 20X. DOX, doxorubicin.

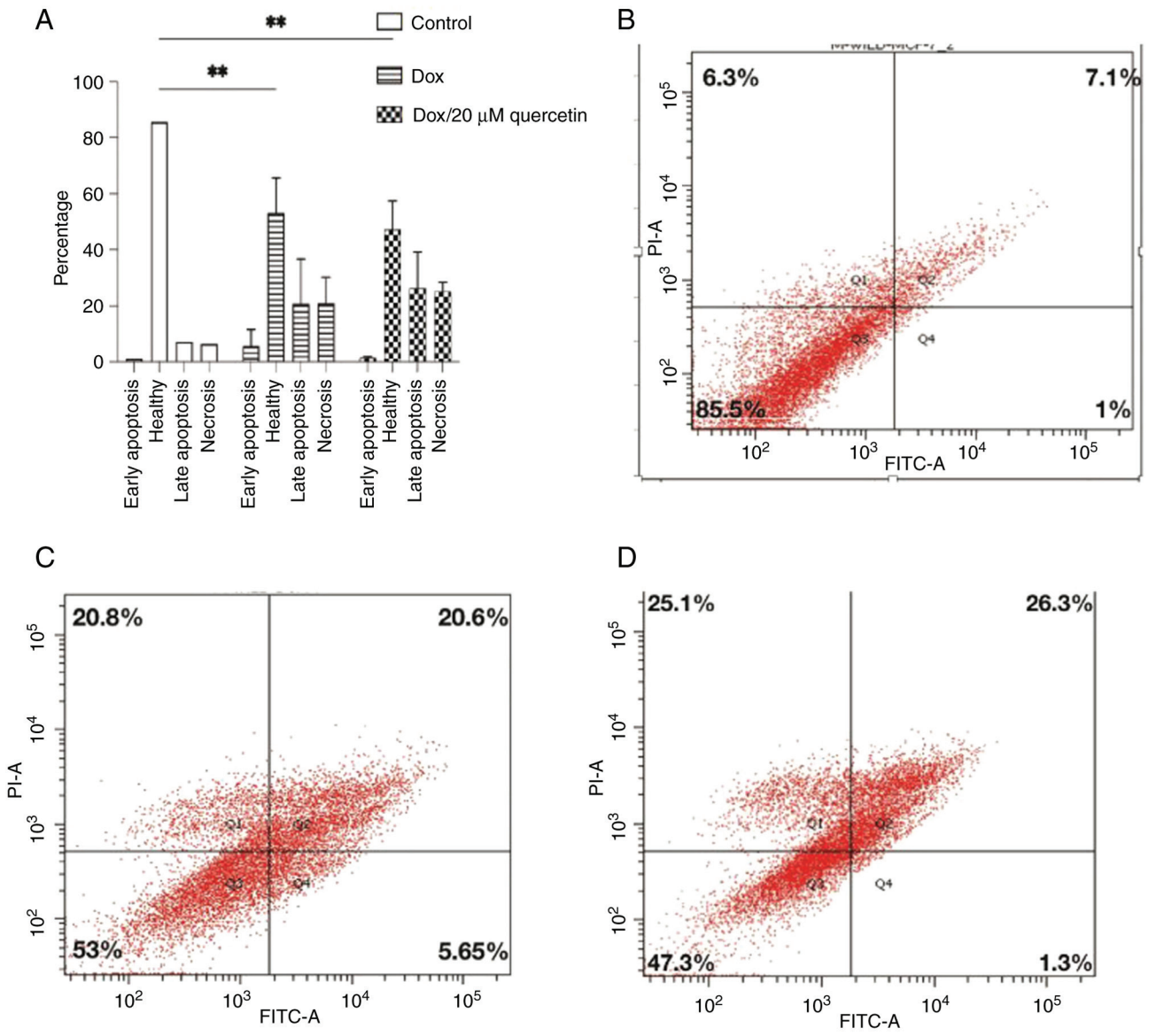

Figure 9. Annexin V-FITC PI binding assay for evaluation of apoptosis for wild MCF-7. (A) The bar chart represents the percentages of the apoptosis assays. (B-D) Cellular death modality by flow cytometry of (B) control wild MCF-7 cells, (C) wild-type MCF-7 cells treated with 1.3 μM Dox and (D) wild-type MCF-7 cells treated with 1.3/20 μM Dox/quercetin combination that had the highest apoptosis rates. Results present the mean ± standard deviation of at least 2 replicates. **P<0.01. PI, propidium iodide; Dox, doxorubicin.

in drug accumulation and enhanced DNA damage (33-35). Notably, the impact of quercetin is more obvious in term of reversing the resistance. Such impact is clear at both the necrosis and apoptosis levels. Verapamil antiproliferation has not been performed on wild-type MCF7 cells as it does not have an effect on these cells by itself nor have an impact on the Doxorubicin IC_{50} when there is no doxorubicin resistance (11). Verapamil impact can only be seen in the resistant MCF7 cells that increased the P-gp expression (36).

The gene expression investigation in the present study revealed that quercetin produced a reversal in the doxorubicin resistance through up- and downregulation of important genes. Those genes are involved in a number of important cellular processes, such as cell cycle, apoptotic pathway, DNA-repair and/or cell migration.

CDKN2A (p16) and CDKN1A are upregulated by 5.21- and 6.11-fold, respectively. The p16 protein binds to two other proteins, CDK4 and CDK6, which assist in cell cycle regulation. CDK4 and CDK6 normally stimulate the cell to continue through the cell cycle and divide; however, binding with p16 blocks cell cycle progression (37). According to AbuHammad and Zihlif (11), p16 regulates the G_1/S cell cycle transition and has the effect of causing cell arrest in the G_1-phase. In the present study, after treatment with quercetin, it was found that CDKN2A (p16) was upregulated by 13.7-fold, which could be due to the effect of quercetin in reversing doxorubicin resistance. Other studies have showed that quercetin causes S phase arrest via decreases in protein expression levels of CDK2 and cyclins A and B while producing an increase in p53 and p57 protein levels (38-40).

In the present study, the CSF1 gene decreased from 35.26- to 17.58-fold after quercetin treatment, thereby leading to a reduction in the expression of anti-apoptotic proteins and rendering cells more sensitive to doxorubicin. In 2016,

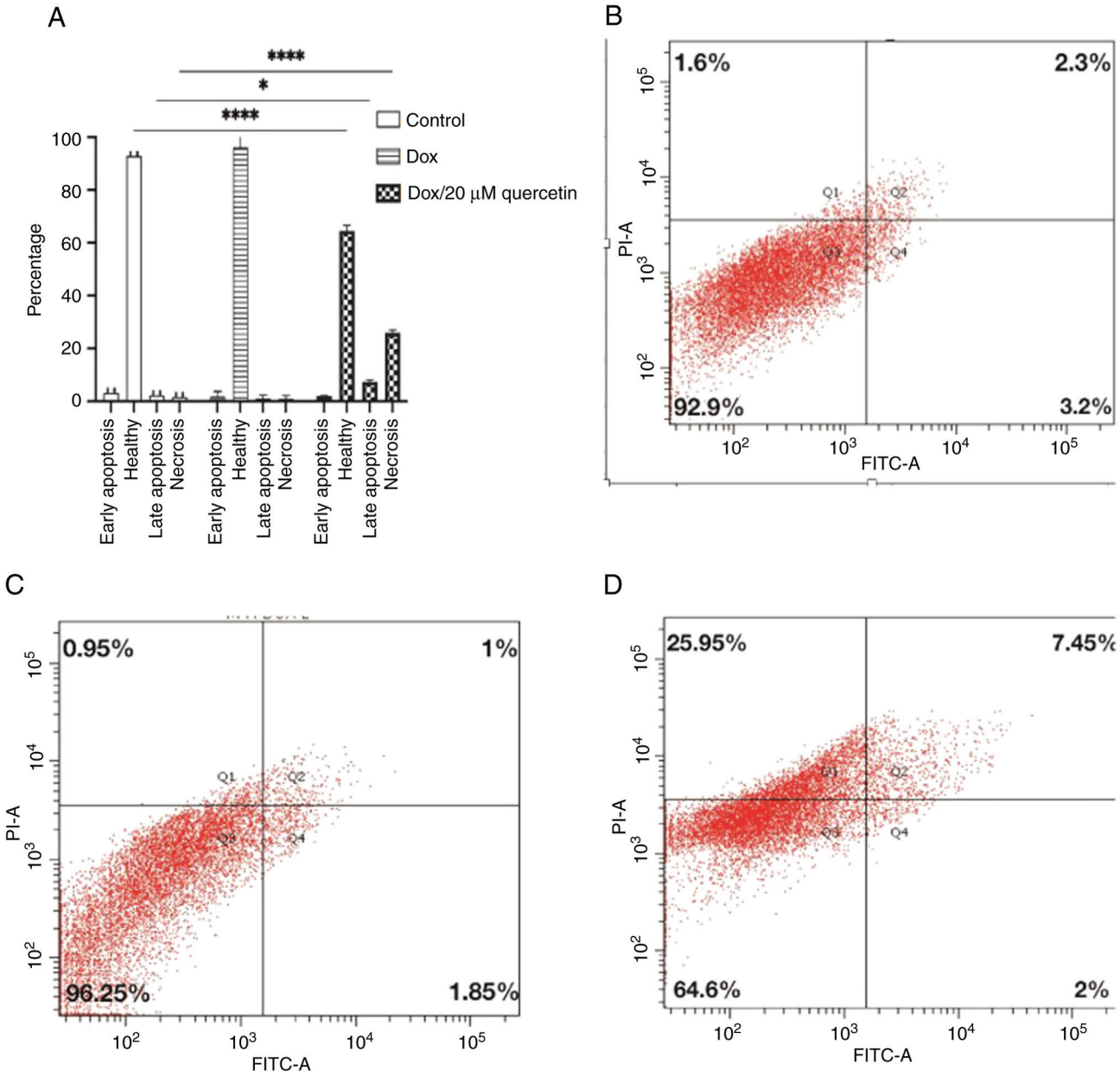

Figure 10. Annexin V-FITC PI binding assay for the evaluation of apoptosis for resistant MCF-7 cells. (A) Percentages of apoptosis assays. Cellular death modality by flow cytometry of (B) control resistant MCF-7 cells, (C) resistant MCF-7 cells treated with Dox and (D) resistant MCF-7 cells treated with Dox/quercetin combination. Results present the mean ± standard deviation of at least 2 replicates. *P<0.05, ****P<0.0001. PI, propidium iodide; Dox, doxorubicin.

Zhang *et al* (41) revealed that the CSF1 gene, which encodes M-CSF protein, induced significant doxorubicin resistance of MCF-7 cell via inhibition of apoptosis through activation of the PI3K/Akt/Survivin pathway.

Another alteration in the apoptotic pathway that occurred in the present study after quercetin addition was upregulation of the ATM gene by 4.02-fold, which further stimulated the p53 pathway. ATM acts as a binary switch by regulating the capability of p53 to induce cell death after chemotherapy. AbuHammad and Zihlif (11) revealed that ATM selectively activates p53, providing a mechanism for controlling the cell cycle and apoptotic responses. Another study showed that treatment with quercetin resulted in p53 upregulation, a finding that was consistent with an earlier report (38).

Since DNA repair mechanisms play an important role in regulating cellular sensitivity to DNA damaging agents, DNA repair plays an important role in the development of resistance to chemotherapy (42).

Breast cancer types 1 (BRCA) and 2 (BRCA2) genes produce proteins that help repair damaged DNA (43). In the present study, BRCA1 and BRCA2 gene expression levels were downregulated in (MCF-7/DOX 53.2 nM) cells by -9.45- and -3.94-fold compared with -2.65 and -4.04-fold in cells treated with quercetin.

Variations in the studies on the role of BRCA1 and BRCA2 proteins in doxorubicin resistance exists. One study found that BRCA1-defective breast cancer cells are significantly less sensitive to doxorubicin when compared with MCF-7 and MDA-MB231 (44), but another study found that reduced

Table II. Gene Ontology groups for upregulated genes (≥2-fold) in (MCF-7/DOX 53.2 nM).

Category	Count	Genes
Protein binding	37	CDKN1C, CDKN1A, NOTCH1, CSF1, GSTP1, SERPINE1, PTEN, TWIST1, NR3C1, GLI1, PTGS2, MAPK8, CCND2, PLAU, CDH1, ADAM23, SFN, SLIT2, JUN, TGFB1, CDKN2A, MMP2, KRT5, IGF1, HIC1, ESR2, NME1, VEGFA, CCNA1, SFRP1, IL6, APC, CCNE1, BCL2, CTNNB1, SNAI2, ABCG2
Negative regulation of cell proliferation	14	CDKN1A, JUN, TGFB1, NOTCH1, CDKN2A, PTEN, PTGS2, NME1, IL6, SFRP1, APC, CDH13, CTNNB1, RARB
Positive regulation of transcription from RNA polymerase II promoter	14	JUN, TGFB1, NOTCH1, CDKN2A, SERPINE1, TWIST1, IGF1, GLI1, NR3C1, VEGFA, IL6, CDH13, CTNNB1, RARB
Positive regulation of transcription, DNA-templated	13	CDKN1C, JUN, TGFB1, NOTCH1, CDKN2A, IGF1, GLI1, ESR2, IL6, SFRP1, CCNE1, CDH1, CTNNB1
Negative regulation of apoptotic process	12	IL6, CDKN1A, SFRP1, MAPK8, CCND2, GSTP1, PTEN, BCL2, RARB, TWIST1, IGF1, VEGFA
Positive regulation of cell proliferation	12	IL6, SFRP1, TGFB1, NOTCH1, CCND2, CSF1, PTEN, BCL2, RARB, IGF1, GLI1, VEGFA
Positive regulation of cell migration	10	TGFB1, NOTCH1, APC, CSF1, PLAU, CDH13, SNAI2, IGF1, GLI1, VEGFA
Negative regulation of transcription from RNA polymerase II promoter	10	CDKN1C, TGFB1, NOTCH1, CTNNB1, RARB, TWIST1, SNAI2, HIC1, ESR2, VEGFA

DOX, doxorubicin.

Table III. Gene ontology groups for downregulated genes (≥2-fold) in (MCF-7/DOX 53.2 nM).

Category	Count	Genes
Positive regulation of transcription from RNA polymerase II promoter	7	RB1, AR, XBP1, PGR, BRCA1, ESR1, MAPK3
Transcription, DNA-templated	7	RB1, AR, BIRC5, PGR, BRCA1, ESR1, MAPK3
Positive regulation of transcription, DNA-templated	6	RB1, AR, BRCA1, BRCA2, ESR1, MAPK3
Regulation of apoptotic process	3	BIRC5, BRCA1, ESR1

DOX, doxorubicin.

Table IV. Pathway analysis for upregulated genes in (MCF-7/DOX 53.2 nM).

Category	Count	Genes
Pathways in cancer	19	CDKN1A, JUN, TGFB1, CDKN2A, GSTP1, MMP2, PTEN, IGF1, GLI1, PTGS2, VEGFA, IL6, MAPK8, APC, CCNE1, CDH1, BCL2, CTNNB1, RARB
MicroRNAs in cancer	11	CDKN1A, NOTCH1, CCND2, APC, PLAU, CCNE1, CDKN2A, PTEN, BCL2, PTGS2, VEGFA
p53 signaling pathway	8	CDKN1A, CCND2, CCNE1, CDKN2A, SERPINE1, PTEN, SFN, IGF1
Cell cycle	8	CDKN1C, CCNA1, CDKN1A, TGFB1, CCND2, CCNE1, CDKN2A, SFN
Signaling pathway	7	IL6, CDKN1A, MAPK8, TGFB1, CCND2, PTEN, IGF1

DOX, doxorubicin.

Table V. Pathway analysis for downregulated genes in (MCF-7/DOX 53.2 nM).

Category	Count	Genes
Pathways in cancer	5	RB1, AR, BIRC5, BRCA2, MAPK3

DOX, doxorubicin.

Table VI. Gene Ontology groups for upregulated genes (≥2-fold) in (MCF-7/DOX 53.2 nM) [Track (2)].

Category	Count	Genes
Negative regulation of cell proliferation	11	IL6, CDKN1A, SFRP1, JUN, APC, CDKN2A, PTEN, CDH13, RARB, PTGS2, NME1
Negative regulation of apoptotic process	10	IL6, CDKN1A, SFRP1, MAPK8, CCND2, GSTP1, PTEN, BCL2, RARB, IGF1
Positive regulation of apoptotic process	9	IL6, SFRP1, MAPK8, APC, CDKN2A, RARB, ATM, SLIT2, PTGS2
Positive regulation of cell proliferation	9	IL6, SFRP1, CCND2, CSF1, PTEN, BCL2, RARB, IGF1, GLI1
Cell cycle arrest	5	CDKN1C, CDKN1A, APC, CDKN2A, ATM
Regulation of cell cycle	4	JUN, CCNE1, PTEN, ATM
DNA damage response, signal transduction by p53 class mediator resulting in cell cycle arrest	3	CDKN1A, ATM, SFN

DOX, doxorubicin.

Table VII. Gene Ontology groups for downregulated genes (≥2-fold) in (MCF-7/DOX 53.2 nM) [Track(2)].

Category	Count	Genes
Positive regulation of cell migration	5	APC, CSF1, PLAU, CDH13, SNAI2
Positive regulation of apoptotic process	4	APC, RARB, SLIT2, PTGS2
Negative regulation of cell proliferation	4	APC, CDH13, RARB, PTGS2
Negative regulation of transcription from RNA polymerase II promoter	4	RARB, SNAI2, HIC1, ESR2
Transcription, DNA-templated	4	RARB, SNAI2, HIC1, ESR2

DOX, doxorubicin.

Table VIII. Pathway analysis for upregulated genes in (MCF-7/DOX 53.2 nM) [Track(2)].

Category	Count	Genes
Pathways in cancer	16	CDKN1A, JUN, CDKN2A, GSTP1, MMP2, PTEN, IGF1, PTGS2, GLI1, IL6, MAPK8, APC, CCNE1, CDH1, BCL2, RARB
MicroRNAs in cancer	10	CDKN1A, CCND2, APC, PLAU, CCNE1, CDKN2A, PTEN, BCL2, ATM, PTGS2
p53 signaling pathway	9	CDKN1A, CCND2, CCNE1, CDKN2A, SERPINE1, PTEN, ATM, SFN, IGF1
Cell cycle	8	CDKN1C, CCNA1, CDKN1A, CCND2, CCNE1, CDKN2A, ATM, SFN

DOX, doxorubicin.

BRCA1 protein expression in ovarian cancer cells leads to an improvement in survival (45). As a result, downregulation of BRCA1 and BRCA2 gene expression levels seen in resistant MCF-7 cells may be one of the main changes seen in the resistant phenotype. The present results showed that quercetin led to an increase in the expression of BRCA1 gene

Table IX. Pathway analysis for downregulated genes in (MCF-7/DOX 53.2 nM) [Track(2)].

Category	Count	Genes
MicroRNAs in cancer	3	APC, PLAU, PTGS2
Pathways in cancer	3	APC, RARB, PTGS2

DOX, doxorubicin.

Table X. Gene Ontology groups for upregulated genes (≥5-fold) in the difference between Track (1) and Track (2).

Category	Count	Genes
Negative regulation of apoptotic process	6	IL6, SFRP1, CCND2, GSTP1, BIRC5, IGF1
Positive regulation of cell proliferation	5	IL6, SFRP1, CCND2, BIRC5, IGF1
Positive regulation of transcription, DNA-templated	5	IL6, SFRP1, CDKN2A, BRCA1, IGF1
Positive regulation of apoptotic process	4	IL6, SFRP1, CDKN2A, ATM
Cellular response to tumor necrosis factor	3	IL6, SFRP1, BRCA1
DNA replication	3	ATM, BRCA1, IGF1
Replicative senescence	2	CDKN2A, ATM

Table XI. Gene Ontology groups for downregulated genes (≥5-fold) in the difference between Track (1) and Track (2).

Category	Count	Genes
Positive regulation of cell migration	5	APC, CSF1, PLAU, CDH13, SNAI2
Positive regulation of apoptotic process	4	APC, RARB, SLIT2, PTGS2
Negative regulation of cell proliferation	4	APC, CDH13, RARB, PTGS2
Transcription, DNA-templated	4	RARB, SNAI2, HIC1, ESR2

Table XII. Pathway analysis for upregulated genes in the difference between Track (1) and Track (2).

Category	Count	Genes
Pathways in cancer	6	IL6, CDKN2A, GSTP1, MMP2, BIRC5, IGF1
p53 signaling pathway	5	CCND2, CDKN2A, SERPINE1, ATM, IGF1
Signaling pathway	4	IL6, CCND2, ATM, IGF1
Cell cycle	3	CCND2, CDKN2A, ATM

Table XIII. Pathway analysis for downregulated genes in the difference between Track (1) and Track (2).

Category	Count	Genes
MicroRNAs in cancer	3	APC, PLAU, PTGS2
Pathways in cancer	3	APC, RARB, PTGS2

by 6.8-fold; this result is consistent with those from a study by Kundur *et al* in 2019 (46), who reported that quercetin and curcumin lead to a dose-dependent enhancement in BRCA1 expression.

In the present study, SNAI2 expression decreased by 30.06-fold to -1.17-fold after combination treatment. This gene encodes a member of the Snail family as described by Alves *et al* (47). The encoded protein represents a transcriptional repressor to E-cadherin transcription in breast carcinoma. It is also involved in epithelial-mesenchymal transitions and has antiapoptotic activity. Another study by Côme *et al* (48) reported that SNAI2 has been found to be upregulated in all endocrine resistant cells when compared with parental cell lines. Reduction of SNAI2 expression levels lead to a disabling of cell migration and an increase in E-cadherin levels in two fulvestrant-resistant breast cancer cell models, proving the role of SNAI2 in controlling cell motility and maintenance of a mesenchymal phenotype in resistant cells (47). Another

valuable observation in the present study was the upregulation of PLAU expression gene in (MCF-7/DOX 53.2 nM) cells by 44.32-fold and downregulated to 13.7-fold after combination treatment.

Lin *et al* (49) showed that downregulation of PLAU expression can suppresses colorectal cancer development via inhibition of colorectal cancer cell growth, cell migration and angiogenesis. Another study by Ai *et al* in 2020 (50) revealed that inhibition of PLAU expression can repress the migratory and invasive capability of cervical cancer cells through downregulation of MMP2. This finding may provide a clue that quercetin can cause reduction in the metastatic phenotype of MCF-7 resistant cells as published by Hoca *et al* (51) who demonstrated that quercetin can lead to inhibition of EMT in cancer cell lines, including oral cancer cells, breast cancer stem cells and prostate cancer cells.

In conclusion, the results of the present study indicated that quercetin could lead to reversal of breast cancer cell doxorubicin resistance via downregulation of the expression of important genes, such as SNAI2, PLAU and CSF1. Such findings may represent a potential strategy for reversing the chemoresistance of breast cancer. Notably, the present study needs a further protein level confirmation for the obtained gene expression changes.

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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

MZ and BAA confirm the authenticity of all the raw data. MZ, RA-D and HZ conceptualized the present study. MZ, BAA and RA-D designed the methodology. All the authors worked on the validation. MZ, BAA and RA-D worked on the formal analysis of data. MZ, BAA and RA-D performed the experiments. All authors worked together on resources, writing-original draft preparation, writing-review and editing. MZ, RA-D and HZ supervised the study. MZ, RA-D and HZ were project administrators. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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