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Enhancement of Doxorubicin Cytotoxicity by Verapamil in Human Breast Cancer Cells

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Authors' contributions

Authors SEA, MAM, HMA, MMS, EAH, HM and AMO sharing in research design, experimental work, data analysis, writing and revision of the manuscript.

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ABSTRACT

Background: Worldwide, breast cancer is a main cause of morbidity and mortality in females. Doxorubicin (DOX) is an anthracycline anticancer drug and most commonly employed in polychemotherapy protocols in the treatment of solid and hematological tumors. Unfortunately, its optimal clinical benefit is limited secondary to the rapid development of DOX resistance and therapeutic failure.
Aim: Therefore, the current study has been initiated to investigate the possible mechanisms whereby the calcium channel blocker Verapamil (VER) could decrease DOX resistance and enhance the cytotoxic activity of DOX against the growth of human breast cancer cells.
Methodology: To achieve the ultimate goal of this study, we have examined DOX-induced cytotoxicity, apoptosis, alteration in the function of multidrug resistance proteins and cell cycle phase distribution against MCF-7 cell line in presence and absence of Verapamil.
Results: Addition of VER enhanced the cytotoxic effect of DOX against the growth of MCF-7 cells which manifested as a significant decrease in the IC50 from 36 µg/ml for DOX alone to 13 µg/ml for

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DOX plus VER. Moreover, combined treatment with VER and DOX significantly increased percentage of early apoptosis and cells arrested in G_0/G_1 phase when compared to DOX alone. In addition, VER significantly increased DOX cellular uptake through inhibition of the function of multidrug resistant proteins.

Conclusion: VER treatment enhanced the cytotoxic activity of DOX against the growth of MCF-7 cells secondary to increase its cellular accumulation. The observed increase in DOX uptake by VER was parallel to increased accumulation of Rho-123 dye which my point to the contribution of inhibition of multidrug resistant proteins by VER in the enhancement of DOX cytotoxicity.

Keywords: Doxorubicin; verapamil; cytotoxicity; potentiation; breast cancer cells.

1. INTRODUCTION

Breast cancer is a main cause of morbidity and mortality in women in both developed and developing countries [1]. Anthracyclines have been used in the treatment of breast cancer for the past five decades, and their use have been associated with improved survival among breast cancer patients [2,3]. However, their use has been limited due to two main problems namely cardiomyopathy and development of drug resistant [4]. In order to circumvent this effect, several strategies have been conducted including producing forms of anthracyclines that could be administered at higher than usual doses, but with fewer adverse events. This led to the production of formulations of liposomal doxorubicin (DOX) [5]. Other investigators conducted a study to determine the antitumor effects of calcium channel blocker VER on multidrug resistance in leukemia cells and in breast cancer patients. They noticed that concentrations of VER at 3 µg/mL and 10 µg/mL enhanced the effects of epirubicin (an anthracycline) on leukemia cells with positive multidrug resistance phenotype by 10 and 19 folds respectively in vitro [6,7] .It has been reported that diltiazem, a calcium channel blocker showed synergestic activity with doxorubicin against the growth of Ehrlich ascites carcinoma cells in experimental animals [8] Therefore, the aim of this study is directed to investigate the effect of the calcium channel blocker verapamil (VER) on the anticancer activity of DOX against the growth of human breast cancer cells using sulfarhodamine-B assay. Apoptosis, cell cycle phase distribution, DOX cellular uptake and the activity of multidrug resistant proteins have also been investigated in the presence and absence of Verapamil.

2. MATERIALS AND METHODS

This research was done at pharmacology Department, Faculty of Medicine, KAU, Jeddah, Saudi Arabia in collaboration with Pharmacology Unit, National Cancer Institute, Cairo University, Egypt from September 2016 to September 2018.

2.1 Drugs and Chemicals

Doxorubicin hydrochloride "Ebewe" was purchased from Ebewe Pharma, Austria and Verapamil hydrochloride (Isoptin®) was purchased from Abbott, USA. Phosphate buffer saline (PBS), Penicillin G and streptomycin antibiotics were purchased from UFC Biotech, SA. Cyto Scan TM SRB Cell Cytotoxicity assay kit was purchased from A Geno Technology, USA and Annexin V-FITC apoptosis detection kit was obtained from Aviscera Bioscience, USA. The cell cycle determination kit was purchased from Cayman Chemical Company, USA and Rhodamine 123 (Rh 123) was purchased from AAT Bioquest, USA.

2.2 Cells and Cell Culture

Human breast cancer cell line MCF-7 used in this study were grown as monolayer cultures and maintained in DMEM tissue culture medium at 37°C in a humidified 5% CO2 air and collected by trypsinization.

2.3 Methods

2.3.1 Assessment of cytotoxicity

Cytotoxicity was determined using sulforhodamine-b (SRB) method as previously described by skehan et al. [9]. In brief, cells were seeded in 96 well microtiter plates at concentration of 40 x 10^3 cells/well in DMEM medium. The cells were kept to attach for 24 hours, then were incubated with various concentrations of DOX and/or VER for 48 hours in the followin range (1.25, 2.5, 5 and 20 µg/ml) for DOX and 24.5 µg/ml for VER (3 wells for each concentration). After 48 hours, 50µl fixative reagent was added onto each well

for 1 hour at 4°C. Then, supernatant was discarded and the plates were washed four times with bidistilled water, air dried, stained for 30 minutes at room temperature in the dark with 0.4% SRB dissolved in 1X dye wash solution. The unbound dye was removed by washing four times with 1X dye wash solution and plates air dried. Then 200 µl SRB solubilization buffer was added to each well. The optical density was read in ELx808 absorbance microplate reader (BioTek, USA.) at wavelength of (490-530) nm. Surviving fraction calculated as the following:

Surviving fraction = optical density of treated cells / optical density of control cells. IC50 (the concentration of DOX necessary to produce 50% inhibition of cells growth) calculated from liner regression equation of the survival fraction curve. Y = mX + b, Where, Y = 0.5 (The surviving fraction where there is a 50% inhibition of cell growth). m= The Slop, X = Dose of DOX induce 50% inhibition, b = the y- intercept.

2.3.2 Apoptosis assay

Cells were seeded in T25 flasks at cell density of 5-8 x 10^5 cells/flask in DMEM media and incubated for 24 hours at humidified air containing 5% CO2. After that the cells were incubated with DOX concentration (5 and 20 µg/ml) alone and combined with verapamil (24.5 ug/ml) for 48 hours (3 flasks for each concentration). Then, the medium was removed and the flasks were washed with PBS and the cells harvested with Trypsin/EDTA. After trypsinization, cells were washed with cold PBS and suspended in 100 µl annexin V incubation reagent. The solution incubated at room temperature in the dark for 15 minutes. Then 400 µl of binding buffer was added to each sample and processed by flow cytometry (NAVIOS Beckman Coulter, U.S.A.).

2.3.3 Cell cycle analysis

Cells were seeded in T25 flasks and proceed as above. Following trypsinization, cells were washed twice with cold assay buffer. The cell pellets were resuspended to a density of 10^6 cells/ml in assay buffer. One ml of fixative agent was added to each sample to fix and permeabilize the cells for at least two hours. The fixed cells pellet was suspended in staining solution and incubated for 30 minutes at room temperature in the dark. Cell cycle analysis was performed by using flow cytometry (Becton DICNSON (BD) FACS Calibur, U.S.A) as previously described [10].

2.3.4 Assessment of doxorubicin cellular uptake

DOX cellular uptake in MCF-7 cells performed according to the method of Bachur et al. [11]. Cells seeded in T25 flasks at cell density of 5-8 x 10^6 cells/flask in DMEM medium and proceed as above. Cells incubated with DOX concentrations (5 and 20 µg/ml) alone and combined with verapamil simultaneously for 48 hours. After 48 hours treatment, medium was removed, wells were washed with PBS, then the cells were harvested with trypsin/EDTA. Following trypsinization, cells were washed with ice PBS and counted. For drug uptake analysis, cells (1 x 10^6) were resuspended in 0.3 N HCl in 50% ethanol and digested by homogenization in ultrasound bath. The 0.3 N HCl in 50% ethanol cell suspensions were centrifuged at 14000 rpm for 10 minutes and collected. The DOX fluorescence intensity of the supernatant was measured by a spectrofluorometer (Synergy HT, BioTek, USA) at excitation and emission wavelengths of 485 nm and 593 nm, respectively.

Determination of the activity of multidrug resistance (MDR) via rhodamine-123 dye: Accumulation of rhodamine-123 in the cells is inversely related to MDR activity [12]. In brief, cells were seeded in T25 flasks at cell density of 5-8 x 10^6 cells/flask in DMEM medium and proceed as above. A 2.62 µM (100 µl) from working solution of Rhodamine-123 was added and keep it in CO2 incubator at 37°C for one hour. Cells were then incubated with DOX concentrations alone and combined with verapamil (24.5 ug/ml) for 10 minutes. Cells were harvested and washed once with iced PBS. For the activity of multidrug resistance, cells (1 x 10^6) were suspended in one ml of PBS to each sample and shaked. Lysed cells were analyzed by spectrofluorometer at wavelength (485-590 nm).

3. STATISTICAL ANALYSIS

Statistical analysis of data was calculated by using statgraphic computer package (Excel, 2010) and computer program package (SPSS, version 18). All date expressed as mean with their standard error of mean (SEM) of three separate experiments, each one in triplicate. One way analysis of variance (ANOVA) was used to test for difference between experimental groups. It was followed by the least significance difference (LSD) test. However, two-sample t-test

and its P-value to analyze the significance of the difference in the sample mean. Differences were considered significant at P <0.05.

4. RESULTS

Effect of verapamil treatment on the cytotoxic activity of doxorubicin: Addition of VER (24.5 µg/ml) to DOX resulted in a significant decrease in the surviving fraction values with IC50 13 µg/ml compared to 36 µg/ml in case of DOX alone and as the concentration of DOX increased, the surviving fraction values gradually decreased (Table 1 and Fig.1).

Effect of DOX and/or VER on induction of apoptosis: Treatment with 5µg/ml DOX showed 26.5% of early apoptotic cells and in combination with VER (24.5 µg/ml) showed 93.5% of early apoptotic cells, (Fig. 2). Similar outcome were observed after treatment with 20µg/ml DOX which showed 40% of early apoptotic cells and in combination with VER showed only 81.35% of early apoptotic cells, (Fig. 2).

Effect of DOX and/or VER on cell cycle phase progression of MCF-7 cells: Combined treatment with DOX (5 µg/ml) and VER (24.5 µg/ml) showed a significant increase in percentage of cells in G_0/G_1 phase (59.45%) compared with 14.80% in case DOX alone. In addition, increasing the concentration of DOX to 20 µg/ml in presence VER showed a significant increase in the percentage of cells in G_0/G_1 phase to 57.40% compared to 16.10% of DOX alone (Fig. 3). Treatment with VER alone, showed a preferential block in G_0/G_1 phase at the expense of S phase and G2/M phase cells, where there was 78.1% accumulation.

Effect of VER treatment on DOX cellular uptake: Table 2 shows DOX level in MCF-7 cells after combination with VER 24.5 µg/ml. DOX level was (0.228 µg/10^6 cells) after treatment with 5 µg/ml DOX, which increased significantly to (1.272 µg/106 cells) in the presence of VER. Moreover, when the concentration of DOX was increased to 20 µg /ml, DOX cellular concentration was (0.842 µg/10^6 cells) and in presence of VER it was (1.740 µg/10^6 cells).

Effect of DOX and/or VER on the activity of multidrug resistance proteins: MCF-7 cells poorly accumulated Rh 123 which reflecting P-glycoprotein efflux of the fluorescent dye (Table 3). DOX 5 and 20 µg /ml increased dye accumulation in MCF-7 (0.104 µg/10^6 cells) and (0.164 µg/10^6 cells), respectively, compared to Rh 123 alone. Addition of 24.5 µg/ml VER to 5 and 20 µg/ml DOX, increased accumulation of Rh dye significantly to (0.794 µg/10^6 cells) and (0.651 µg/10^6 cells), respectively , compared to corresponding DOX treatment.

5. DISCUSSION

Doxorubicin (DOX) is the most active cytotoxic agent used in the management of several human solid tumors either alone or in combination with other cytocidal agents. However, it's clinical uses

Table 1. Effect of doxorubicin and/or verapamil on the growth of MCF-7 cells

Treatment	IC50 (µg/ml)
Doxorubicin	36 ± 0.025
Doxorubicin and Verapamil	13 ± 0.067 a

IC50: the concentration of Doxorubicin necessary to produce 50% inhibition of cell growth. Data are presented as the mean ± S.E.M of three independent experiments each one in triplicate and analyzed using student t-test. a Significantly different from DOX at P-Value < 0.05

Table 2. Effect of VER treatment on the cellular uptake of DOX in MCF-7 cells

Treatment	DOX concentration (µg/106 cells)
DOX (5µg/ml)	0.228± 0.01
DOX (5µg/ml)+ VER (24.5 µg/ml)	1.272± 0.03a
DOX (20µg/ml)	0.842±0.02 a
DOX (20µg/ml)+ VER (24.5 µg/ml)	1.740±0.05 a

The MCF-7 cells were treated with DOX and /or with VER. The cells were analyzed for DOX level after 48 hours of exposure to drugs. Data expressed as mean ± S.E.M of three independent experiments each one in triplicate.
[a] Significantly different from corresponding DOX at P-value < 0.05

Table 3. Effect of DOX and/or VER on the P-glycoprotein efflux of the fluorescent dye

Treatment	Rh 123 concentration (µg/106 cells)
Rh 123 (100µl/ml)	0.01±.01
VER(24.5 µg/ml)	0.548±0.04 a
DOX (5 µg/ml)	0.104±0.02a
DOX (5 µg/ml)+ VER (24.5 µg/ml)	0.794± 0.05a,b
DOX (20 µg/ml)	0.164±0.01a
DOX (20 µg/ml)+ VER (24.5 µg/ml)	0.651±0.03a,b

The MCF-7 cells were exposed to 100 µl/ml of Rh 123 for 30 minutes at 37°C. Data expressed as mean ± S.E.M of three independent experiments each one in triplicate. [a] Significant different from Rh 123 at P-value < 0.05. [b] Significantly different from corresponding DOX at P-value < 0.05

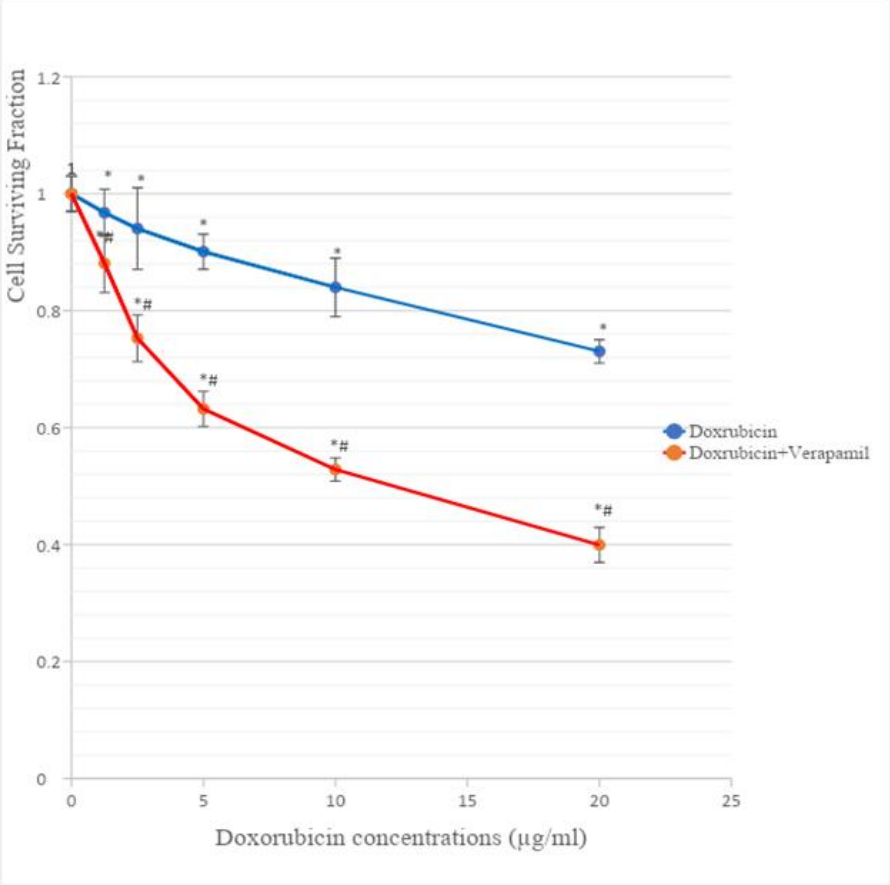

Fig. 1. Cytotoxic effect of DOX and /or VER treatment on the growth of MCF-7 cells

Cell survival was determined after 48 hours of exposure to drugs. Each point is the mean ± S.E.M of three independent experiments each one in triplicate. Significant different from control at P-value < 0.05. # Significant different from the corresponding DOX at P-value < 0.05*

are limited by its detrimental adverse effects including cardiotoxicity [13,14,15]. Chemosensitization is one strategy that can be used to lower the anti-tumor dose and toxicity. A variety of approaches have been examined to enhance the cytotoxic effects of chemotherapeutic agents, and at the same time decrease their toxicity. Among the potential chemosensitizer is calcium channel blocker which has cytotoxic activity against solid tumors [16] and has chemopreventive effect [17,18]. The present study focused on investigating the possible mechanisms whereby VER could enhance DOX cytotoxicity against the growth of MCF-7 through studying the changes of apoptosis induction, cell cycle phase distribution,

DOX cellular uptake and the activity of multidrug resistance proteins after treatment with different DOX concentrations in the presence and absence of Verapamil. Results from the current study showed that treatment of MCF-7 cells with 24.5 µg/ml VER, significantly enhanced the cytotoxic activity of DOX against the growth of MCF-7 cells by 2.8 fold decrease in survival compared with cells treated with DOX alone (Table 1). These findings are in harmony with previous study of Jensen et al. [19] who showed the inhibitory effect of calcium channel antagonists such as VER on meningioma cells. In addition, the previous study of Zheng et al. [20] showed that VER increases cytotoxicity of DOX in human ovarian cancer cells in-vitro, compared with cells treated with DOX alone.

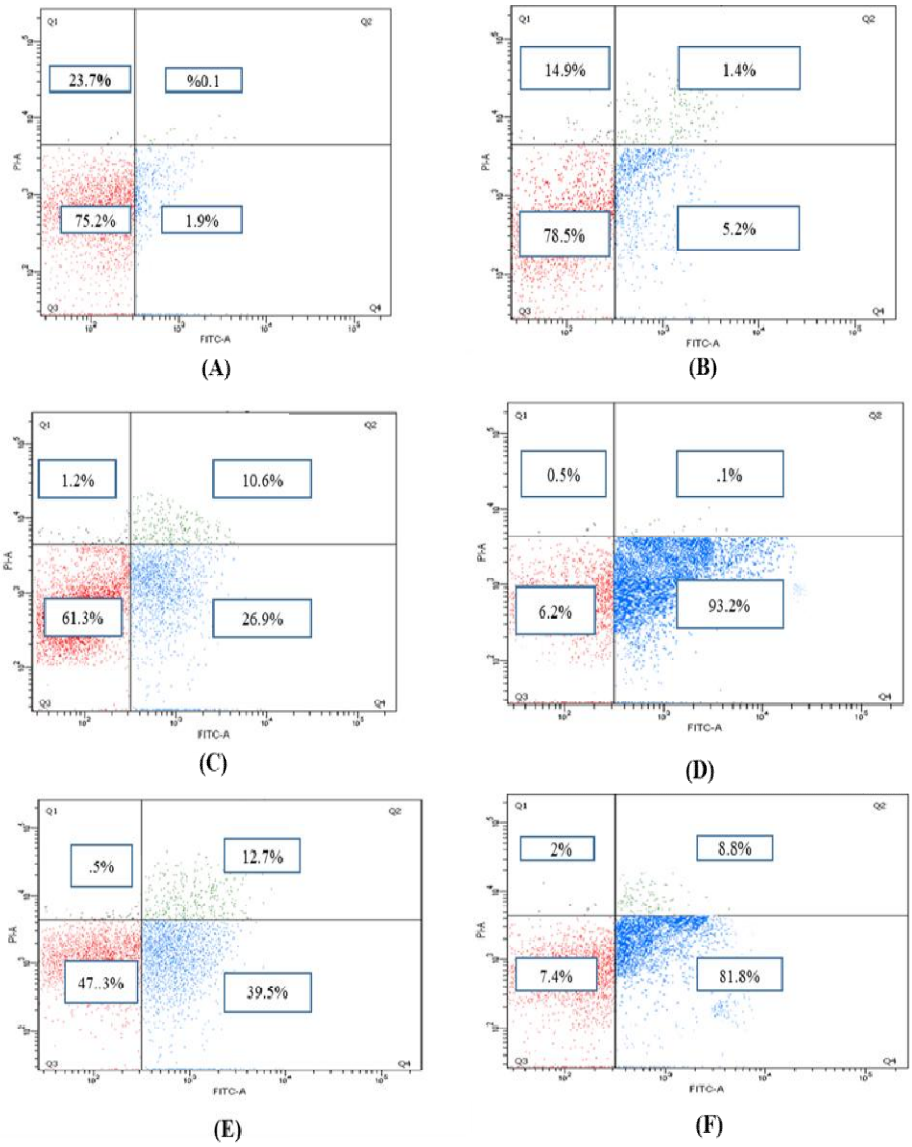

Fig. 2. Effect of DOX and /or VER treatment on induction of apoptosis in MCF-7 cells

Apoptosis was analyzed after 48 hours of exposure to drugs by staining with Annexin V FITC-A and Propidium iodide (PI). (A) control, (B) cells treated with VER, (C) cells treated with 5 µg /ml DOX, (D) cells treated with 5 µg /ml DOX plus verapamil 24.5 µg/ml, E) cells treated with 20 µg /ml DOX, (F) cells treated with 20 µg /ml DOX plus VER 24.5 µg/ml. The percentage of cells in each is indicated early apoptosis. All data from three independent experiments each one in triplicate

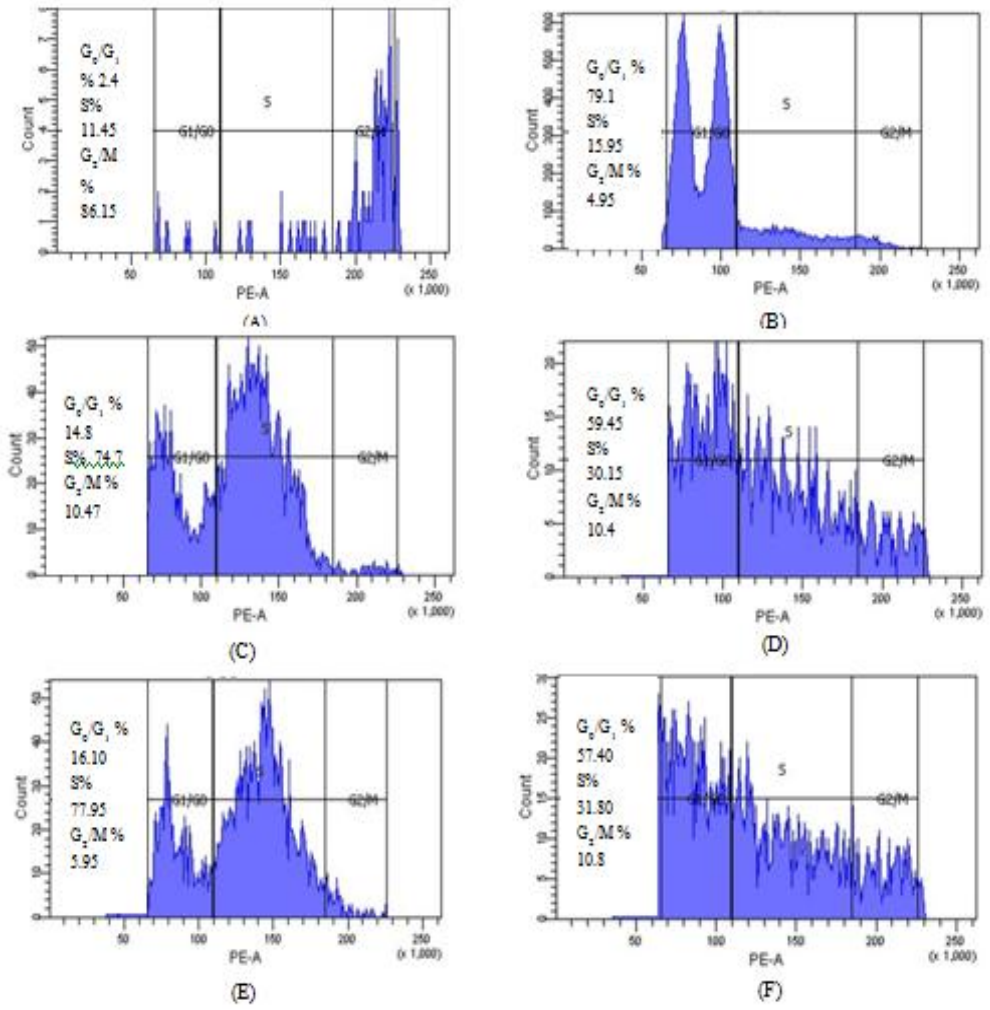

Fig. 3. Effect of DOX and / or VER on cell cycle phase distribution of MCF-7 cells

Cell cycle phase distribution was analyzed after 48 hours of exposure to drugs by staining with Propidium iodide (PI). (A) control, (B) cells treated with VER 24.5 µg/ml (C) cells treated with 5 µg/ml DOX, (D) cells treated with 5 µg/ml DOX plus VER 24.5 µg/ml (E) cells treated with 20 µg/ml DOX, (F) cells treated with 20 µg/ml DOX plus VER 24.5 µg/ml. The independent experiments was repeated twice each one in duplicate

The current study showed that treatment of the MCF-7 cells with VER and DOX resulted in a significant increase of the cells that arrested in G_0/G_1 phase compared with cells treated with DOX alone (Fig. 3). Previous studies on the effect of VER on the cell cycle of many human tumor cell lines including MCF-7 cells, demonstrated the ability of VER to block the cells inflowing from G_0/G_1 into S phase transition, resulting in a concentration-dependent accumulation of cells in G_0/G_1 phase [21]. It's worthy noted that cell cycle arrest in G_0/G_1 phase after treatment with VER might be due to inhibition of the enzymes used for DNA replication and synthesis. This consequently led to the interference of DNA replication within these cells. These findings comply with the previous study of Cao et al. [22], in which VER blocked cell cycle, resulting in increased G_0/G_1 phase and decline in S phase in human colonic tumor. Authors found an inverse relationship between VER concentrations and the percentage of S-phase cells and a direct one between the calcium channel antagonist and the percentage of G_0/G_1 phase cells, suggesting that VER could block the transformation of human colonic cells from G_0/G_1 phase into S phase. Worth mentioning is that damaged DNA could induce

cell cycle arrest at G_1, S, or G_2, thereby avoiding replication of damaged DNA which if not repaired, may result in either tumorigenesis or apoptosis [23,24].

It has been reported that single treatment of DOX induced apoptosis in MCF-7 cells [25]. The current study showed a significant increase in percentages of early apoptosis in the MCF-7 cells treated with DOX and VER, compared with cells treated with DOX alone (Fig. 2). In line with current results, the previous study of Cao et al. [22] reported that VER could sensitize cancer cells to the apoptosis or growth arrest and synergistically increase the cytotoxicity of antineoplastic agents against human colonic tumor cells line. The present study showed a significant increase of DOX cellular uptake in presence of VER. There were a 5.5 and 2-fold increase in DOX cellular uptake for cells treated with 5 and 20 µg/ml DOX and VER, compared with cells treated with DOX alone (Table 2). Therefore, VER not only exposed higher proportion of MCF-7 cells to DOX by arresting cells in G0/G1 phase, but also increased the DOX available inside the cells. This could explain why VER/DOX combination has more cytotoxicity than DOX alone. The obtained results are in harmony with the data presented by Balakrishnan et al. [25] which have reported that VER increased cyclophosphamide cellular uptake in MCF-7 cells. In order for chemotherapeutic agents such as DOX to exert their cytotoxic activity against cancer, it is necessary for the drug to be available in the cellular compartments and be distributed to the action site [26]. Thus, it is expected that sensitivity to agents such as DOX should be greater in cells with a higher uptake of the drug.

In reality, cancer cells develop mechanisms against the cytotoxic effect of drugs like DOX [27,28,29]. One of these mechanisms is cell expression of multidrug resistant proteins, including P-glycoprotein [30,31,32]. This protein is an ATP-dependent drug efflux pump which is responsible for expelling drug molecules from the intracellular area, consequently, causing a decrease in the concentration of the accumulated anti-cancer agent [12]. So, the cancer cells develop drug resistance as the chemotherapy agent cannot reach the action site. Data presented in the current study showed that treatment with VER resulted in a significant 79.4 and 65.1-fold increase in accumulation of fluorescent dye inside the cells which reflect inhibition of P-glycoprotein activity level and lead to more accumulation of DOX in the presence of VER. In the present study, P-glycoprotein activity in MCF-7 cells was significantly inhibited in the presence of VER. Therefore, the observed increase in cytotoxicity of DOX by VER was parallel to the increase in its cellular uptake. It is well documented that rhodamine-123 dye is a substrate for multidrug resistance (MDR) genes and the proteins codified by these genes including P-glycoprotein. In the current study, the accumulation of rhodamine-123 in the cells measures the contribution of P-gp in the uptake of DOX. The accumulation of rhodamine-123 in breast cancer cells was increased by combined treatment with VER and DOX compared with DOX alone. These results suggest that VER may down regulate MDR proteins or inhibits their catalytic activity with the consequent increase in the accumulation of DOX in the presence of VER.

6. CONCLUSION

In conclusion Verapamil enhanced DOX cytotoxicity against the proliferation of MCF-7 humane breast cancer cells. This could be explained by induction of apoptosis and enhanced DOX cellular uptake. The observed increase in DOX uptake by VER was parallel to increased accumulation of Rho-123 dye which my point to the contribution of inhibition of multidrug resistant proteins by VER in the enhancement of DOX cytotoxicity.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All studies were approved by the ethical research committee unit at the College of Medicine, King Abdulaziz University (Reference No.165-19).

AVAILABILITY OF DATA AND MATERIALS

All relevant data are within the paper and its supporting information file.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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