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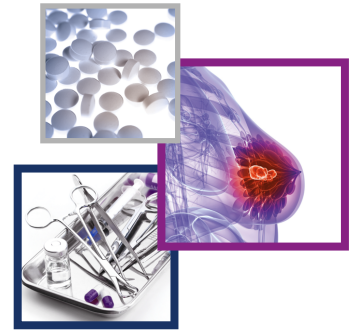
Breast Cancer Management



Diltiazem potentiation of doxorubicin cytotoxicity and cellular uptake in human breast cancer cells

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Aim: Breast cancer is the most common cancer among Arab women and also around the world. Chronic cardiotoxicity and multidrug resistance are potential limiting factors of doxorubicin (DOX), a known anthracycline antibiotic. **Materials & methods:** DOX cytotoxicity was evaluated by the sulforhodamine method. DOX cellular uptake, detection of P-glycoprotein activity and the photomicrograph of MCF-7 cells were also determined. **Results:** Diltiazem (DIL) treatment improved DOX cytotoxic activity and increased the cellular uptake of DOX significantly and aggregation of rhodamine 123, reflecting inhibition of P-glycoprotein pump. Cytopathological investigation of MCF-7 cells revealed marked cytotoxic activity of DOX in the presence of DIL. **Conclusion:** DIL treatment enhanced DOX cytotoxic effect and reduced multidrug resistance, which increased the drug accumulation intracellularly.

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Keywords: activity enhancement • diltiazem • doxorubicin • MCF-7 cells • multidrug resistance

Breast cancer is the most common cancer among Arab women and in women around the world. This cancer is also prevalent among young age women in western countries [1]. The anthracycline chemotherapy drug doxorubicin (DOX) has been considered as the most effective anticancer drug for the treatment of breast malignancy [2]. However, DOX causes chronic cardiotoxicity by intercalation between DNA base pairs on the double helix and degrading topoisomerase II, which is the major limiting factor in the use of DOX [3].

In addition, various other forms of cancer are unresponsive against DOX and those that were responding efficiently, ultimately become resistant causing a major threat to the treatment of breast cancer. Furthermore, overexpression of P-glycoprotein (P-gp) has been shown to prevent the entry of DOX molecules into murine breast cancer cells (4T1-R), hence causing DOX chemoresistance [4].

In many studies, P-gp expression has been shown to be associated strongly with resistance to drug treatment. These studies reported that the therapeutic failure in addition to relapse may be a result of an increasing degree of P-gp expression, either intrinsic or acquired [5]. Through studying the function of P-gp in resistance to chemotherapy, several attempts have been implicated that inhibit the function of P-gp, which enhances the efficacy of chemotherapeutic agents through increasing their concentrations. These studies also confirmed that combination remedy could be utilized as an approach to defeat multidrug resistance (MDR) [6,7].

This study investigated several approaches to overcome the side effects and resistance related to the use of such drug. Searching for a natural or synthetic alternative with anticancer or chemopreventive properties that can be used in combination with DOX was one of the major objectives.

Based on our previous work on potentiating effect of diltiazem (DIL) on DOX activity in an experimental tumor [8], we directed this study to further investigate the DIL–DOX interaction on human breast cell lines and the possible mechanism of this interaction. We also recently investigated the underlying molecular mechanisms of

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DIL-induced enhancement in the cytotoxic activity of DOX by measuring the expression of genes responsible for drug resistance, apoptosis induction and cell cycle disturbance [9].

Therefore, this study completed many branches for our previous work and was directed to explore whether the calcium channel blocker DIL could improve the cytotoxicity of DOX against the growth of human breast cancer cells (MCF-7 cell line). The current study investigated cytotoxic P-gp activity of DOX, its cellular uptake in cancer cells and the activity of P-gp pump in presence and absence of DIL.

Materials & methods

Drugs & chemicals

DOX, DIL, SulfoRhodamine-B (SRB), dimethylsulfoxide 99.9% (DMSO), Triton (X-100), AO A6014 and trypan blue powder were purchased from Sigma-Aldrich Co. (MO, USA), while Verapamil was purchased from Abbott (IL, USA). Fetal bovine serum, Dulbecco's Modified Eagle Medium (DMEM), Trypsin–EDTA (0.05%) and phosphate-buffered saline (PBS, pH 7.4) were purchased from Thermo Fisher Scientific Inc. (MA, USA).

Cells & cell cultures

The MCF-7 cell line used in this study was provided by the National Cancer Institute (Cairo University, Egypt). The adherent cells were grown as a single layer in the DMEM with penicillin (100 IU/ml), streptomycin (100 µg/ml) and 10% fetal bovine serum supplementations. Furthermore, these cells were cultured at 37°C in a humidified 5% CO_2 atmosphere and were subcultured every 4–5 days.

Assessment of cytotoxicity

Based on the description by Skehan *et al.,* the SRB method was used to determine the cytotoxicity [10]. Cells were seeded in 96-well microtiter plates at a concentration of 40×10^3 cells/well in DMEM and after 24 h, they were incubated in a CO_2 incubator with increasing concentrations of DOX and DIL simultaneously for 48 h. After 48 h, 50% cold trichloroacetic acid was added to the cells for fixation at 4°C for 1 h. The supernatant was then removed and the plates were washed five-times using distilled water. The plates were then air dried and stained for 30 min at room temperature with 0.4% SRB dissolved in 1% acetic acid. The unbound dye was then removed by washing with 1% acetic acid five-times and was air dried. However, the bound stain was solubilized with 100 µl/well 10 mM Tris base (pH 10.5) for 10 min. The optical density was read under the ELx808 absorbance microplate reader (BioTek, VR, USA) at 490–530 nm.

Surviving fraction = Optical density of treated cells/optical density of control cells

IC_{50} (the concentration of DOX necessary to produce 50% inhibition of cell growth) was calculated from linear regression equation of the survival fraction curve.

$$Y = mX + b$$

where Y = 0.5 (the surviving fraction when there is a 50% inhibition of cell growth), m = the slope, X = dose of DOX induces 50% inhibition and b = the *y*-intercept.

DOX cellular uptake

The measurement of cellular DOX content was done using a spectrofluorometer based on the Kitagawa *et al.* method [11]. Cells were seeded in six-well plates at cell density $6–8 \times 10^5$ cells/well in DMEM and were then allowed to attach for 24 h, followed by treatment with DOX concentrations (0.25 and 1 µg/ml) alone or with DIL 20 µg/ml for 48 h. After trypsinization of the cells, pellets were washed once with PBS, then resuspended (1×10^6 cells) in 1 ml of DMSO. The DMSO cell suspensions were centrifuged at 5000 rpm for 20 min and the clear supernatants were collected for the assay. The clear supernatant was measured by a spectrofluorometery (Synergy HT; BioTek) at excitation of an emission wavelength ex = 496 nm and em = 592 nm, respectively, for determination of DOX concentration.

Detection of P-gp activity using rhodamine 123

Mechanism of P-gp in MCF-7 cell line was determined using the Ludescher *et al.* method [12] based on the fluorescent properties of rhodamine 123 (Rho 123), transported through the membrane efflux pump P-gp. Flow cytometric assay was used to analyze the function of MDR in breast cancer cells, which enabled demonstration of

significant differences in Rho 123 efflux and further aggregation inside cells. MCF-7 cells were seeded in six-well plates at density of 1×10^5–10^6 cells/well in DMEM. These cells were then cultured in a CO_2 incubator for 24 h before treatment. The cells were stained with 1 µl/ml of Rho 123 and kept in a dark area for 30 min to 1 h. After that, the cells were treated with DOX concentrations (0.25 and 1 µg/ml) alone or with DIL 20 µg/ml for 1 h. The medium was then removed, and cells were harvested with trypsin/EDTA. Following trypsinization, cells were washed with PBS, centrifuged and then the supernatant was discarded. The intracellular Rho 123 concentration was determined against a standard curve following the cell lysis with 2% (v/v) Triton X-100 by spectrofluorometery (Synergy HT; BioTek).

Fluorescence microscopic analysis of apoptosis

A stock solution of acridine orange (AO) Sigma A6014 (MO, USA; 1 mg/ml) was prepared according to manufacture protocol. The use of AO alone has been previously described as an inexpensive method to study lysosomal vacuolation, autophagy and apoptosis [13]. AO can penetrate the cell membrane and emits orange fluorescence on binding to RNA and green fluorescence on binding to DNA. This conduct varies as it binds to live, apoptotic or fixed permeabilized cells [14]. It has been confirmed that AO, because of green emission related to DNA alteration, and without the combination with another stain, can differentiate between different mechanisms of cellular death: apoptosis and necrosis [15]. In a recent study, Zhao *et al.* documented that AO staining is an efficient method to evaluate the cell apoptosis of zebrafish embryos [16].

This solution was aliquoted and stored at 4°C in the dark. With the dilution of the stock solution in PBS (1 µg/ml), a working solution was prepared and the cells were then seeded in the 12-well plates at a density of 5×10^4 cells per well, followed by overnight incubation under a CO_2 incubator. Subsequently, the cells were then exposed to different concentrations of DOX (0.25 and 1 µg/ml) alone or with DIL 20 µg/ml, followed by 24-h incubation. The cells were harvested by trypsin and centrifuged at 700× *g*. After that, 20 µl of AO was added to the cell plaque. The stock solution of dyes was then directly added to the culture media and before the imaging, the cells were incubated with the dye for 5–15 min at room temperature and the cells viewed under a fluorescence microscope (Moticam Pro 2828; AO × 400)

Nuclear cytoplasmic structural study

Giemsa stain (GS) was used to study the structural alterations in the cells under the effect of different concentrations of medication and the experiment followed the specified protocol (Procedure no.: GS-10 Sigma). The cells were arranged for staining by removing the media, washing each well two-times with PBS and fixing the cells with absolute methanol as a monolayer for 5 min. After that, the methanol was removed and the plate was air-dried. The cells were then stained with 1 ml of a 1:20 dilution of modified Giemsa reagent in PBS. At room temperature, the plate was incubated for 1 h, rinsed extensively with distilled water and air-dried. Slides were examined under light microscope (Olympus light microscope; BX51TF-Japan) and photographed with GS ×200 [17].

Results

Effect of DIL treatment on the cytotoxicity of DOX

The addition of DIL to DOX demonstrated a remarkable decline in surviving fraction values in comparison to with DOX alone. Also, as the concentration of DOX increased, the surviving fraction values decreased in a gradual way from 0.84 to 0.23 and from 0.79 to 0.17 for cells treated with 0.0156–1 µg/ml DOX simultaneously with DIL 10 and 20 µg/ml, respectively (Figure 1). Cytotoxicity was shown as a percentage of surviving fraction of treated cells by untreated control cells. DOX treatment alone showed IC_{50} value of 0.89 µg/ml and when DIL was added at 10 and 20 µg/ml, the IC_{50} of 0.28 and 0.21 µg/ml, respectively, was observed (Table 1).

Effect of DOX &/or DIL on the P-gp efflux of the fluorescent dye

As shown in Figure 2, MCF-7 cells poorly accumulated Rho 123 by showing P-gp-dependent efflux of the fluorescent dye. DOX 0.25 and 1 µg/ml increased dye accumulation in MCF-7 cells by 17- and tenfold, respectively, compared with Rho 123 (1 µg/ml). The addition of DIL (20 µg/ml) to DOX either 0.25 or 1 µg/ml restored and increased accumulation of dye significantly by 2.2- and 2.1-fold, respectively, compared with corresponding DOX treatment.

Effect of DIL on DOX cellular uptake

Table 2 and Figure 3 show DOX concentration (µg/1 × 10^6) in MCF-7 cells in presence and absence of DIL.

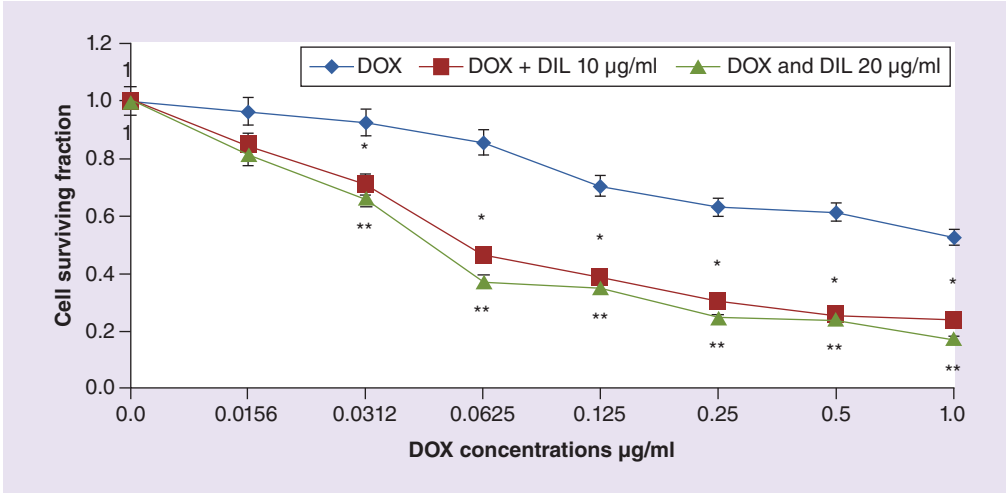

Figure 1. Effect of DOX and/or DIL (10 or 20 µg/ml) treatment on the growth of MCF-7 cells. Data are expressed as mean ± standard deviation of two experiments, each one in duplicate.
*p = 0.05 after the addition of DIL 10 µg/ml, compared with DOX alone
**p = 0.05 after the addition of DIL 20 µg/ml, compared with DOX alone.
DIL: Diltiazem; DOX: Doxorubicin.

Table 1. Effect of doxorubicin and/or diltiazem treatment on the growth of MCF-7 cells (IC_{50}).

Treatment IC_{50}	(µg/ml)
DOX	0.89 ± 0.8
DOX and DIL	(10 µg/ml) 0.28 ± 0.63† (supplied simultaneously)
DOX and DIL	(20 µg/ml) 0.21 ± 0.35†,‡ (supplied simultaneously)

Data are expressed as mean ± standard deviation of two experiments each one in duplicate.
†Remarkably different from the DOX at p-value < 0.05.
‡Insignificantly different between DIL either 10 or 20 µg/ml.
DIL: Diltiazem; DOX: Doxorubicin; IC50: the concentration of DOX necessary to generate 50% inhibition in the growth of cells.

Table 2. Effect of diltiazem treatment on doxorubicin cellular uptake in MCF-7 cells.

Treatment	DOX concentration (µg/1 × 10^6)
DOX 0.25 µg/ml	0.037 ± 0.01†
DOX 0.25 µg/ml + DIL 20 µg/ml	0.144 ± 0.13‡
DOX 1 µg/ml	0.113 ± 0.01†
DOX 1 µg/ml + DIL 20 µg/ml	0.302 ± 0.01‡

†The growing cells were treated with DOX and/or 20 µg/ml DIL for 48 h. Cells washed once with phosphate-buffered saline and were harvested, counted and resuspended in 1 ml of 20% DMSO. The DMSO cell suspensions were centrifuged and the clear supernatant was collected for the assay. Data are expressed as mean ± standard deviation of the experiment (n = 2).
‡Significantly different from corresponding DOX at p-value < 0.05.
DIL: Diltiazem; DMSO: Dimethylsulfoxide; DOX: Doxorubicin.

DOX cellular concentration were 0.037 and 0.113 (µg/1 × 10^6) after respective treatment with 0.25 and 1 µg/ml DOX. The addition of DIL at a dose of 20 µg/ml with DOX increased its concentration by 3.9- and 2.7-fold, respectively.

Fluorescence microscopic analysis of apoptosis & nuclear cytoplasmic structural after DOX &/or DIL treatment

Photomicrographs of MCF-7 breast cancer cells treated with DOX and/or DIL displayed a control with a confluent monolayer of typical spindle-shaped cells with well-defined outline and abundant cytoplasm (Figure 4A). Cells treated with DIL 20 µg/ml showed few shrunken cells that were markedly increased on treatment with DOX 0.25 µg/ml only few cells retained their shape (Figure 4B & C). In addition, cells treated with higher dose of DOX

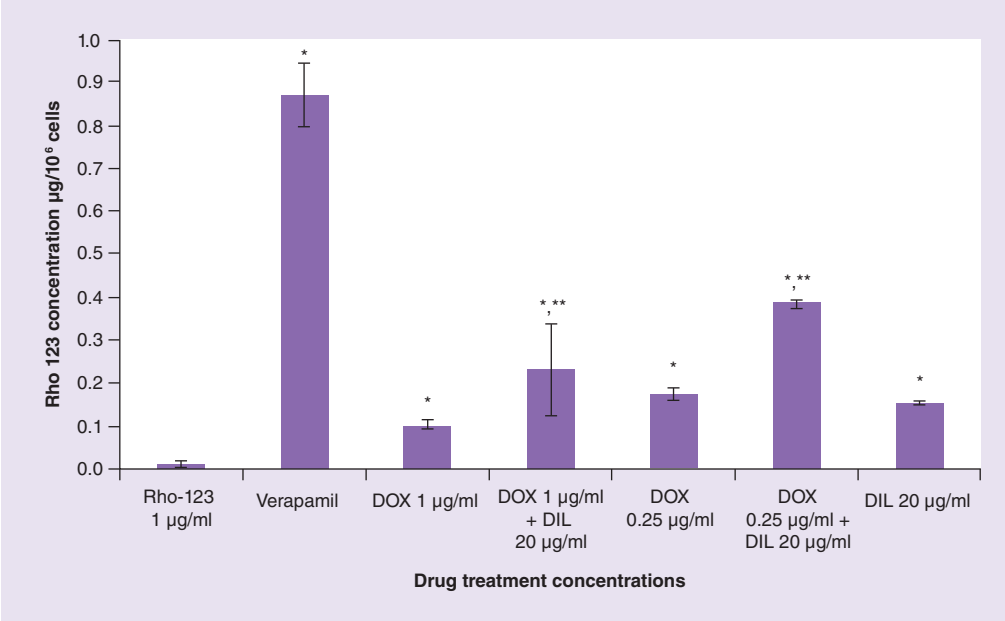

Figure 2. MCF-7 cells were exposed to 1 µl/ml rhodamine 123 for 30 min at 37°C. Rho 123 accumulations were quantified by spectrofluorometry after washing. Data are expressed as ratio of fluorescent dye accumulation in MCF-7 cells with the means ± standard deviation of two experiments each one in duplicate.
*$p < 0.05$ compared with Rho 123.
**Compared with corresponding DOX.
DIL: Diltiazem; DOX: Doxorubicin; Rho 123: Rhodamine 123.

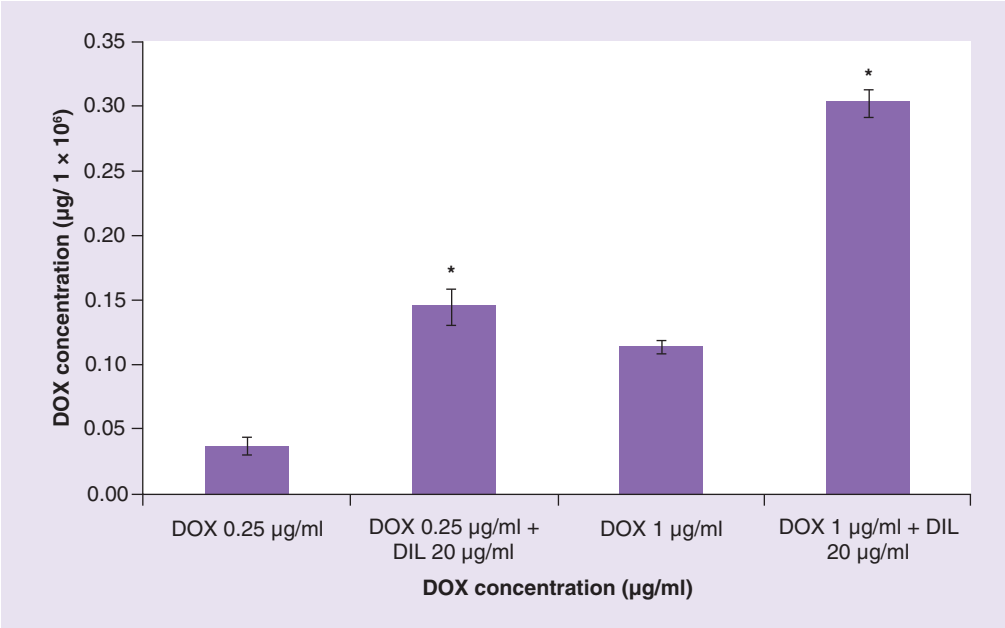

Figure 3. Effect of diltiazem treatment on doxorubicin cellular uptake in MCF-7 cells. Cells were treated with DOX and/or with 20 µg/ml DIL for 48 h, followed by washing with phosphate-buffered saline. The cells were then harvested, counted and resuspended in 1 ml of dimethylsulfoxide. The dimethylsulfoxide cell suspensions were centrifuged and the clear supernatant was collected for the assay with the means ± standard deviation of two experiments each one in duplicate.
*$p < 0.05$ compared with corresponding DOX.
DIL: Diltiazem; DOX: Doxorubicin.

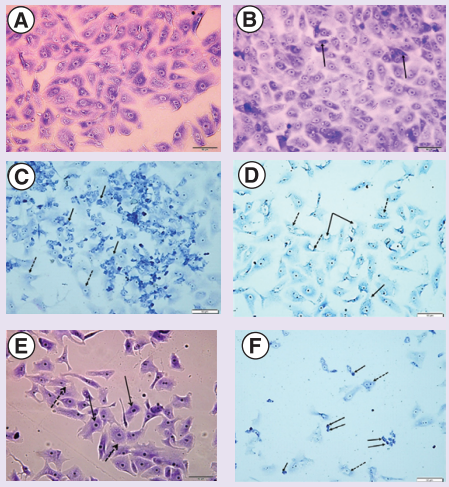

Figure 4. Photomicrographs of MCF-7 breast cancer cells. (A) Control. **(B)** Cells treated with DIL 20 μg/ml. **(C)** Cells treated with DOX 0.25 μg/ml. **(D)** Cells treated with DOX 0.25 μg/ml + DIL 20 μg/ml. **(E)** Cells treated with DOX 1 μg/ml. **(F)** Cells treated with DOX 1 μg/ml + DIL 20 μg/ml (GS ×400). DIL: Diltiazem; DOX: Doxorubicin; GS: Giemsa stain.

1 μg/ml revealed condensed nuclear chromatin and perinuclear halo and some cells showed cytoplasmic vacuoles (Figure 4E). The addition of DIL to DOX induced multiple cytoplasmic vacuoles (more enhancements toward apoptosis) of different sizes and more nuclear fragmentations were also noted in the field (Figure 4D & F).

Figure 5 showed cells stained with vital stain (AO) and visualized by florescent microscope. In the control group, cells revealed green intact nuclei of viable cells (arrows). In case of cells subjected to DIL 20 μg/ml, some nuclei revealed dense green areas of chromatin condensation presenting early apoptosis (dashed arrows). The increase in these cells was DOX dose-dependent and on simultaneous use of DOX and DIL. DOX 1 μg/ml and DIL-treated cells demonstrated a reduced count in the field, with condensed chromatin.

Discussion

Our study was focused on investigating whether DIL (a calcium channel blocker [CCB]) can overcome the resistance to DOX therapy as well as enhancement of its cytotoxic effects against the growth of MCF-7 human breast cancer cell line. The idea of using CCBs with chemotherapy drugs for the treatment of cancer is supported by many literatures (e.g., vincristine and adriamycin), which results in enhanced cytotoxicity in multiple cell lines (e.g., glioma, prostate and lung) [18,19].

In our results, DIL dose showed no direct cytotoxic activity against breast cancer cells by DIL alone, however, it improved the DOX uptake into the tumor cells. A good correlation was reported between the high cellular level of DOX in MCF-7 cells in presence of DIL and the increased in the cytotoxicity of DOX was observed by a decrease in IC_{50} (Figure 1 & Table 1). In agreement with our result, Al-Shabanah *et al.* reported that the DIL potentiates DOX cytotoxicity and cellular uptake in Ehrlich ascites carcinoma cells in mice. They found that pretreatment with DIL increased the survival rate of Ehrlich ascites carcinoma-bearing mice [8]. In addition, Chiu *et al.* compared DIL and nifedipine with verapamil in their ability to reverse DOX resistance in A549/D16 human lung cancer cells lines [20]. These results indirectly agree with the work of Nguyen *et al.*, where they found that concurrent treatment of DOX with calcium caused decline in cytotoxic effect of DOX [21]. MDR continues to be a major clinical obstacle to effective cancer chemotherapy. The hallmark of MDR is the expression of P-gp, which acts as a transmembrane drug exporter [22]. It plays a role in the development of drug resistance in several cancers including those of the lung, breast, prostate, ovarian cancers and myeloid leukemias as well as childhood neuroblastoma [23,24].

To examine the function of P-gp in altering the usefulness of the agents, we studied the effect of DOX and/or DIL on the P-gp efflux of the fluorescent dye. We used verapamil as a reference that inhibits the activity of P-gp [25]. A significant increase in accumulation of Rho 123 dye in MCF-7 cells has been observed after the addition of DIL to DOX by more than eightfold (Figure 2) indicating inhibition of P-gp efflux pump that leads to more accumulation of DOX with more cytotoxicity. These results have been further confirmed by the observed increase in DOX cellular uptake in MCF-7 cells after simultaneous treatment with DIL in a dose-dependent manner (Figure 3 & Table 2). However, a low dose of DOX showed more accumulation of Rho 123 in MCF-7 cells than a high dose. This result is consistent with work of Riganti *et al.*, where they found two repeated low doses of DOX is

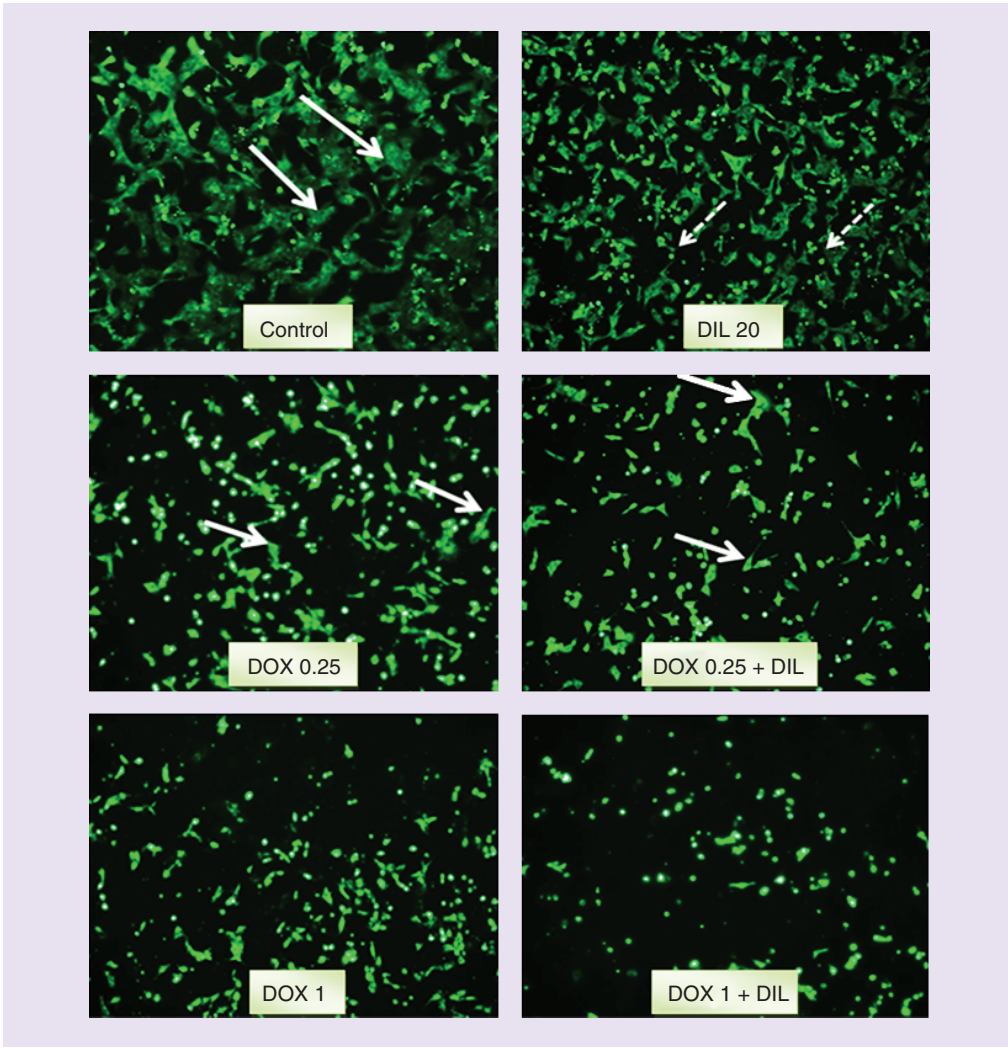

Figure 5. Photomicrographs of MCF-7 breast cancer cells stained with vital stain (acridine orange) and visualized by fluorescent microscope. In the control group, cells revealed green intact nuclei of viable cells (arrows). In case of cells subjected to DIL 20 μg/ml, some nuclei revealed dense green areas of chromatin condensation presenting early apoptosis (dashed arrows). The DOX 1 μg/ml and DIL-treated cells showed few cells in the field, all of which revealed condensed chromatin (GS ×400).
DIL: Diltiazem; DOX: Doxorubicin.

more effective than a single high dose against tumors overexpressing P-gp [26]. They reported that the two repeated low-dose treatment reduced cell proliferation and increased the cleavage of caspase 3, suggesting that it activated the proapoptotic machinery in resistant cells. In addition, they showed a significant increase of intracellular ROS levels paralleled by typical signs of oxidative damages, such as a decrease in Gluthathione levels and increased lipid peroxidation. It has been reported that low doses of liposomal DOX produced greater clinical benefits in patients with breast metastatic cancers than standard chemotherapy protocols based on maximum tolerated doses [27].

In the present study, the increased DOX cellular uptake inside MCF-7 cells can be explained based on inhibition of P-gp and MDR. In many studies, the treatment with cytotoxic agents directly effects on MDR-1 promoter [28,29].

These agents lead to MDR-1/P-gp expression by transcriptional and translational activation. Moreover, chemosensitization of tumor cells is one of objectives for research to find an adjuvant therapy working via inhibition of P-gp, which results to increase the concentration of chemotherapeutic agents intracellularly [10,11].

Light microscopic study confirmed the biochemical data in MCF-7 cells, where a shrunken appearance of the cells with condensed nuclear chromatin after DOX treatment has been observed. In addition, a marked condensation of the nuclear chromatin and perinuclear halo were detected with some cells showing cytoplasmic vacuoles when

DOX concentration was increased (Figure 4C & E). After the addition of DIL, highly condensed nuclear fragments and a rim of cytoplasmic were scattered (Figure 4F).

These results may suggest that the combination treatment of DOX and DIL induces apoptosis of the MCF-7 cells in a time-dependent manner, which may lead to a reduced dose of chemotherapy and consequently reduced side effects. Hence, depending on the current results, it can be said that the DIL inhibits P-gp when it is provided in combination with DOX, followed by increase in the DOX cellular uptake. However, the exact reasons behind the cytotoxicity improvement by CCBs are unclear, because of the difficulty in assessing the relation of drug accumulation and cytotoxicity. This is because drug accumulation might not be the only factor that influences the cytotoxicity of chemotherapeutic agents. Studies are needed in which basic questions concerning tumor enhancement or suppression functions can be addressed using robust and clinically relevant cancer models.

Ikeda *et al.* reported that blockage of CCB-attenuated DOX induced cardiomyocyte apoptosis by suppression the intracellular Ca^{2+} abnormalities through suppression Ca^{2+}/calmodulin-dependent protein kinase II, which might be beneficial in DOX-induced cardiomyopathy. Therefore, together with present work, it seems interesting to use DIL, a CCB with DOX to increase its activity and decrease toxicity [30]. The use of these models to investigate DOX-induced cardiotoxicity will hopefully help to reduce the gap in basic research. Patient-based studies, other than those focused on preclinical models, may contribute toward the field. Until now, early detection of cardiotoxicity and mechanisms of pathophysiology have not been examined.

In summary, this study suggests the possible novel use of DIL to enhance the antitumor activity of DOX, allowing its dose and consequently the serious side effects, to be reduced.

Conclusion

CCB, DIL treatment enhanced DOX cytotoxic effect and reduced MDR. As such, increasing the drug accumulation inside cancer cells may predict a good chance to improve the cytotoxic activity of DOX. Such improvement may lead to a decrease in the dose and, consequentally, its side effects. This could result in improvements in the general performance of cancer patients and could limit the cost of treatment.

Summary points

- Doxorubicin (DOX) has been considered an effective drug against breast cancer, to which chronic cardiotoxicity and multidrug resistance are potential limiting factors.
- Resistance to chemotherapeutic drugs may be considered one of the more important reasons for cancer return, deterioration and metastasis.
- Investigating whether diltiazem can overcome the resistance to DOX therapy.
- Enhancement of DOX cytotoxic effects against the growth of MCF-7 human breast cancer cell line.
- Diltiazem could improve the cytotoxicity of DOX against the growth of human breast cancer cells (MCF-7 cell line).
- Increasing the drug accumulation inside cancer cells may predict a good chance to improve the cytotoxic activity of DOX.

Author contributions

AM Osman, HS Al-malky and AA Al-Qahtani shared the experimental work and WS Ramadan did cytopathological investigation, and others read and shared their interpretation of data and contributed in the writing of manuscript.

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

This study was approved by the Institutional Ethical Committee of King Abdulaziz University Hospital (reference no. 190-18).

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- **Demonstrates the role of P-gp expression in MDR and development of drug resistance.**

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