Original Research Article

Potentiation of Doxorubicin Cytotoxicity by Calcium Channel Blocker Verapamil in Human Breast Cancer Cells

Running title: Potentiation of the anticancer activity of doxorubicin by verapamil.

Abstract

Background: Breast cancer is a main cause of morbidity and mortality in females in the Arab universe and classified first among Saudi Arabia. Doxorubicin (DOX) is one of the treatment protocol modalities used in treatment of solid tumors such as Hodgkin’s and non-Hodgkin’s lymphoma, sarcomas, breast cancers, and lung cancers. Its medical effectiveness has been, however disadvantaged by harmful effects, including chronic cardiotoxicity and development of DOX resistance. Aim: Therefore, this work is directed to potentiate the cytotoxic activity of DOX and to ameliorate the tumor cell resistant by using a calcium channel blocker Verapamil (VER). Methodology: To verify this, we have examined DOX cytotoxicity, apoptosis, and cell cycle phase distributing effects of DOX against MCF-7 cell line in presence and absence of calcium channel blocker Verapamil. DOX cellular uptake and P-gp activity has also been examined. Results: Addition of VER enhanced the cytotoxic effect of DOX against the growth of human Breast cancer cell line (MCF-7) cells with IC50 13 µg/ml, compared to 36 µg/ml when DOX used alone. Moreover, combination therapy significantly increased percentage of early apoptosis and cells arrested in G0/G1 phase when compared to DOX alone. In addition, VER significantly increased DOX cellular uptake through inhibition of P-gp activity. Conclusion: VER treatment improved the cytotoxic activity of DOX against the growth of MCF-7 cells and increased its cellular uptake through inhibition of P-gp activity.

Key words: Doxorubicin, Verapamil, cytotoxicity, potentiation, breast cancer cells.
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 have been limited due to their potential to cause cardiac toxicity (4). In order to circumvent this effect, several strategies have been used, these included, for example, 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 found that agents with antioxidant activity decreased DOX cardiotoxicity (6,7). So, larger dose can be used for increasing the cytotoxic activity of doxorubicin. One of these agents is diltiazem, a calcium channel blocker which showed synergistic activity with doxorubicin against 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 P-gp activity have also been investigated in the presence and absence of Verapamil.

Materials and Methods

This research was done at pharmacology department, Faculty of Medicine, KAU, Jeddah in collaboration with Pharmacology Unit, National Cancer Institute Cairo University. It started September 2016-2018.
Drugs and Chemicals:

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

Cells and Cell Culture:

Human breast cancer cell line MCF-7 used in this study. Cells 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.

Methods:

Assessment of Cytotoxicity:

Cytotoxicity determined using sulforhodamine-b (SRB) method as previously described by skehan et al.(9). Cells seeded in 96 well microtiter plates at concentration of 40 x10^3 cells/well in DMEM medium. The cells kept to attach for 24 hours, then the cells incubated with various concentrations of DOX and/or VER for 48 hours in the following 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. The supernatant was discarded, then the plates 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 removed by washing four times with 1X dye wash solution and plates air dried. Add 200μl SRB solubilization buffer 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:

\[
\text{Surviving fraction} = \frac{\text{optical density of treated cells}}{\text{optical density of control cells}}.
\]

IC\text{50} (the concentration of DOX necessary to produce 50% inhibition of cells growth) calculated from linear 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.

**Apoptosis assay:**

Cells seeded in T25 flasks at cell density of 5-8 x10\(^5\) cells/flask in DMEM media and incubated for 24 hours at humidified air containing 5% CO\(_2\). After that the cells incubated with DOX concentration (5 and 20µg/ml) alone and combined with verapamil (24.5 ug/ml) for 48 hours (3 flasks for each concentration). Medium removed and the flasks washed with PBS and the cells harvested with Trypsin/EDTA. After trypsinization, cells 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 added to each sample and process by flow cytometry (NAVIOS Beckman Coulter, U.S.A.).

**Cell Cycle Analysis:**

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

**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 $\times 10^6$ 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 removed, wells washed with PBS, then the cells harvested with trypsin/EDTA. Following trypsinization, cells washed with ice PBS and counted. For drug uptake analysis, cells (1 x $10^6$) resuspended in 0.3 N HCl in 50% ethanol and digested by homogenized in ultrasound bath. The 0.3 N HCl in 50% ethanol cell suspensions centrifuged at 14000 rpm for 10 minutes and collected. The DOX fluorescence intensity of the supernatant measured by a spectrofluorometer (Synergy HT, BioTek, USA) at excitation and emission wavelengths of 485 nm and 593 nm, respectively to determine DOX concentration.

**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 seeded in T25 flasks at cell density of 5-8 $\times 10^6$ cells/flask in DMEM medium and proceed as above. Add 2.62 µM (100 µl from working solution) of Rhodamine-123 and keep...
it in CO₂ incubator at 37°C for one hour. Cells incubated with DOX concentrations alone and combined with verapamil (24.5 µg/ml) for 10 minutes. Cells harvested and washed once with iced PBS. For p-glycoprotein analysis, cells (1x10⁶) suspended in one ml of PBS to each sample and shaking. Lysed cells were analyzed by spectrofluorometer at wavelength (485-590 nm).

**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 means. Differences were considered significant at P <0.05.

**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 Figure1).
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, (Figure 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, (figure 2).

Effect of DOX and/or VER on cell cycle phase progression of MCF-7 cells

Combination treatment of 5 µg/ml DOX with VER 24.5 µg/ml showed a significant increase in percentage of cells in G0/G1 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 increased percentage of cells in G0/G1 phase to 57.40% compared to 16.10% of DOX alone (figure 3). Treatment with VER alone, showed a preferential block in G0/G1 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 show DOX level in MCF-7 cells after combination with VER 24.5 µg/ml. DOX level was (0.228 µg/10⁶ 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/10⁶ cells) and in presence of VER it was (1.740 µg/10⁶ cells).
**Effect of DOX and/or VER on the P-glycoprotein Efflux of the fluorescent dye**

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 dye significantly (0.794 µg/10^6 cells) and (0.651 µg/10^6 cells), respectively , compared to corresponding DOX treatment.

**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 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 whether VER which is one of the calcium channel blocker would enhance DOX cytotoxic effect against the growth of human breast cancer cell line MCF-7 and the possible modulatory mechanisms were also explored by studying the changes of apoptosis induction, cell cycle phase distribution, DOX cellular uptake and P-gp activity after treatment with different DOX concentrations in the presence and absence of...
Verapamil. The current study showed that treatment of MCF-7 cells with 24.5\(\mu\)g/ml VER, significantly enhances the cytotoxic activity of DOX against the growth of MCF-7 cells by 2.8 fold decrease in survivors compared with cells treated with DOX alone (table 1). The 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.

The current study showed that treatment of the MCF-7 cells with VER and DOX had a significant increase of the cells the arrested in G\(0/G1\) phase compared with cells treated with DOX alone (figure 3). Previous studies on the effect of VER on the cell cycle of many cell lines including MCF-7 cells, demonstrated the ability of VER to block the cells inflowing from G\(0/G1\) in - to S phase transition, resulting in a concentration-dependent accumulation of cells in G\(0/G1\) phase (21). It's worthy noted that cell cycle arrest in G\(0/G1\) 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\(0/G1\) phase and decline in S phase in human colonic tumor. They 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\(0/G1\) phase cells, suggesting that VER could block the transformation of human colonic cells from G\(0/G1\) phase into S phase. It is worth mentioning 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 (figure 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 previous study of Balakrishnan et al. (25) who showed that VER increase 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 proteins, amongst which is 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. In the present study, the P-glycoprotein showed that treatment with VER lead to almost 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 measure 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.

In conclusion Verapamil chemosensitizes DOX cytotoxicity against the proliferation of MCF-7 humane breast cancer cells. This could be explained by induction of apoptosis, enhanced DOX cellular uptake and inhibition of P-glycoprotein activity.

**Compliance with ethical standards**

**Competing interests**

The authors have declared that they have no competing interests

**Ethical approval**

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

All authors approved the publication of this article

Authors’ contribution

Availability of data and material

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

References


Table 1: Effect of Doxorubicin and/or Verapamil on the growth of MCF-7 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IC_{50} (µg/ml)</th>
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<tbody>
<tr>
<td>Doxorubicin</td>
<td>36 ± 0.025</td>
</tr>
<tr>
<td>Doxorubicin and Verapamil</td>
<td>13 ± 0.067{a}</td>
</tr>
</tbody>
</table>

Each data is mean ± S.E.M of two independent experiments each one in triplicate.

IC_{50}: the concentration of Doxorubicin necessary to produce 50% inhibition of cell growth.

{a} Significantly different from MCF-7 cells at P-Value < 0.05.
Figure 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 two 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.

Figure 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. The independent experiments was repeated twice each one in duplicate.
Figure 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.

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DOX concentration (µg/10⁶ cells)</th>
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</thead>
<tbody>
<tr>
<td>DOX (5µg/ml)</td>
<td>0.228± 0.01</td>
</tr>
<tr>
<td>DOX (5µg/ml)+ VER (24.5 µg/ml)</td>
<td>1.272± 0.03</td>
</tr>
<tr>
<td>DOX (20µg/ml)</td>
<td>0.842±0.02</td>
</tr>
<tr>
<td>DOX (20µg/ml)+ VER (24.5 µg/ml)</td>
<td>1.740±0.05</td>
</tr>
</tbody>
</table>

The MCF-7 cells were treated with DOX and /or with VER. The cells were analyzed after 48 hours of exposure to drugs. Data is the mean ± S.E.M of two independent experiments each one in duplicate. * 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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Rh 123 concentration (µg/10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rh 123 (100µl/ml)</td>
<td>0.01±0.01</td>
</tr>
<tr>
<td>VER (24.5 µg/ml)</td>
<td>0.548±0.04 a</td>
</tr>
<tr>
<td>DOX (5 µg/ml)</td>
<td>0.104±0.02 a</td>
</tr>
<tr>
<td>DOX (5 µg/ml)+ VER (24.5 µg/ml)</td>
<td>0.794± 0.05 a,b</td>
</tr>
<tr>
<td>DOX (20 µg/ml)</td>
<td>0.164±0.01 a</td>
</tr>
<tr>
<td>DOX (20 µg/ml)+ VER (24.5 µg/ml)</td>
<td>0.651±0.03 a,b</td>
</tr>
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</table>

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