PHARMACOKINETIC AND PHARMACOLOGIC STUDY OF TWO P-GLYCOPROTEIN MODULATING AGENTS COMBINED WITH DOXORUBICIN

Samia S. Sokar, Thanaa A. El-Masry, Magda E. El-Sayad & Sherin R. El-Afify*
Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Tanta, Egypt

ABSTRACT

Overexpression of the transmembrane drug efflux pump p-glycoprotein (p-gp) is one of the major mechanisms by which cancer cells develop multidrug resistance (MDR) against anticancer drugs including doxorubicin.

Objectives: This study was carried out to assess the possible effects of two p-gp modulating agents, verapamil and tamoxifen on cytotoxicity of doxorubicin in Ehrlich ascites carcinoma (EAC), the probable mechanism(s) underlying the possible interaction between verapamil and tamoxifen with doxorubicin was investigated.

Methods: To achieve these objects the study was divided into two parts, in vivo study which was carried out on Swiss albino mice and in which the following parameters were determined: Time course effect of verapamil (5mg/kg) and tamoxifen (1mg/kg) on doxorubicin (10mg/kg) concentration in Solid Ehrlich Carcinoma (SEC) and semi quantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis of multiple drug resistance gene. The in vitro study is the second part, it was carried out on Ehrlich ascites carcinoma cells and in which evaluation of doxorubicin cytotoxic activity in the presence and absence of verapamil and tamoxifen and detection of p-glycoprotein activity on Ehrlich Ascites Carcinoma (EAC) using rhodamine 123 uptake and efflux techniques were investigated. In both in vivo and in vitro studies groups were classified into: group1 (saline), group2 (ethanol), group3 (doxorubicin), group4 (verapamil), group5 (tamoxifen), group6 (a combination of doxorubicin and verapamil) and group7 (a combination of doxorubicin and tamoxifen).

Results and Conclusions: It was found that both verapamil and tamoxifen caused a significant increase in doxorubicin cytotoxicity in EAC. The modulatory effect of both verapamil and tamoxifen on doxorubicin cytotoxicity was due to the ability of both verapamil and tamoxifen to cause a significant increase in doxorubicin intracellular concentration in SEC. Increased sensitivity of cancer cells to doxorubicin caused by verapamil and tamoxifen was explained on the basis of inhibition of p-gp function. The mechanism by which verapamil caused an inhibition of p-gp transport activity involves downregulation of mdr1a gene expression in EAC cells. On the other hand tamoxifen inhibited p-gp transport activity via a mechanism independent on mdr1a gene expression.

Key Words: Mammary tumor; tamoxifen; doxorubicin; p-glycoprotein; verapamil.

1. INTRODUCTION

Chemotherapy plays an important role in the management of cancer. As an important example, doxorubicin, an anthracycline antibiotic is considered among the most active chemotherapeutic agents [1]. However, clinical usefulness of doxorubicin in treatment of cancer is often limited by the development of a type of drug resistance known as multidrug resistance (MDR) [2]. MDR is a term used to describe a phenomenon characterized by the ability of some tumors to exhibit simultaneous resistance to a number of structurally and functionally unrelated chemotherapeutic agents [3]. The mechanism of MDR is not fully understood, but it is frequently associated with overexpression of membrane bound proteins that efflux drugs from the cells thus decreasing the intracellular concentration of the drugs [3]. Attention has been focused on agents to reverse MDR and so enhance the response of tumors to chemotherapeutic agents.

Although hundreds of compounds have been found to be able to modulate MDR in vitro, their clinical application was limited by dose limiting toxicities [4]. Accordingly, searching for compounds able to modulate the MDR phenotype and have low toxicity is an important issue.

Verapamil is a calcium channel blocker widely used in cardiovascular therapy as anti-arrhythmic agent; and in the treatment of hypertension, angina and certain cardiomyopathies [5]. Cardiovascular diseases are considered one of the most common types of diseases that can easily affect cancer patients. Accordingly, both verapamil and chemotherapeutic drugs could be concomitantly administered by cancer patients. Tamoxifen is a non-steroidal anti-estrogen which is commonly employed in the treatment of advanced as well as early breast cancer as a hormonal therapy [6], so patients suffering from breast cancer could receive a combination of both tamoxifen and chemotherapy in their treatment regimens. Therefore, this study aimed at investigating the possible role and mechanisms of two p-glycoprotein modulating agents, verapamil and tamoxifen on the efficacy of the cytotoxic
drug, doxorubicin on mouse mammary carcinoma model: Solid Ehrlich Carcinoma model (SEC) and Ehrlich Ascites Carcinoma model (EAC).

2. MATERIAL AND METHODS

Animals

Adult female Swiss albino mice weighing 18 - 20 gm purchased from the animal house of the National Institute of Ophthalmology, Cairo, Egypt were used. Experimental procedures started after one-week acclimatization period. Animals had free access to food (standard mouse chow) and water (ad libitum).

Cell line

A line of Ehrlich Ascites Carcinoma (EAC) cells was used. The EAC tumor cells were obtained from the Pharmacology and Experimental Oncology Unit of the National Cancer Institute (NCI), Cairo University, Egypt. EAC appeared firstly as a spontaneous breast cancer in female mice.

Drugs

Verapamil: A calcium channel blocker (Isoptin, Knoll, Ludwigshafen)
Doxorubicin: Cytotoxic drug that belongs to the anthracycline group (Manufactured by Tedec-Meiji Farma and imported by Al Delta Pharm. Trading, Egypt).
Tamoxifen: A non-steroidal antiestrogen (Memphis chemical company, Egypt).

Part I: Pharmacokinetic study

Tumor model

A model of Solid Ehrlich Carcinoma (SEC) was used for in vivo experiments, where $2.5 \times 10^6$ EAC cells were implanted subcutaneously (S.C.) into the right thigh of the lower limb of mice. A palpable solid tumor mass developed within 12 days.

Assessment of the time-course effects of verapamil and tamoxifen on doxorubicin intracellular concentration of SEC using high performance liquid chromatographic assay (HPLC)

Drug treatments

Treatment with either verapamil in a dose of (5mg/kg, I.P.) or tamoxifen in a dose of (1mg/kg, I.P.) or control vehicles(saline and ethanol) were initiated one day before the implantation of the EAC cells, and continued for one month on the basis of daily I.P. injections. However, Doxorubicin (10mg/kg) was administered I.P as a bolus dose on day 30 after tumor inoculation. SEC tissue samples were taken from mice at 1, 3, 24 and 48 hours from the dose of doxorubicin.

Determination of intracellular doxorubicin concentration of SEC

Intracellular doxorubicin concentration of SEC was determined using high performance liquid chromatographic assay (HPLC) [7]. The chromatographic equipment comprised a Kontron pump (Model 420) coupled to a fluorescence detector (Shimadzu, Model RF-10AXL). Data collection was accomplished with a Varian integrator (Model 4270). Detection being accomplished at 470 excitation wave length and 555 emission wave length.

Briefly 10 gram of SEC tumor samples were weighed and homogenized in 100 mL saline to produce 10% homogenate. SEC tumor homogenate was diluted with 5 mL 1/15 M phosphate buffer (pH 7.4± 0.1). Protein denaturation and precipitation procedures were carried out for the tumor homogenate samples before injection into the chromatograph as follows, 200μl of a 50:50 (v/v) mixture of methanol and 40% ZnSO4 were added to 150μl of sample. After 1 min of vigorous vortex mixing, the fluid was centrifuged at 1500g for 10 min. The supernatant obtained was injected directly into the chromatograph with a 200μl fixed volume.

Calculations

The concentration of doxorubicin in tumor homogenate samples was expressed as μg/g tissue using a standard curve. The following equation is used:

$$Y = 17661.14 + 22387.1X$$

Where:

22387.1: is the slop of doxorubicin standard curve.
17661.14: is the intercept of doxorubicin standard curve.
X: is the concentration of doxorubicin in μg/g tissue .
Y: area under curve (AUC).
Part II: Pharmacologic study
Cell culture
Ehrlich Acites carcinoma cells (2.5 x 10^5) were grown as a monolayer in RPMI 1640 culture medium supplemented with 10% fetal calf serum, 1% penicillin and 1% streptomycin. The cells were incubated in a humidified atmosphere containing 5% carbon dioxide at 37°C.

Determination of the concentration of doxorubicin that produces 50% growth inhibition in EAC cell line (IC50) by using cytotoxic assay
EAC cells were seeded into flat bottom 96 well plate at concentration of 5x10^5 cells / well. Each well contains 400μL of RPMI 1640 culture medium supplemented with 10% fetal calf serum. Cells were grown for 24 hours [8]. To determine the concentration of doxorubicin that produces 50% of growth inhibition, cells were incubated for 48 hours with ascending concentrations of doxorubicin. The doxorubicin concentrations are (0.5 μg/mL, 1 μg/mL, 1.5 μg/mL, 2 μg/mL, 2.5 μg/mL, 3 μg/mL, 3.5 μg/mL, 4 μg/mL, 4.5 μg/mL, 5 μg/mL) then 40 μL of (3-(4,5 dimethylthiazol-2-yl) 2,5 diphenyl tetrazolium bromide) MTT dye was added. The plate was incubated at 37°C for 4 hours. The plate was left for a few minutes at room temperature. The absorption was measured at 450nm using spectrophotometer [9]. Growth inhibition percentage was calculated according to the following formula [10]:
\[
\text{Growth inhibition} \% = \left( \frac{1}{1 - \text{AT}} \right) \times 100
\]
AT: absorption of treated cells
AU: absorption of untreated cells

Assessment of the influence of verapamil and tamoxifen on cytotoxicity of doxorubicin
Either verapamil in a concentration of (1 μmol/L, 3 μmol/L, 10 μmol/L, 30 μmol/L) or tamoxifen in a concentration of (1 μmol/L, 3 μmol/L, 10 μmol/L, 30 μmol/L), in combination with the measured IC50 of doxorubicin were incubated with EAC cells for 24 hours. All experiments were done in triplicate.

Determination of the sensitization ratio
Sensitization ratio is determined to assess the stronger chemosensitizer. Sensitization ratio is determined by dividing the absorption of the IC50 of doxorubicin in the absence of the chemosensitizer by the absorption of IC50 of doxorubicin in the presence of maximum doses chemosensitizer [10].

Assessment of p-glycoprotein activity in EAC cells using rhodamine 123
Assessment of p-glycoprotein activity in EAC cells was done by using rhodamine 123 accumulation and retention techniques [11, 12].

Preparation of EAC cell suspension
The ascites fluid (containing EAC cells) was collected on the seventh day after I.P. transplantation of 2.5x10^6 cells in adult female Swiss albino mice. The viability of EAC cells was confirmed by trypan blue dye exclusion method to be more than 95%. Cell viability was determined by trypan blue dye exclusion technique [13].

Rhodamine 123 accumulation
In order to measure rhodamine 123 uptake by EAC cells, the tested drugs, verapamil (50μmol/L) and tamoxifen (10 μmol/L) or their control vehicles were added to 5 mL aliquots of 2x10^6 /mL EAC cell suspension. This was followed by addition of rhodamine 123 (200 ng / mL) to each drug-treated or control vehicle treated cell suspension, and incubation was done for 30 minutes at 37°C. Uptake was then stopped by putting samples on ice for 10 minutes. Cells were then pelleted by centrifugation at 1300 rpm for 5 minutes at 4°C, washed twice with ice-cold sterile saline, and then lysed in 3 mL of 0.8 % Triton X-100 at 4°C for 15 minutes. Centrifugation was then done at 3000 rpm for 10 minutes to separate the cell debris from the supernatant (containing rhodamine 123). The fluorescence intensity of the supernatant from each cell suspension was then measured at 485 nm and 535 nm for excitation and emission respectively using spectrofluorimeter (Schimazu RF 5301-PC, Japan).

Rhodamine 123 retention
Aliquots of 5 mL of 2x10^6 cells / mL EAC cell suspension previously treated with verapamil (50μmol/L) and tamoxifen (10 μmol/L) or their control vehicles were incubated with rhodamine 123 (200 ng / mL) for 30 minutes at 37°C, to load rhodamine 123 into EAC. Loading the EAC cells with rhodamine 123 was then stopped by transferring samples on ice for 10 minutes. Cells were then pelleted by centrifugation at 1300 rpm for 5 minutes at 4°C, washed twice with ice-cold sterile saline. Efflux of rhodamine 123 from EAC cells was initiated by resuspending the cell pellets in 5 mL of Hanks balanced salt solution (HBSS) solution containing the tested drugs.
(verapamil 50 μmol/L, tamoxifen 10 μmol/L and control vehicles) without adding rhodamine 123, and incubation for 30 minutes at 37°C. Efflux was then stopped by transferring samples on ice for 10 minutes. Cells were lysed in 3 mL of 0.8 % Triton X-100 at 4 C0. Centrifugation was then done at 1300 rpm for 5 minutes at 4C0, and the fluorescence intensity of the supernatant from each cell suspension was then measured at 485 nm and 535 nm for excitation and emission respectively using spectrofluorimeter (Schimazu RF 5301-PC, Japan).

Calculations
Data were presented as mean ± SD of fluorescence intensities of drug-treated or control vehicle-treated samples. The following equation was used for calculating the p-glycoprotein function in EAC cells [14]

% Function of P-glycoprotein = (Rhodamine 123 accumulation- Rhodamine 123 retention/ Rhodamine 123 accumulation) x100

Assessment of multiple drug resistance 1a gene in EAC by reverse transcriptase polymerase chain reaction (RT-PCR)
Drug treatments
Female Swiss Albino mice were divided into four groups each group consisting of seven animal. All mice was injected I.P. with EAC cells (2x10⁶ cells / mouse). Twenty four hours later, Group (I) was injected I.P with saline and served as the saline control group. Group (II) was injected I.P with ethanol and served as the ethanol control group. Group (III) was injected I.P with verapamil (20 mg/kg). Group (IV) was injected I.P with tamoxifen (20 mg/kg), both verapamil and tamoxifen were injected once per day for 10 days, control groups received equal volumes of saline or ethanol. All mice of the four groups were injected I.P. with a single dose of doxorubicin (20 mg/kg) in the tenth day of injection. After 6 hours of doxorubicin injection, EAC cells were withdrawn from each group and washed twice with PBS and prepared for RT-PCR

Extraction and isolation of total RNA from EAC: Total RNA (TRNA) was isolated from EAC using TRIZOL reagent according to the manufacturer instructions. This method is an improvement to the single step RNA isolation method development by Chomczynski and Sacchi (1987) [15]. Expression of mdr1a gene was detected using one step RT-PCR procedure where both cDNA synthesis and PCR steps were performed in a single tube. Primers for mdr1a gene and β actin gene as a control is amplified in the same tube. MDR1a primer sense (5′-TTTGCAAAACGTGCCACC-3′) Antisense (5′-GGAACAACCTGTAAGAGCAG-3′) β-actin primer sense (5′-CAACAAAGCTGAGGGAATCGTCTGTA-3′) Antisense (5′-AATTGCCTGACGATGGGCGACT-3′), cDNA strands are mixed with gentel vortex and 25 pmoL of each MDR1a sense and antisense primers or β-actin primers and 1μL of 4 mM of MgCl2 were added. Tubes were gently vortexed and centrifuged to collect all drops to the bottom. Samples were placed in a thermocycler and program was started as follows: The program for both MDR1a and β-actin is 30 cycles at 94°C for 1 min, 30 cycles at 60°C for 1 min and 30 cycles at 72°C for 1 min [16]. The amplified DNA was separated by agarose gel electrophoresis, stained with ethidium bromide then photographed. The density of the stained bands was measured by scion image program for windows. Relative band intensities were calculated as a ratio to β actin.

Statistical Analysis
Results were expressed as the mean ± SD for all experiments. Regression analysis and correlation coefficient were done for standard curves. A comparison between different groups was carried out one-way analysis of variance (ANOVA). The Statistical Package for Social (SPSS) computer software (version 10) was used to carry out the statistical analysis. The level of significance was set at P < 0.05.

3. RESULTS
Pharmacokinetic study

Time course effect of verapamil and tamoxifen on doxorubicin intracellular concentration in SEC
Treatment of mice with a combination of verapamil (5 mg/Kg) /doxorubicin (10 mg/Kg) resulted in a significant increase in doxorubicin intracellular concentration of SEC after 3 and 24 hours of doxorubicin injection by 57.2 % and 38.2 % respectively compared to mice treated with doxorubicin alone as shown in (figure1). Treatment of mice with a combination of tamoxifen (1 mg/Kg) /doxorubicin (10 mg/Kg) resulted in a significant increase in doxorubicin intracellular concentration of SEC after 3 and 24 hours of doxorubicin injection by 35.5 % and 23.1 % respectively compared to mice treated with doxorubicin alone as shown in (figure1). AUCs in SEC of female mice treated with doxorubicin alone or doxorubicin /verapamil combination and doxorubicin/tamoxifen combination are shown in (table 1).
Pharmacologic study

Effect of verapamil and tamoxifen on IC50 of doxorubicin in EAC cell

Doxorubicin concentration that produced 50% growth inhibition in EAC cells was 3 μg/ml with a mean absorption 0.846 ± 0.00253. Doxorubicin in a concentration of 1 μg/ml produced 13.2% growth inhibition with a mean absorption 1.468 ± 0.00744 whereas 5 μg/ml doxorubicin produced 97.5% growth inhibition with a mean absorption 0.0432 ± 0.00169 in EAC.

Effect of verapamil and tamoxifen on the growth inhibition of EAC

Treatment of EAC cells with verapamil alone in a dose of (1, 3, 10 and 30 μmol/L) resulted in no significant growth inhibition compared with saline control group as shown in (figure2). Also treatment of EAC cells with tamoxifen alone in a dose of (1, 3, 10 and 30 μmol/L) resulted in no significant growth inhibition compared with ethanol control group as shown in (figure2).

Effect of verapamil and tamoxifen on cytotoxic activity of doxorubicin in EAC cells.

Treatment of EAC cells with a combination of doxorubicin 3 μg/ml and verapamil 10 μmol/L or 30 μmol/L resulted in a significant increase in growth inhibition by 40.18% and 51.6% respectively compared to doxorubicin alone as shown in (figure 3 & 5). Treatment of EAC cells with a combination of doxorubicin 3 μg/ml and tamoxifen 10 μmol/L or 30 μmol/L resulted in a significant increase in growth inhibition by 20.2% and 39% respectively compared to doxorubicin alone as shown in (figure 4 & 5). However, Treatment of EAC cells with doxorubicin 3 μg/ml in combination with either verapamil (1 μmol/L or 3 μmol/L) or tamoxifen (1 μmol/L or 3 μmol/L) resulted in no significant change in growth inhibition compared to doxorubicin alone as shown in (figure 3 & 4). Both verapamil 30 μmol/L and tamoxifen 30 μmol/L reduced IC50 of doxorubicin from 3 μg/mL without chemosensitizer to 0.741 μg/mL for (doxorubicin/verapamil) and 0.915 μg/mL for (doxorubicin/tamoxifen).

Chemosensitizing effect of verapamil and tamoxifen

In correlation with growth inhibition verapamil (30 μmol/L) was a stronger chemosensitizer than tamoxifen (30 μmol/L) as shown in (table 2).

Effect of verapamil and tamoxifen on p-glycoprotein function in EAC using rhodamine 123

The accumulation of rhodamine 123 from EAC cells were significantly increased by verapamil (50 μmol/L) by 21.8% compared to control saline (figure 6). Treatment of EAC cells with verapamil (50 μmol/L) produced a significant increase in the retention of rhodamine 123 by 70% compared to control saline (figure 6). Treatment of EAC cells with tamoxifen (10 μmol/L) produced a significant increase in the retention of rhodamine 123 by 30% compared to control ethanol (figure 6). On the other hand treatment of EAC cells with tamoxifen (10 μmol/L) resulted in a non-significant increase in the accumulation of rhodamine 123 compared to control ethanol (figure 6). The function of P-glycoprotein in EAC was found to be significantly reduced by both verapamil (50 μmol/L) and tamoxifen (10 μmol/L) by 56.8% and 36.8% respectively compared to control saline and ethanol (figure 7).

Effect of verapamil and tamoxifen on mdr1a gene expression in EAC

In control groups, the ratio of mdr1a gene to β actin was 2.12, mdr1a mRNA showed an increase in doxorubicin treated group over control group by 97.6% (Table 3). Pretreatment of mice with verapamil (20mg/Kg) before administration of doxorubicin (20 mg/Kg) caused a significant decrease in mdr1a gene expression by 30.3% compared to that of doxorubicin alone (Table 3). On the other hand, pretreatment of mice with tamoxifen (20mg/Kg) before administration of doxorubicin (20 mg/Kg) resulted in a non-significant change in mdr1a gene expression compared to that of doxorubicin alone (table 3).
Fig (1): Time course effect of verapamil and tamoxifen on doxorubicin concentration in Solid Ehrlich Carcinoma
Mice were pretreated with doxorubicin alone or in combination with verapamil (5 mg/kg) or tamoxifen (1 mg/kg). Data are presented as mean ± SD of the concentration of doxorubicin (n = 7). * indicates significant difference from doxorubicin group at p< 0.05.

Fig (2): Effect of verapamil and tamoxifen on growth inhibition of EAC
EAC cells were treated with verapamil (1, 3, 10, 30 μmol/L) or tamoxifen (1, 3, 10, 30 μmol/L). Mean absorption of EAC cells treated with control vehicles, verapamil and tamoxifen was determined. Data are presented as mean ±SD of absorption intensities.

Fig (3): Effect of verapamil on IC50 of doxorubicin in EAC cells
EAC cells were treated with a fixed dose of doxorubicin (3 μg/mL) alone and in combination with verapamil (1, 3, 10, 30 μmol/L). The mean absorption of EAC cells was determined. Data are presented as mean ± SD of absorption intensities. * indicates significant difference from doxorubicin at p<0.05.

Fig (4): Effect of tamoxifen on IC50 activity of doxorubicin in EAC cells
EAC cells were treated with a fixed dose of doxorubicin (3 μg/mL) alone and in combination with different concentrations of tamoxifen (1, 3, 10, 30 μmol/L). The mean absorption of EAC cells was determined. Data are presented as mean ± SD of absorption intensities. * indicates significant difference from doxorubicin at p<0.05.
Fig (5) Effect of verapamil and tamoxifen on cytotoxic activity of doxorubicin in Ehrlich Ascites Carcinoma cells.

EAC cells were treated with a fixed dose of doxorubicin alone and in combination with different concentrations of either verapamil (10, 30 μmol/L) or tamoxifen. (10, 30 μmol/L). Growth rates of EAC cells were assessed. Data are presented as the mean % of control ± SD of triplicate samples.

* indicates significant difference from control saline at p<0.05

** indicates significant difference from control and doxorubicin at p<0.05

Figure (6): Effect of verapamil and tamoxifen on p-glycoprotein function in Ehrlich Ascites Carcinoma cells using rhodamine 123.

EAC cell suspensions were incubated with verapamil (50 μmol/L) or tamoxifen (10 μmol/L), rhodamine 123 accumulation and retention were assessed and the % function of p-glycoprotein was calculated. Data are presented as mean ± SD of fluorescence intensities.

* indicates significant difference from control saline at p<0.05.

** indicates significant difference from control ethanol at p<0.05.

Fig (7): Effect of verapamil and tamoxifen on p-glycoprotein function in Ehrlich Ascites Carcinoma cells using rhodamine 123.

EAC cell suspensions were incubated with verapamil (50 μmol/L) or tamoxifen (10 μmol/L), rhodamine 123 accumulation and retention were assessed and the % function of p-glycoprotein was calculated. Data are presented as mean ± SD of fluorescence intensities.

* indicates significant difference from control saline p< 0.05

** indicates significant difference from control ethanol p< 0.05
Table (1): AUCs in SEC of female mice treated with doxorubicin alone or doxorubicin plus verapamil or tamoxifen

<table>
<thead>
<tr>
<th>Mean area under curve (AUC) of doxorubicin (µg.g⁻¹.h) of SEC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin (10 mg/kg)</td>
</tr>
<tr>
<td>-----------------------</td>
</tr>
<tr>
<td>3 hours</td>
</tr>
<tr>
<td>4512.99±270.72</td>
</tr>
<tr>
<td>24 hours</td>
</tr>
<tr>
<td>2950.371±206.5</td>
</tr>
</tbody>
</table>

*indicates significant difference from doxorubicin group at p< 0.05.

Table (2): Chemosensitizing effect of verapamil and tamoxifen

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Relative optical density of mdr1a gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control groups</td>
<td>2.12 ± 0.0153</td>
</tr>
<tr>
<td>Doxorubicin (20 mg/kg)</td>
<td>4.19 ± 0.0353</td>
</tr>
<tr>
<td>Doxorubicin/ verapamil (20 mg/kg)/(20 mg/kg)</td>
<td>2.92 ± 0.0612*</td>
</tr>
<tr>
<td>Doxorubicin/ tamoxifen (20 mg/kg)/(20 mg/kg)</td>
<td>3.83± 0.0841</td>
</tr>
</tbody>
</table>

Sensitization ratio is determined by using the maximum doses of verapamil and tamoxifen, by dividing the absorption of the IC₅₀ of doxorubicin in the absence of the chemosensitizer by the absorption of IC50 of doxorubicin in the presence of chemosensitizer.

Table (3) Effect of verapamil and tamoxifen on mdr1a gene expression in EAC

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Absorption of Ic₅₀</th>
<th>Sensitation ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin (3 μg/mL)</td>
<td>0.846 ± 0.00253</td>
<td>--</td>
</tr>
<tr>
<td>Doxorubicin/ verapamil (3 μg/mL) / (30 μmol/L)</td>
<td>0.204 ± 0.0051</td>
<td>4.1</td>
</tr>
<tr>
<td>Doxorubicin/ tamoxifen (3 μg/mL) / (30 μmol/L)</td>
<td>0.360 ± 0.0108</td>
<td>3.2</td>
</tr>
</tbody>
</table>

Mice were treated with either verapamil (20 mg/kg) or tamoxifen (20 mg/kg) for 5 days. Doxorubicin (20 mg/kg) was administered in the last day. Data are presented as mean ± SD of relative optical density of triplicate samples. * indicates significant difference from doxorubicin p<0.05.
4. DISCUSSION
Multidrug resistance is one of the most important problems in cancer chemotherapy, MDR involves the expression of transport proteins such as p-glycoprotein, which act as energy dependent efflux pumps and reduce the intracellular concentration of several anticancer agents among which doxorubicin. It has been shown that doxorubicin is one of the most effective anthracycline antibiotics with broad antitumor spectrum. It is effective against wide range of cancers including solid tumors, leukemias and lymphomas [17, 18]. Doxorubicin is considered one of the most important anti-cancer drugs known to be a substrate of p-gp transporter [19].

In our study, treatment of EAC cells with a combination of doxorubicin (3µmol/L) with either verapamil (10 µmol/L) or (30 µmol/L) resulted in a significant increase in growth inhibition of EAC by 40.18 % and 51.6 % respectively compared to doxorubicin alone. The study also showed that verapamil (30 µmol/L) reduced IC50 of doxorubicin from (3 µmol /L) to (0.741 µmol /L). The present study also showed that treatment of EAC cells with a combination of doxorubicin (3µmol/L) with either tamoxifen (10 µmol/L) or (30 µmol/L) resulted in a significant increase in growth inhibition of EAC by 20.2 % and 39 % respectively compared to doxorubicin alone. The study also showed that tamoxifen (30 µmol /L) reduced IC50 of doxorubicin from (3µmol/L) to (0.915 µmol /L). These results were in agreement with the previously published data which revealed that treatment of human leukemia cells with a combination of verapamil and paclitaxel (an anti-cancer drug and a p-gp substrate) resulted in a significant increase in cancer cell growth inhibition percentage in vitro compared to that produced by paclitaxel alone [20]. Studies also showed that treatment of metastatic lung carcinoma cells with a combination of verapamil and daunorubicin (cytotoxic drug acts as substrate of p-gp) generated 2 to3 fold reduction in IC50 values of daunorubicin in vitro compared to that of daunorubicin alone [21]. It was reported that tamoxifen and toremifene( an analogue of tamoxifen) potentated the cytotoxicity of mitoxantrone (an anti-cancer drug and a substrate of p-gp) on human myelogenous leukemia cells in vitro[22]. Devalapally et al (2008) showed that Tamoxifen produced a 3 fold reduction in IC50 value of paclitaxel in human ovarian adenocarcinoma cell line [23].

To determine if the modulatory effect of both verapamil and tamoxifen on doxorubicin cytotoxicity was mediated by a change in its intracellular concentration, the current study determined doxorubicin level in SEC. Results of the current study revealed that treatment of mice with a combination of verapamil (5 mg/Kg) /doxorubicin (10 mg/Kg) resulted in a significant increase in doxorubicin intracellular concentration of SEC after 3 and 24 hours of doxorubicin injection by 57.2 % and 38.2 % respectively. The present study also showed that treatment of mice with a combination of tamoxifen (1 mg/Kg) /doxorubicin (10 mg/Kg) resulted in a significant increase in doxorubicin intracellular concentration of SEC after 3 and 24 hours of doxorubicin injection by 35% and 23.1% respectively.

Wang et al (2006) showed that verapamil caused a significant increase in area under curve (AUC) of doxorubicin in brain tissues of rats; the increase in AUC of doxorubicin indicates an increase in its intracellular concentration. AUC of doxorubicin was determined by using HPLC [24]. Darvari and Boroujerdi (2005) reported that tamoxifen increased the accumulation of doxorubicin in renal tissues of rats. The concentration of doxorubicin in kidney tissue is determined by HPLC [25].

According to the previously mentioned results, the ability of both verapamil and tamoxifen to potentiate doxorubicin cytotoxic activity in tumor cells could be explained on the basis of the ability of these two drugs to cause a significant increase in doxorubicin concentration in tumor tissues. Accordingly, we tried to explore if the increase in intracellular doxorubicin concentration is related to a decrease in p-gp function caused by verapamil and tamoxifen.

P-gp is expressed in numerous tissues, including the gastro-intestinal tract, the liver and the kidney [26]. Many of the chemotherapeutic agents used in clinical practice are substrates of p-gp e.g. paclitaxel, epirubicin, doxorubicin, daunorubicin, vinblastine and vincristine[27]. The present study demonstrated that the function of p-glycoprotein in EAC was found to be significantly reduced by both verapamil (50 µmol) and tamoxifen (10 µmol) by 56.8 % and 36.8 % respectively compared to control saline and ethanol.

So the enhancement of doxorubicin antitumor activity caused by its combined administration with either verapamil or tamoxifen in the current study can be explained on the basis of the hypothesis that: The ability of both verapamil and tamoxifen to act as a multidrug resistance reversing agents and p-gp modulators. Treatment of EAC cells with either verapamil or tamoxifen inhibited p-gp transport activity in EAC cells in SEC bearing mice, thereby resulting in an increase in intracellular doxorubicin concentration within EAC cells through the inhibition of doxorubicin efflux with consequent increase in its cytotoxic activity. Accordingly, we tried to explore if the increase in intracellular doxorubicin concentration and cytotoxic activity is related to a decrease in the expression of p-gp function, which is the most consistent morphological finding in the MDR phenotype. P-gp was first described as the product of the MDR1 gene in human and mdr1a and mdr1b genes in rodents [28]. Studies demonstrated that over expression of MDR genes caused cancer cells to become resistant to a variety of anticancer drugs e.g. vinblastine, vincristine, daunorubicin and paclitaxel [29]. The current study revealed that mdr1a mRNA showed an increase in
doxorubicin treated group over control group by 97.6%. Pretreatment of mice with verapamil (20 mg/kg) before administration of doxorubicin (20 mg/kg) caused a significant decrease in mdr1a gene expression by 30.3% compared to that of doxorubicin alone. On the other hand, pretreatment of mice with tamoxifen (20 mg/kg) before administration of doxorubicin (20 mg/kg) caused a non significant decrease in mdr1a gene expression compared to that of doxorubicin alone. Some studies showed that mice made null for the mdr1a and mdr1b genes shown a decreased ability to excrete vinblastine and digoxin (two p-gp substrates) resulting in a longer half life and increased accumulation, particularly in the brain. This leads to a 4- fold reduction in the LD50 of vinblastine in these mice [30].

The ability of verapamil to inhibit p-gp transport activity can be explained on the basis of the hypothesis that: Since p-gp transport activity is dependent on its expression in tumor cells and that p-gp expression is increased through up regulation of MDR1 mRNA , as p-gp is encoded by this gene. So down regulation of MDR1 mRNA exerted by verapamil is considered the mechanism through which verapamil inhibited p-gp in EAC cells. ATP dependent function of p-gp requires phosphatidyl ethanolamine as phosphatidyl ethanolamine is necessary for the function of ATPase enzyme. Some studies demonstrated that tamoxifen inhibited phosphatidyl ethanolamine in human breast carcinoma cells. These studies showed that tamoxifen stimulated phosphatidyl ethanolamine hydrolysis and caused inhibition of its synthesis resulting in inhibition of p-gp mediated efflux of cytotoxic drugs [31, 32]. The finding of the current study states that tamoxifen inhibits p-gp transport activity via a mechanism independent on inhibition of MDR1 mRNA. The ability of tamoxifen to inhibit p-gp transport activity could be explained as follows: As p-gp is an ATP utilizing protein, the increase in ATPase activity increase substrate transporting by p-gp. Tamoxifen have the ability to interact with p-gp with a high binding affinity, and inhibits ATPase activity resulting in inhibition of ATP hydrolysis and energy production required for substrate efflux by p-gp.

5. CONCLUSION
Combined administration of either verapamil or tamoxifen with doxorubicin significantly increased doxorubicin intracellular concentration in SEC and significantly enhanced doxorubicin cytotoxicity in EAC cells. Verapamil is a stronger chemosenstizer than tamoxifen. The ability of both verapamil and tamoxifen to inhibit p-gp activity is assumed to be responsible for enhancement of doxorubicin cytotoxicity. Enhancement of p-gp activity in EAC tumor cells could result from an increase in mdr1a gene expression in the tumor cells. Verapamil inhibits p-gp activity in EAC cells via down regulation of mdr1a gene expression. On the other hand tamoxifen inhibits p-gp activity in EAC cells via a mechanism independent on down regulation of mdr1a gene expression.

6. REFERENCES