Journal of the Egyptian Nat. Cancer Inst., Vol. 12, No. 3, September: 221-227, 2000

Modulation by Verapamil of Doxorubicin Induced Expression of Multidrug Resistance Gene (MDR-1/P-Glycoprotein) in Murine Tumour Cells

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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 natural product anticancer drugs including Doxorubicin (DOX). In this study, the effects of DOX and/or DOX plus the calcium channel blocker, Verapamil, on mdr-1/p-gp expression in Ehrlich ascites carcinoma cells (EAC-cells) were studied using Rhodamine 123 (RD 123), Western blotting and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). Tumor-bearing mice treated with DOX (2 mg/kg I.P. every other day for a total of 3 doses) showed 34.1 days median survival time with 20% long-term survivors. However, pretreatment with Verapamil (20 mg/kg I.P. daily for 5 days), before DOX increased the long-term survivors to 60% with median survival time of 44.4 days. Verapamil pretreatment increased the cellular levels of DOX at all time points tested with maximum level appeared at 6 hours after treatment (5.5 µg/10^8 cells compared to 3.4 µg/10^8 for DOX alone). The expression of mdr-1/p-gp was significantly increased 6 hours after treatment with DOX. Verapamil pretreatment (20 mg/kg) for 5 days before DOX significantly decreased mdr-1/p-gp expression and decreased p-gp function from 37% to 16%. On the basis of our findings we may conclude: (1) DOX induced mdr-1/p-gp expression in sensitive EAC-cells at both transcriptional and translational levels at only 6 hours post treatment. (2) Verapamil block DOX-induced mdr-1/p-gp expression with the consequent increase in DOX cellular uptake and cytotoxicity. (3) Verapamil can potentiate the cytotoxic activity of DOX against the growth of EAC cells by mdr-1/p-gp independent mechanism.

Key Words: *Doxorubicin - Multidrug resistance - Calcium channel blocker - Drug-drug interaction.*

INTRODUCTION

The anthracycline antibiotic, Doxorubicin (DOX), has been widely and successfully employed for the treatment of various types of cancer. Unfortunately, many types of cancer are refractory to DOX and others that initially was responding favorably later become resistant. Multidrug resistance (MDR) is the most widely studied manifestations of tumour cell resistance to DOX [3]. This spectrum of resistance results from expression of mdr-1 gene, which encodes a protein termed p-glycoprotein (P-gp) that acts as a membrane-bound ATP-consuming drug efflux pump that reduces cellular drug accumulation and confers a drug resistant state [11,12,24]. Using A540 human lung adenocarcinoma, Chevillard et al. [6] reported that DOX induces expression of P-gp after only 24 hrs contact with sensitive cells. In five series of EAC-cell lines selected for resistance to DOX, Nielsen et al. [20] demonstrated that the degree of resistance and the rate of development of p-gp and its maximal content depended on the dose of DOX used for selection (selection pressure). In contrast, Volme et al. [26], Devien and Melera [9] reported that increased expression of p-gp and mdr 1 amplification were independent on the dose of DOX used for selection. Results from these studies should be carefully reviewed since they were performed in-vitro in which the entire biological environment and drug metabolizing enzyme systems were lacking. Therefore, the present study has been initiated with the following specific aims: (1) to investigate whether p-gp could be expressed in sensitive EAC-cells after in vivo treatment with a single dose of DOX and if so, what are the effects of cellular contents and various time interval after DOX treatment on mdr-1/p-gp expression and (2) to determine whether pretreatment of EAC-cells with the calcium channel blocker, Verapamil

(VER), could affect mdr-1/p-gp expression at the transcriptional and/or translational levels.

MATERIAL AND METHODS

Drugs:

Doxorubicin (Farmitalia, Carlo Erba, Italy) and Verapamil (Isoptin, Knoll, Ludwighafen, FRG) were supplied from National Cancer Institute (NCI) Cairo, Egypt. All other reagents and chemicals were of high analytical grade.

Animals and tumor:

Female Swiss albino mice, weighing 20-22g were obtained from the animal house of NCI, Cairo, Egypt. Animals were allowed free access to standard diet and water ad. libitum. A line of EAC cells was supplied through the courtesy of Dr.C.Benckhuijsen, Amsterdam, the Netherlands and maintained in our laboratory by weekly I.P. transplantation of 2.5 X 10^6 cells/ mouse.

Evaluation of antitumor activity:

The effect of Verapamil on the antitumor activity of DOX was evaluated using the regimen of Donenko et al. [10] with slight modification. In brief, EAC cells were inoculated I.P. into female swiss albino mice (2 x 106 cells/mouse). Twenty four hours later, Verapamil (20 mg/kg) was injected I.P. once per day for 5 days. A control group received equal volumes of saline. Equal numbers of cells (2.5 x 10^6) from either saline or Verapamil-pretreated mice were injected I.P. into naive mice. DOX treatment started 24 hours after tumor inoculation in a dose of 2 mg/kg every other day for a total of 3 doses.

Effect of verapamil-pretreatment on DOX uptake in EAC-cells:

Ehrlich ascites carcinoma cells were inoculated as described above at 2 x 10^6 cells/mouse. Twenty four hours later, Verapamil (20 mg/kg) or an equal volume of saline was injected I.P. once per day for 5 days. On the sixth day, DOX was injected I.P. in a single dose (20 mg/kg). After doxorubicin therapy, EAC-cells were withdrawn from each group, counted, homogenized and extraction of DOX was performed according to the method of Bachur et al. [2]. The concentration of DOX was measured spectrofluorometrically (SFM 25 Kontron, Italy) with excitation and emission wave lengths 470 and 585 nm, respectively.

Detection of MDR-1 gene using reverse transcriptase-polymerase chain reaction (RT-PCR):

Total RNA was isolated from EAC-cells according to the single step RNA isolation method developed by Chomezymski and Saccho [8] using guanidine isothiocyanate, Trizol kit (life technology, Gibco, BRL). Expression of mdr-1 gene was detected using one-step RT-PCR procedure where both cDNA synthesis and PCR steps were performed in a single tube. Primers for mdr-1 gene and beta-actin gene as a control are amplified in the same tube. mdr-1 gene product is 196-bp and B-actin gene product is 306-bp as described by Nooman et al. [22]. Parameters for amplification were performed as 1 cycle of 45˚C for 30 min and 94˚C for 2 min as predenature for cDNA synthesis followed by 40 cycles of 94˚C for 15 seconds, 60˚C for 30 seconds and 72˚C for 1 min. The PCR products were separated by 12% polyacrylamide gel electrophoresis, stained with ethidum bromide, photographed using polaroid films 667. The mdr-gene expression was calculated relative to that of control gene (beta-actin in each reaction).

P-glycoprotein expression, western blot analysis:

The expression of p-gp in EAC cells 6 hours after treatment with DOX and Verapamil plus DOX was done according to Nielsen et al. [21]. Cells were withdrawn from each group (1 x 10^6) and then solubilized in cell lysis buffer contained 50 mM Hepes (pH 7.0), 500 mM NaCl, 1% Np40, 10 µg/ml each aprotinine and leupeptin, 1 mM phenyl methyl sulfonyl fluoride, 1 mM NaVO4 and 1 mM EDTA, kept on ice for 30 min, frozen at-80˚C and thawed on ice. Cell lysate were centrifuged at 13.600 xg at 4˚C for 15 min and postnuclear supernatants were collected. Equal amounts of protein lysates (50 µg) were separated on 7% SDS-polacrylamide gels and transferred to nitrocellulose (Trans Plot Transfer Medium, Bio Rad Laboratories, Richmond, CA, USA). After blocking, filters were saturated with 5% non-fat dry milk, incubated for 12 hours at 4˚C with Anti-P-170 monoclonal antibody (Oncogene Science, USA), then were washed, incubated with sheep antimouse IgG linked to horseradish peroxidase. Bound proteins were visualized using Diaminobenzidine and hydrogen peroxide.

Detection of p-glycoprotein activity using rhodamine 123 (RD-123):

The activity of p-gp in EAC-cells was determined 6 hours after DOX therapy according to Ludescher et al. [**16**]. This method depends on the fluorescent properties of RD 123 dye which is transported by the membrane efflux pump, p-gp. In brief, 2 x 10^6 EAC-cells from each group were withdrawn, washed, resuspended in 2 ml RPMI media and incubated with 200 ng/ml RD 123 at 37˚C for 1 hour. At the end of the incubation time, cells were washed twice with 2 ml cold PBS and divided into two parts. For accumulation experiments, the first part of EAC-cells were resuspended in 2 ml cold PBS and the fluorescence intensity was quantitated spectrofluorometrically (SFM 25 Kontron, Italy) using excitation and emission wave lengths 470 and 590 nm, respectively. For efflux/retention experiments, the second part of EAC-cells were resuspended in 2 ml RD 123-free RPMI medium for additional 30 min at 37˚C. Thereafter, RD 123 efflux/retention was determined as previously mentioned in the accumulation experiments. The function of p-gp was then calculated as the amount of RD 123 efflux per 30 min using the following formula:

$$\text{Function of p-gp (\%)} = \frac{\text{RD 123 accumulation - RD 123 retention}}{\text{RD 123 Accumulation}}$$

RESULTS

Fig. (1) shows the effect of Verapamil pretreatment on the cytotoxic activity of DOX against the growth of EAC cells inoculated I.P. into female Swiss albino mice. Control tumor-bearing mice showed a median survival time (MST) of 15 days, whereas, administration of DOX (2 mg/kg) for 3 doses increased the MST to 34.1 days, with 20% long-term survivors. Verapamil pretreatment (20 mg/kg) daily for 5 days, increased the MST of tumor-bearing mice treated with DOX to 44.4 days with 60% long-term survivors.

Fig. (2) shows the cellular level of DOX in EAC-cells after treatment with a single dose of DOX (20 mg/kg) and/or 5 doses of Verapamil (20 mg/kg). Verapamil pretreatment significantly increased the cellular level of DOX at nearly all the time points tested, with the maximum level appeared at 6 hours after treatment (5.5 ug/10^8 cells compared to 3.4 ug/10^8 cells for DOX alone).

The effects of time intervals after a single dose of DOX on mdr-1 and P-glycoprotein experiment are shown in Figs. (3 & 4). Treatment of EAC-bearing mice with DOX revealed non significant expression of mdr-1 and P-gp expression in tumor cells at all time points tested except at 6 hours, where, the expression was statistically significant. Interestingly, pretreatment of EAC cells with verapamil completely suppressed the expression of mdr-1 and p-gp induced by DOX.

Fig. (5) shows the effect of DOX and DOX plus verapamil on the accumulation/uptake and efflux/retention of RD 123 in EAC cells. DOX-treated cells clearly accumulated less amount of RD 123 when being compared with those pretreated with verapamil (6.62 ng/10^6 cells for Verapamil plus DOX versus 5.2 ng/10^6 cells for DOX alone). In order to prove whether the decrease in RD 123 accumulation was in fact caused by an active outword transport, efflux experiment was carried out, as shown in Fig. (6) a rapid decrease of intracellular RD 123 was observed in DOX-treated cells manifested as 1.9 ng RD 123 efflux/30 min and 37% p-gp function. In verapamil pretreated cells, RD 123 was retained and the efflux was inhibited (1.1 ng RD 123 efflux/30 min and 16% p-gp function, 5.5 ug/10^8 cells for Verapamil plus DOX versus 3.4 ug/10^8 cells for DOX alone).

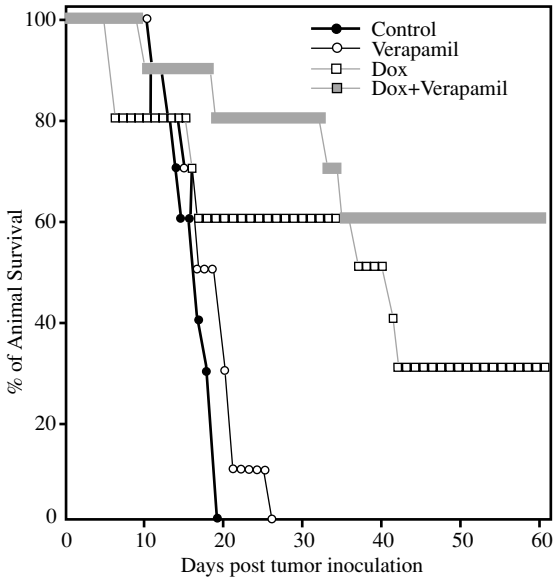


Fig. (1): Effect of verapamil pretreatment on the antitumour activity of doxorubicin in mice-bearing Ehrlich ascites carcinoma cells. MST (Median Survival Time) = average survival days of mice. LTS (Lond-term survivors) are defined as the mice survived to the end of the experiment (60 days) without an apparent evidence of tumour cell growth on microscopic examination.

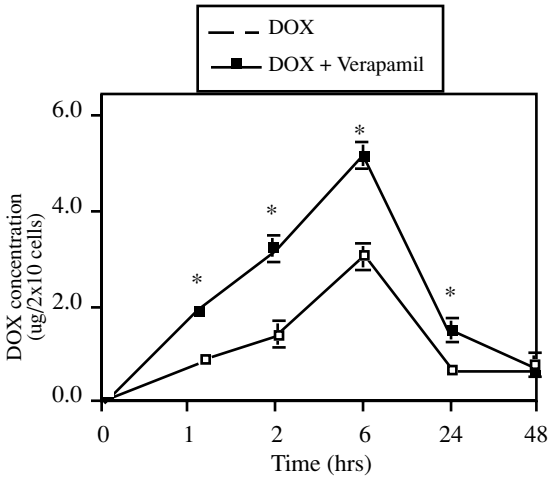


Fig. (2): Effect of verapamil pretreatment on DOX level in Ehrlich ascites carcinoma cells. DOX was injected (20 mg/kg I.P.) in tumour bearing mice pretreated either with verapamil or saline. Values are presented as mean ± SEM (n=6). * indicate significant change of DOX plus verapamil versus DOX ($p < 0.05$).

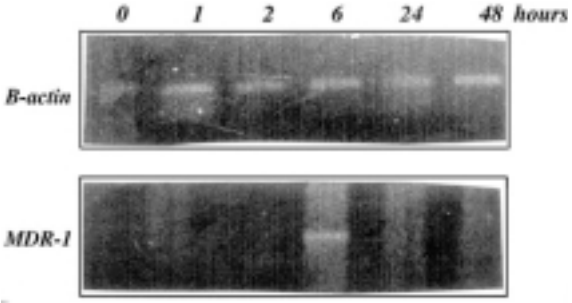
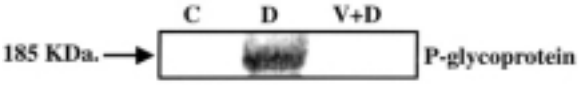


Fig. (3): Effect of verapamil pretreatment on MDR-1 mRNA expression in Ehrlich ascites carcinoma cells measured by Reverse Transcriptase-Polymerase Chain Reaction (Rt-PCR). RT-PCR at different time intervals (0,1,2,6,24 and 48 hours) after doxorubicin treatment. DOX was injected (20 mg/kg I.P.) in tumour bearing mice pretreated either with verapamil or saline. The expression of MDR-1 gene was shown after 6 hours only, the lower band. Expression of B-actin control gene, the upper bands.



Effect of verapamil pretreatment on expression of P-glycoprotein 6 hrs after doxorubicin treatment.
C, Control
V, Verapamil (VER)
D, Doxorubicin (DOX)

Fig. (4): Effect of verapamil pretreatment on p-glycoprotein expression in Ehrlich ascites carcinoma cells measured by Western blotting six hours after doxorubicin treatment. DOX was injected (20 mg/kg I.P.) in tumour bearing mice pretreated either with verapamil or saline. C, D and V+D represents control, doxorubicin and verapamil plus DOX, respectively.

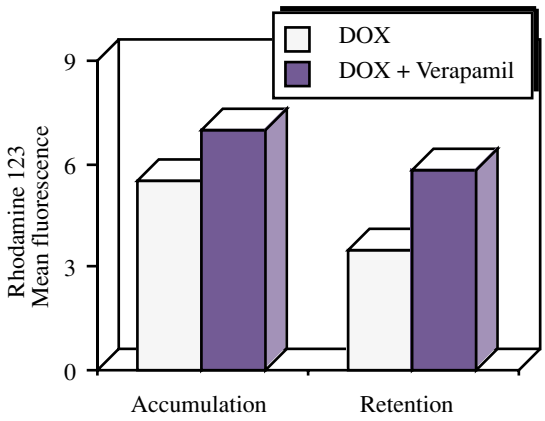


Fig. (5): Effect DOX and DOX plus verapamil on rhodamine 123 accumulation and retention in Ehrlich ascites carcinoma cells. DOX was injected (20 mg/kg I.P.) in tumour bearing mice pretreated either with verapamil or saline. Values are presented as mean ± SEM (n=6).

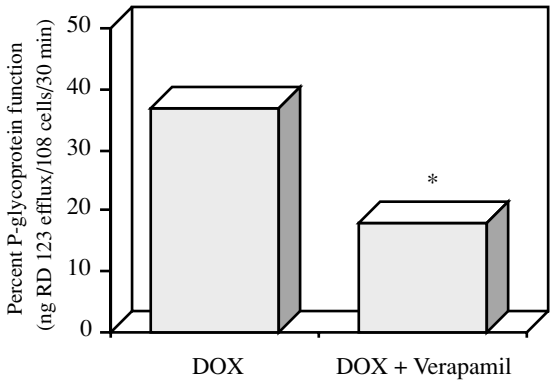


Fig. (6): Effect DOX and DOX plus verapamil on p-glycoprotein function in Ehrlich ascites carcinoma cels. DOX was injected (20 mg/kg I.P.) in tumor bearing mice pretreated either with verapamil or saline. Values are presened as mean ± SEM (n=6).

Function of p-gp (%) =

$$\frac{\text{RD 123 Accumulation - RD 123 retention X 100}}{\text{RD 123 Accumulation}}$$

* Indicates significant changes of DOX plus Verapamil versus DOX ($p < 0.05$).

DISCUSSION

The usefulness of DOX as an important anticancer drug in the treatment of cancer is usually limited by its severe cardiotoxicity and the development of MDR phenotype. In order to control and/or prevent MDR, a number of pharmaceutical agents, not antitumour drugs in their own right, are known to modulate the MDR

phenotype and to increase cellular sensitivity to DOX and other anticancer drugs [**1,13,23**]. Among these agents, calcium channel blockers including verapamil have attracted much attention in recent years [**24**]. In this study, pretreatment of EAC-bearing mice with calcium channel blocker, Verapamil, before administration of DOX produced 3-fold increase in long-term survivors as compared to those treated with DOX alone (Fig. 1). This is in a good agreement with the report by Al-Shabanah et al. [**1**], who found enhancement in the antitumor effect of DOX against mammary carcinoma by prior administration of calcium channel blocker Verapamil. It also agrees with results of Hori et al. [**14**], who found that Verapamil enhanced the antitumor activity of DOX on both KB and KB-re cells. The precise mechanism of enhanced DOX cytotoxicity by calcium channel blockers are not clear, however, Tusuro et al. [**25**] and Al-shabanah et al. [**1**] reported that calcium channel blockers including Verapamil, exert their effect by inhibiting the efflux of anticancer drugs from tumor cells leading the accumulation of chemotherapeutic agents and enhanced cytotoxicity. The increased cellular accumulation of anticancer drugs caused by calcium channel blockers was reported to be due to the blockage of the expression of P-glycoprotein which is responsible for MDR phenotype [**19**].

The lack of mdr-1 expression during our experiment at time interval (1 and 2 hours) after DOX therapy (Fig. 3) is consistent with the data presented by Chevillard et al. [**6**] which have demonstrated that mdr-1/p-gp is not expressed in human lung adenocarcinoma after 2 h contact with DOX. On the other hand, the increase in DOX uptake by Verapamil at time points (1,2 and 24 hrs) despite the lack of mdr-1 expression suggests that the inhibitory effect of Verapamil on mdr-1 expression is not the only mechanism by which Verapamil modulate DOX resistance. In our study, DOX induced mdr-1 expression in EAC-cells 6 hours after treatment (Fig. 3). These results disagree with those previously reported by Volme et al. [26] and Chevillard et al. [**5,6**] who claimed that DOX induced mdr-1 amplification and p-gp expression after only a single 24 hrs contact time with A540 human lung adenocarcinoma. This discrepancy could be explained by the difference in tumour types, the nature of the treatment protocol and the experimental system used. Unlike our in vivo study, Volme et al. [**26**] and Chevillard [**6**] studies have been performed in vitro in which the entire biological environment and drug metabolizing enzyme system which are necessary for DOX metabolism were lacking. Furthermore, Nielsen et al. [**20**] reported that 3-fold resistance in vitro correspond to no resistance in vivo, whereas, 10-30 fold resistance in vitro correspond to complete resistance in vivo. It has been reported that the maximum DOX metabolism occurs from 6-9 hrs [**4**]. DOX is metabolized to more hydrophilic metabolites as doxorubicinol which is easily transported outside the cell. This indirectly support the expression of mdr-1/p-gp by DOX after 6 hrs contact with EAC-cells with the consequent increase in DOX efflux.

The increase in mdr-1 expression in EAC-cells 6 hrs after treatment with DOX is confirmed by the increase in p-gp expression assessed by western blot analysis (Fig. 4). These results suggests that MDR phenotype induced by DOX could be due to both transcriptional and translational activation. The mdr-1 promoter has been shown to respond directly to treatment with cytotoxic agents including DOX [**7,17**]. Like other proteins, p-gp may undergoes post-translational modification (e.g. phosphorylation) which alter its function as membrane bound drug efflux pump. Therefore, it was of necessary to monitor p-gp expression using RD 123 which provide informations concerning its function. The decrease in RD 123 accumulation in DOX-treated cells (Figs. 5 & 6) could be a secondary event following the increase in the expression and function (37%) of p-gp with the consequent increase RD 123 efflux. Interestingly, Verapamil pretreatment increased the accumulation of RD 123 by decreasing p-gp function (16%).

A critical question remains: Does the calcium environment plays a role in the active efflux of antineoplastic drugs?. Although this is possible, Mazzoni and Trave [**18**], have reported that no relationship between calcium efflux and transport of anticancer drug DOX. The discrepancy of our work and Mazzoni and Trave work could be explained by the presence of many intracellular mechanisms of protection and detoxification in tumor cells which may lead to drug resistance and variations in the response to chemotherapy [**18**]. Moreover, different calcium

influx blockers posses different specificity for organs or cells and one calcium influx blocker showed also different response in organs and cells [**15**]. On the basis of our findings we may conclude: (1) DOX induced mdr-1/p-gp expression in sensitive EAC-cells at both transcriptional and translational levels after only 6 hours after treatment. (2) Verapamil block DOX-induced mdr-1/p-gp expression with the consequent increase in DOX cellular uptake and cytotoxicity. (3) Verapamil can potentiate the cytotoxic activity of DOX against the growth of EAC cells by mdr-1/p-gp-independent mechanism. Further studies are in progress in our laboratory to investigate the effect of Verapamil on DOX-induced cardiototxicity.

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