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Biochemical Pharmacology 70 (2005) 725–732
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Modulation of epirubicin cytotoxicity by tamoxifen in human breast cancer cell lines

Samar S. Azab [a], Ebtehal El-Demerdash [a,*], Ashraf B. Abdel-Naim [a], Ekram Youssef [b], Nahla El-Sharkawy [c], Abdel-Moneim M.Osman [d]

[a] *Department of Pharmacology and Toxicology, Faculty of Pharmacy, Ain Shams University, Egypt*
[b] *Unit of Cell Biochemistry, Department of Cancer Biology, National Cancer Institute, Cairo University, Egypt*
[c] *Department of Clinical Pathology, National Cancer Institute, Cairo University, Egypt*
[d] *Unit of Pharmacology, Department of Cancer Biology, National Cancer Institute, Cairo University, Egypt*

Received 23 January 2005; accepted 7 March 2005

Abstract

The present study was designed to investigate the modulatory effect of the anti-estrogen, tamoxifen (Tam) on epirubicin (Epi) cytotoxicity in breast cancer cell lines; MCF-7 and NCI-adr. Using sulphorhodamine-B assay, NCI-adr cell line was found to be five-folds more resistant to the cytotoxic effect of Epi as compared to MCF-7 cell line. Pretreatment of cells with Tam was observed to enhance Epi cytotoxicity by 4.3- and 6.5-folds in MCF-7 and NCI-adr cells, respectively. Tam–Epi interaction was found to be additive in MCF-7 cells and synergistic in NCI-adr cells. Flowcytometric DNA ploidy analysis revealed that, Epi induced cell arrest at G_2/M phase. Tam pretreatment enhanced the blocking activity of low dose of Epi in MCF-7 and induced nearly two-fold increase in the percentage of S phase in NCI-adr cells. Determination of cellular Epi level revealed that Tam induced a significant increase in intracellular Epi accumulation only in NCI-adr cells after 48 h. However, analysis of P-gp function revealed that Tam failed to modulate P-gp function in both cell lines. Also, assessment of topoisomerae IIα gene expression showed that neither Epi nor Tam managed to change its expression level. In conclusion, Tam potentiates Epi cytotoxicity in sensitive and resistant breast cancer cell lines. This potentiation can be explained by an enhancement of cell accumulation in S and G_2/M phase, at which the cells are most sensitive to the cytotoxic effect of Epi as well as an increase in the intracellular level of Epi in resistant cell line.

Keywords: Epirubicin; Multi-drug resistance; Tamoxifen; Cell cycle; P-glycoprotein; Topoisomerase IIα

1. Introduction

Chemotherapy plays an important role in the management of breast cancer. As an important example, Epirubicin (Epi), an anthracycline antibiotic, is considered among the most active chemotherapeutic agents for patients with advanced breast cancer [1]. However, clinical usefulness of Epi in treatment of breast cancer is often limited by the development of drug resistance. Acquired drug resistance to the anthracyclines is associated with cross-resistance to other chemotherapeutic drugs. This phenomenon is commonly known as multi-drug resistance (MDR). The mechanism of MDR is not fully understood, but is frequently associated with over-expression of a membrane protein, P-glycoprotein (P-gp), along with a variety of other intracellular events [2].

In the last two decades, attention has been focused on agents to reverse MDR and so enhances the response of tumors to chemotherapeutic agents. Although hundreds of compounds have been found in vitro to be able to modulate the MDR phenotype, their clinical application was limited by dose-limiting toxicities [3]. For instance, verapamil, one of the most investigated modulators of MDR, is currently being used to assist doxorubicin treatment, even though its cardiotoxicity prevents the administration of adequate doses [4]. Accordingly, searching for compounds able to modulate the MDR phenotype and have low toxicity is an important issue.

Among these chemosensitizers, antiestrogens constitute a class of great interest. The non-steroidal antiestrogen tamoxifen (Tam) has been shown to potentiate the in vitro

* Corresponding author. Fax: +202 2876271; mobile: +202 0101925375.
E-mail address: ebtehal_dm@yahoo.com (E. El-Demerdash).

0006-2952/$ – see front matter © 2005 Published by Elsevier Inc.
doi:10.1016/j.bcp.2005.03.036

cytotoxicity of several chemotherapeutic agents [5,6]. In addition, several clinical studies have proposed the potential usefulness of adding Tam as modulator to chemotherapeutic regimens including anthracycline antibiotics [7,8]. However, until now there is no data about the possible modulating effect of Tam on Epi cell resistance in human breast cancer cells.

Therefore, this study was designed to find solutions for two questions:

1. Can Tam modulate the cytotoxic effect of Epi in human breast cancer cells MCF-7 and NCI-adr?
2. If so, what are the possible mechanisms for this modulatory effect?

2. Materials and methods

2.1. Drugs

Epi was purchased as Farmorubicin vials from Pharmacia and Upjohn Co. (Italy). Each vial contains 10 mg red orange of Epi hydrochloride. The content was freshly dissolved in sterile water for injection to yield a stock solution of 2 mg/ml, then serially diluted with RPMI-1640 medium and was used at concentrations varying from 8 ng/ml to 5 μg/ml. Tam was obtained from Memphis Chemical Co. (Egypt). It is solubilized in absolute ethanol, and then diluted in RPMI 1640 medium. The final concentration of ethanol was <0.5% (v/v). In all experimental studies, Tam was used in a single concentration of 10 μM.

2.2. Cell lines

Human breast cancer cell lines; MCF-7 wild type and doxorubicin resistant NCI-adr were used in this study. They were obtained frozen in liquid nitrogen from the American Type Culture Collection. The tumor cell lines were maintained in the National Cancer Institute, Cairo, Egypt, by serial sub-culturing. The cells were grown as ''monolayer culture'' in RPMI-1640 medium supplemented with 10% fetal bovine serum and 100 units/ml penicillin and 2 mg/ml streptomycin. Cells of both cell lines were divided into four treated groups. The first group served as the control and was treated with vehicle (0.5% ethanol in RPMI-1640 medium). The second and third group of cells was treated with either Epi or Tam while the last group was pre-treated with Tam 2 h before Epi treatment.

2.3. Assay of cytotoxic activity

Cytotoxicity was determined using SulphoRhodamine-B (SRB) method as previously described by Skehan et al. [9]. Cells were seeded in 96 well microtiter plates at a concentration of 5×10^4–10^5 cells/well in RPMI-1640 supplemented medium. After 24 h, cells were incubated for 48 h with various concentrations of Epi in the following ranges: 8–167 ng/ml for MCF-7 cell line and 50–1000 ng/ml for NCI-adr cell line (3 wells for each concentration). Following 48 h treatment, the cells were fixed with 50% trichloroacetic acid for 1 h at 4 °C. Wells were washed five times with water, stained for 30 min at room temperature with 0.4% SRB dissolved in 1% acetic acid and then washed four times with 1% acetic acid. The plates were air dried and the dye was solubilized with 100 μl/well of 10 mM tris base (PH 10.5) for 5 min on a shaker at 1600 rpm. The optical density (OD) of each well was measured spectrophotometrically at 564 nm with an ELIZA microplate reader (Meter tech. Σ960, USA). The IC_{50} values were calculated using sigmoidal concentration–response curve fitting models (Graph Pad, Prizm software).

2.4. Evaluation of drug interaction

Dose-response curves for Tam alone in both cell lines were first generated. The extent of the effect of the combined treatment was analyzed by applying isobologram equation [10]: $I = d_1/D_1 + d_2/D_2$, where d_1 and d_2 are the respective concentrations of Epi and Tam used in the combination required to produce a fixed level of inhibition IC_{50}, while D_1 and D_2 are their concentrations able to produce alone the same magnitude of effect (50% inhibition of cell growth). If ''I'' (interaction index) is less than 1, the effect of combination is synergestic, whereas if $I = 1$ or $I > 1$ the effect is additive or antagonistic, respectively.

2.5. Cell cycle analysis

The method was carried out according to that of Li et al. [3]. Cells were plated at a density of 5×10^6 cells/ml in RPMI-164 supplemented medium. After 24 h the medium was replaced with fresh medium containing Epi (0.02 or 0.2 μg/ml) and/or Tam (10 μM). The cells were harvested after 48 h and washed twice with phosphate buffered saline (PBS), then fixed with 70% (v/v) ethanol. The sample was concentrated by removing ethanol and staining of cellular DNA was performed using ready made kit (DNA prep reagent, Beckman Coulter Corp., USA). Flow cytometric DNA ploidy analysis was performed by acquiring a minimum of 200,000 nuclei. Cell cycle analysis was performed with the Epics XL coulter software package (Beckman Coulter Corp., USA). The percentage of cells with DNA content corresponding to G_0/G_1, S and G_2/M phases, respectively were computed by the planimetry of the histogram and then compared for both cell lines.

2.6. Assay of cellular Epi concentration

Epi uptake in MCF-7 and NCI-adr cells was performed using HPLC according to the method of

Seymour et al. [11]. Cells were plated at a density of 5×10^5 cells/ml and divided into two treatment groups: the first group was treated with 5 μg/ml Epi, while the second was treated with combination Tam followed by Epi 2 h later. Epi intracellular concentration was determined after 2, 4, 24 and 48 h of incubation. In brief, cells were harvested by trypsinization and resuspended in PBS. Suitable samples are taken for cell counting. A double volume of extraction medium consisting of chloroform:methanol (75:25%, v/v) was added, samples were vortexed and mixed three times during 30 min period and finally centrifuged at $1000 \times g$ for 10 min. The lower organic layer was separated and recentrifuged for maximal purification. The samples were evaporated to dryness, redissolved in 29% aqueous isopropanol and analyzed by HPLC system (Kontron instruments, Italy) using Hypersil BDS C18 column and eluted at a flow rate of 1 ml/min with a mobile phase of aqueous isopropanol (29%) adjusted to pH 3.2 with orthophosphoric acid. Detection was performed using flourimetric detector at excitation wave length 480 nm and emission wave length 560 nm.

2.7. Flowcytometric analysis of P-gp function

The uptake-efflux of Epi was monitored using the method of DeVincenzo et al. [12]. Cells were incubated for 2 h at 37 °C in the presence of 5 μg/ml Epi alone or with Tam. Tam was added 2 h prior to Epi treatment. Cells were then placed in ice to block the reaction (the energy requirement for Pgp function) until analysis. Rhodamine-123 (Rh-123) efflux was monitored as the following: cells were incubated in complete medium with Rh-123 (10 μg/ml) at 37 °C for 30 min. After the accumulation period, cells were placed in ice. Cells in half the number of tubes were collected and re-suspended in 1 ml ice cold PBS and kept in ice in the dark until flowcytometric analysis. Efflux was initiated in the other half of the tubes by sedimentation of cells in a cooling centrifuge and re-suspension in Rh-123 free medium. Cells were incubated at 37 °C for 1 h in the absence of Tam in case of control cells and in the presence of Tam in case of tested cells. At the end of the efflux period, cells were sedimented in a cooling centrifuge, re-suspended in 1 ml ice cold PBS and kept on ice in the dark until flowcytometric analysis. The same procedures of the accumulation and retention studies were carried out without using Rh-123 but instead depending on the fluorescent property of Epi alone. In all instances, propidium iodide 5 μg/ml was added to identify non-viable cells, a potential source of error in this kind of study. Ten thousands viable cells were analyzed for each sample using an Epics XL flowcytometer. The function of P-gp was calculated according to Osman et al. [13] using the following formula: Function of P-gp (%) = Rh-123 accumulation − Rh-123 efflux/Rh-123 accumulation.

2.8. Semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis of topoisomerase IIα (topo IIα) gene expression

Cells were plated at a density of 5×10^6 cells/ml. Epi was used at concentration of 0.5 μg/ml and the incubation lasted for 24 and 48 h time interval. Total RNA was extracted from the cells using Total RNA Isolation kit (TPIR, Abgene House, UK), in accordance with the manufacturer's instructions. The concentration and purity of RNA were quantified spectrophotometrically by measuring A_{260} and A_{280}; the ratio A_{260}/A_{280} of pure RNA is approximately 1.8. Reverse transcription reaction was carried out using Super Script One Step RT-PCR with Platinum Taq kit (Invitrogen Corporation, USA), in accordance with the manufacturer's instructions. PCR was set up as described previously [3]. Two sets of primers (Promega Biotech, Germany) were used in separate reactions, the first to yield the amplification of the specific target of interest, which is the topo IIα gene (200 base pair). The second is for the amplification of an endogenous control β-actin gene (621 base pair). Amplification of β-actin gene was performed to set a base line for each sample that enables the evaluation of the expression of the target gene and to confirm successfulness of the RT-PCR.

2.9. Statistical analysis

Data are presented as mean ± S.E.M. Unpaired *t*-test was used to compare two different treatment groups. Multiple comparisons were carried out using one way analysis of variance (ANOVA) followed by LSD (least significant difference) for post hoc analysis. Statistical significance was acceptable to a level of $p < 0.05$. Data analysis was achieved using software program SPSS (statistical package of social sciences, version 9).

3. Results

3.1. Assessment of cytotoxic activity

Cytotoxicity was expressed as the percentage of survival fraction compared with untreated control cells. SRB assay revealed that IC_{50} values for MCF-7 and NCI-adr cells treated with Epi were 67 and 314 ng/ml, respectively. Thus, NCI-adr cells were observed to be five-folds more resistant to the effects of Epi as compared to MCF-7 cells (Fig. 1; Table 1). Addition of 10 μM Tam 2 h prior to Epi treatment was found to sensitize both cell lines to the cytotoxic effect of Epi. It was found that Epi IC_{50} in the presence of Tam reached 15.5 ng/ml (for MCF-7) and 48 ng/ml (for NCI-adr) i.e. Tam induced 4.3- and 6.5-fold increase in sensitivity of MCF-7 and NCI-ad cells, respectively to the cytotoxic effect of Epi (Fig. 1; Table 1). To verify the type of interaction between Tam and Epi,

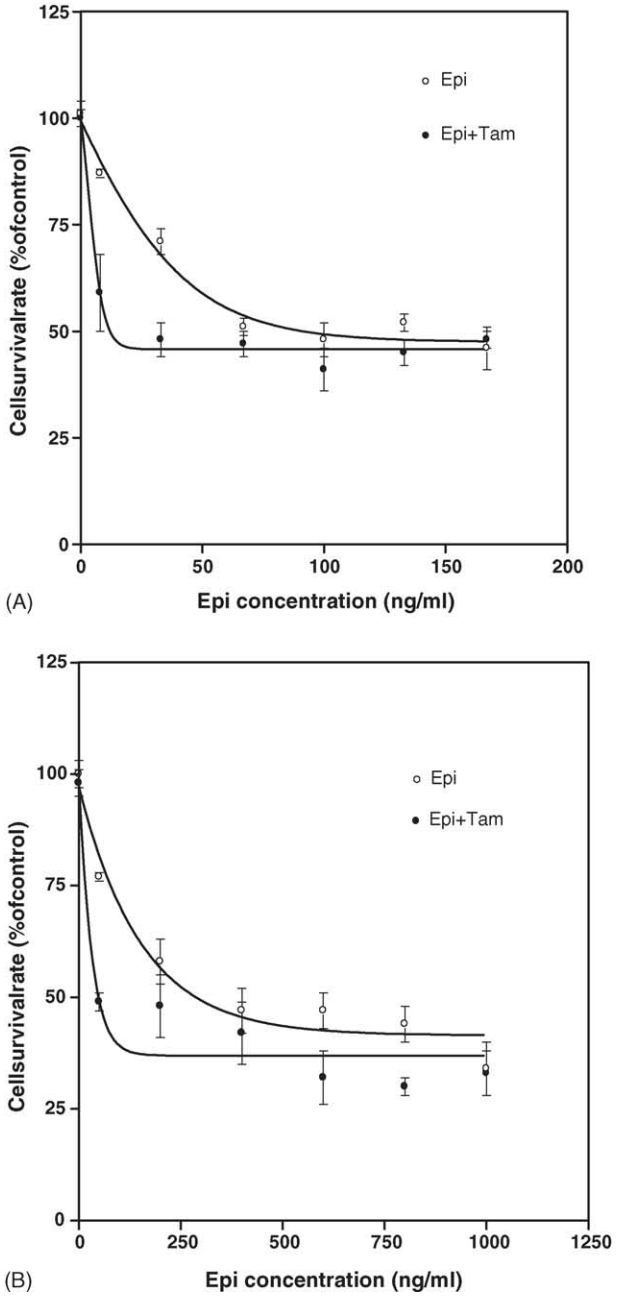

Fig. 1. Cytotoxic effect of Epi alone (○) or in the presence of Tam (●) on (A) MCF-7 cells or (B) NCI-adr cells. Cell counts were performed after 48 h of exposure to drugs. Each point is the mean ± S.E.M. of three separate experiments.

isobologram analysis was carried out and revealed that the interaction between Tam and Epi was additive in MCF-7 cell line ($I = 1.1$) and synergistic in NCI-adr cell line ($I = 0.55$) (Table 1).

3.2. Cell cycle analysis

Analysis of cell cycle distribution after Epi treatment presented evidence of a preferential block of breast cancer cells (either MCF-7 or NCI-adr) in G_2/M phase (Fig. 2; Table 2). Regarding MCF-7, when Epi concentration is increased from 0.02 to 0.2 μg/ml, the cell accumulation in G_2/M phase increased from approximately 9 to 42% at the expense of G_0/G_1 phase cells. On the contrary, in NCI-adr cells, upon increasing the dose of Epi from 0.02 to 0.2 μg/ml, a plateau arrest was detected in the percentage of G_2/M cells (Table 2). Tam pretreatment enhanced the blocking activity of low dose of Epi (0.02 μg/ml) by increasing in percentage of accumulation of MCF-7 cells in G_2/M phase (Fig. 2; Table 2). Regarding NCI-adr cells, addition of Tam prior to 0.2 μg/ml Epi induced an increase in the percentage of cells in S phase (nearly two-fold) at the expense of G_0/G_1 cells (Table 2).

3.3. Assessment of cellular Epi concentration

HPLC analysis of cellular Epi concentration revealed that there was a time-dependent increase in Epi concentration reaching its maximal level after 4 and 24 h of incubation for NCI-adr and MCF-7 cell line, respectively. Then, intracellular Epi concentration decreased after 48 h of incubation. Pre-treatment of cells with Tam did not alter the pattern of cellular uptake of Epi except of that Epi uptake reaches maximum level after 4 h (instead of 24 h) in MCF-7 cell line. Regarding NCI-adr cells, Tam pretreatment induced a significant increase in cellular Epi level only after 48 h of incubation (Fig. 3).

3.4. Assessment of P-gp function

By using Rh-123 as tracer, it was found that the percentage of functional activity of P-gp pump in MCF-7 cells was 16%. Upon treatment of cells with Epi, the percentage efflux significantly increased to 33% as compared with the

Table 1
Effect of Tam on Epi cytotoxicity in MCF-7 and NCI-adr cells

Drugs	MCF-7 cell line			NCI-adr cell line		
	IC_{50} (ng/ml)	MDR reversal fold	Interaction index (I)	IC_{50} (ng/ml)	MDR reversal fold	Interaction index (I)
Epi	67	4.3	1.1	314	6.5	0.55
Epi + Tam	15.5			48		

IC_{50}: The concentration of Epi necessary to produce 50% inhibition of cell growth, MDR reversal fold: IC_{50} of Epi/IC_{50} of Epi + Tam, interaction index: $I = d_1/D_1 + d_2/D_2$, where d_1 and d_2 are the respective concentrations of Epi and Tam used in the combination required to produce a fixed level of inhibition IC_{50}, while D_1 and D_2 are their concentrations able to produce alone the same magnitude of effect.

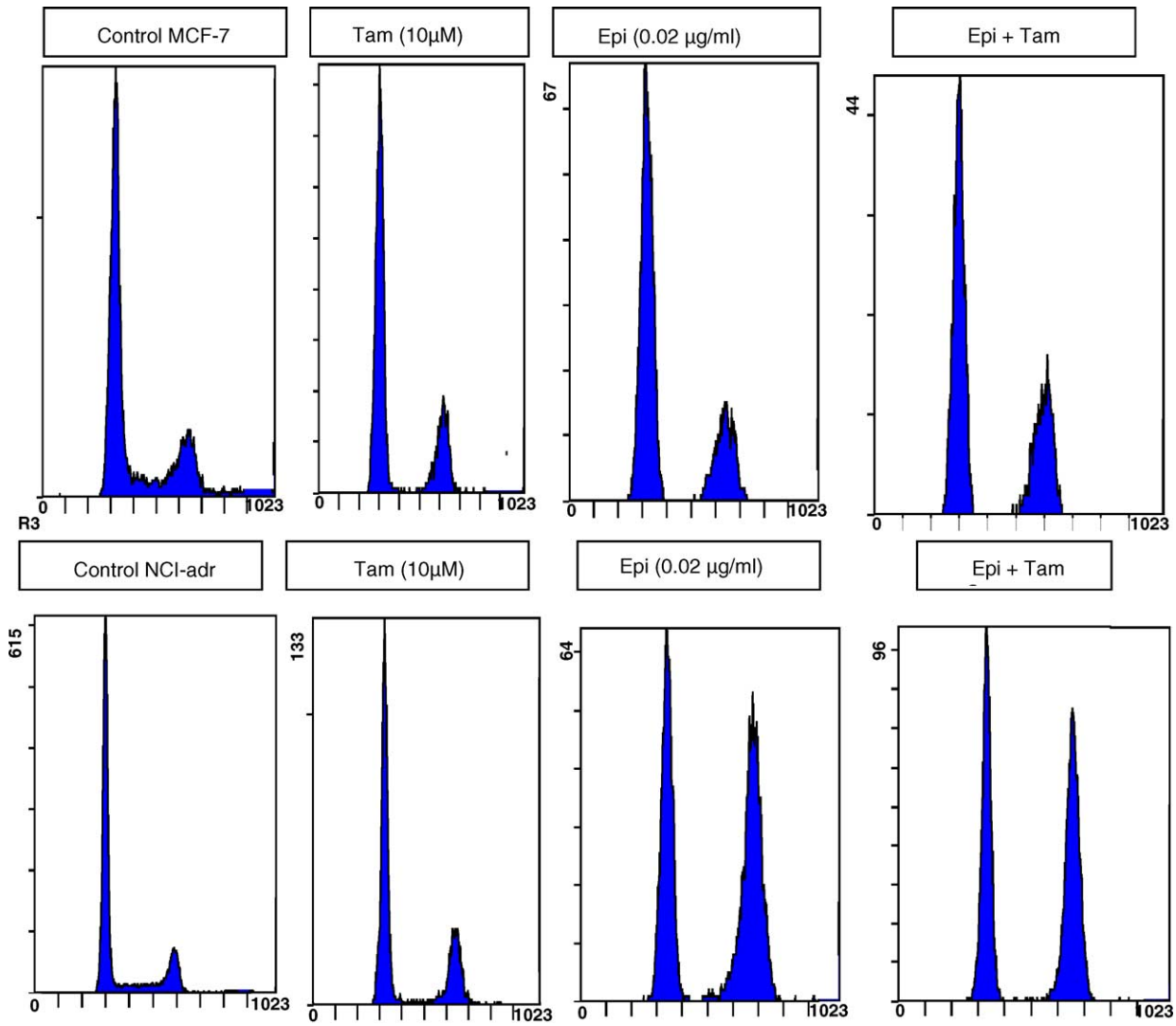

Fig. 2. Cell cycle analysis of MCF-7 cell and NCI-adr cells treated with Epi (0.02 μg/ml) and/or Tam (10 μg/ml). The percentage of cells in the G_0/G_1, S and G_2/M phases are detailed in Table 2. Data refer to two experiments.

control group (Fig. 4). Pretreatment of cells with Tam decreased this percentage to reach 27%. However, the percentage was still significantly higher than the control value. Moreover, treatment of cells with Tam alone was found to induce a significant increase in percentage efflux to 34% as compared to the control group (Fig. 4). These results were confirmed by using Epi as tracer (data not shown). In addition, as expected, the NCI-adr cell line exhibited significantly higher percentage of P-gp efflux when compared to MCF-7 cell line, 40% for the former and 16% for the later (Fig. 4). However treatment of NCI-adr cells with Epi, Tam or their combination did not alter significantly the percentage of efflux of Rh-123 (Fig. 4). These results were confirmed using Epi as a racer (data not shown). Moreover, comparing to cyclosporine A, a classical P-gp blocking agent, Tam was found to be ineffective (Fig. 4).

Table 2
Effect of Epi and/or Tam on on cell cycle in MCF-7 and NCI-adr cells

Groups	MCF-7 cell line			NCI-adr cell line		
	G_0/G_1	S	G_2/M	G_0/G_1	S	G_2/M
Control	80.3	10.1	9.6	72.9	17.7	9.4
Epi (0.02 μg/ml)	87.5	3.8	8.7	50.8	3.6	45.6
Epi (0.2 μg/ml)	55.8	2.1	42.1	42.4	5.4	52.2
Tam (10 μM)	92.3	3.5	4.2	83	6.5	10.5
Epi (0.02 μg/ml) + Tam	71	3.3	25.7	50	3.5	46.5
Epi (0.2 μg/ml) + Tam	55.7	3.8	40.5	38.6	9.4	52

Values are represented as percentages of cells at the indicated cell cycle phases.

3.5. Assessment of topo IIα gene expression

The effects of Epi, Tam and their combination on topo IIα gene mRNA level were evaluated by calculating the ratio of its expression to that of β-actin by semiquantitative analysis. In the control MCF-7 and NCI-adr cells, topo IIα gene was expressed positively (Fig. 5). However, no

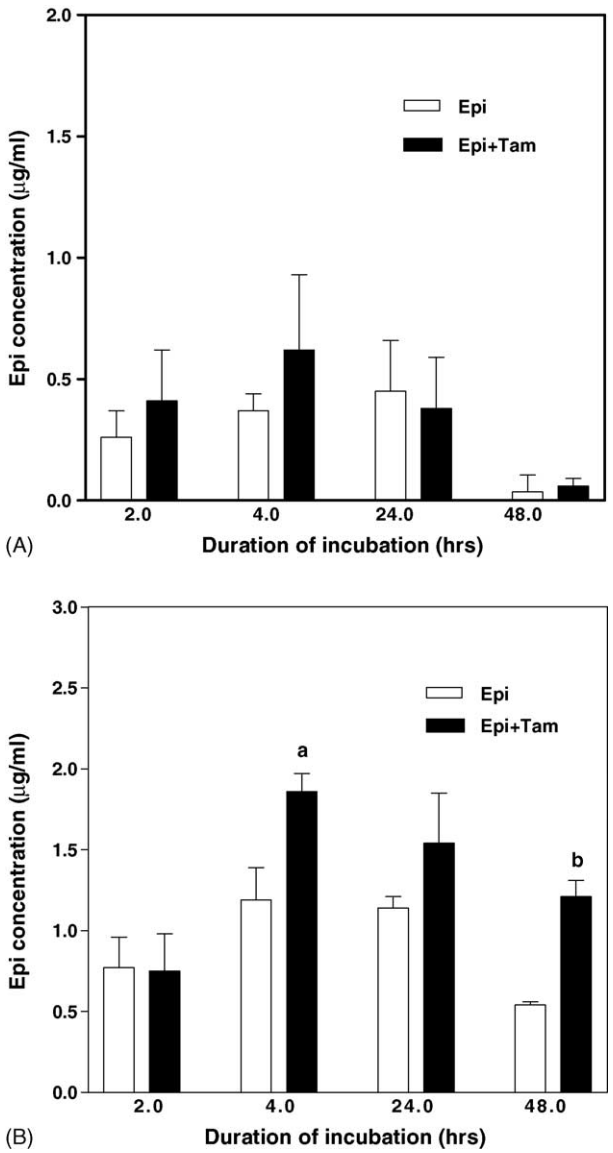

Fig. 3. Time course uptake of Epi (5 μgm/ml) in absence (□) and presence (■) of Tam (10 μM) in (A) MCF-7 cells and (B) NCI-adr cells. a: Statistical significance as compared to 2 h time interval using unpaired *t*-test. b: Statistical significance as compared to corresponding Epi group using unpaired *t*-test.

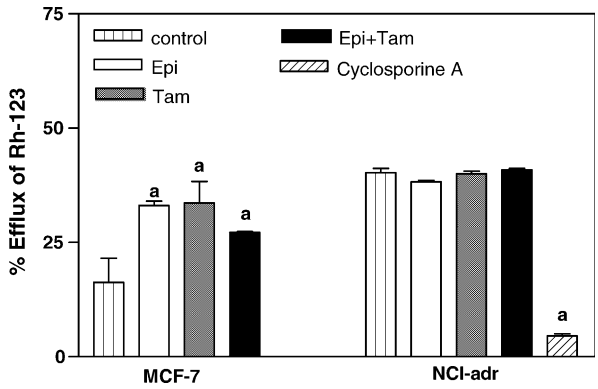

Fig. 4. Effect of Epi (5 μgm/ml) and/or Tam (10 μM) on P-gp function expressed as % efflux of Rh-123 tracer in MCF-7 and NCI-adr cells. Cyclosporine was used as a classical P-gp blocking agent. a: Statistical significance as compared to corresponding control group using one way ANOVA followed by LSD as post hoc test.

significant difference was found between the expressions of topo IIα gene in control cells of both cell lines (Table 3). Moreover, treatment of both sensitive and resistant cell line with Epi and/or Tam resulted in non-significant change in the expression level of topo IIα gene as compared to the control values (Table 3).

4. Discussion

The current study is the first in vitro trial to investigate the modulatory effect of anti-estrogen, Tam on Epi cytotoxicity in breast cancer cell lines; MCF-7 and NCI-adr. The possible modulatory mechanisms were explored by studying the cell cycle perturbation, changes in intracellular Epi accumulation, P-gp functional analysis as well as topo IIα expression. In the present study, Tam was used at a concentration of 10 μM that resembles the plasma level achieved when Tam is used clinically [14,15].

At the beginning we studied the sensitivity of breast cancer MCF-7 and NCI-adr cells to the cytotoxic effect of Epi and its modulation by Tam pretreatment. From dose survival curves, NCI-adr cell line was found to be five

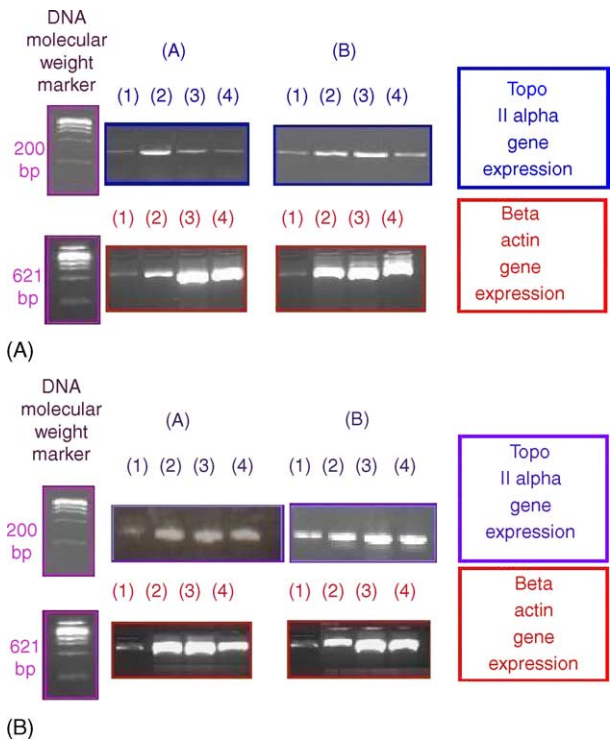

Fig. 5. RT-PCR analysis of topo IIα gene in (A) MCF-7 cells and (B) NCI-adr cells, using β-actin gene as an endogenous control. Lane (1): control cells, Lane (2): Tam (10 μM) treated cells, Lane (3): Epi (0.5 μg/ml) treated cells and Lane (4): Tam (10 μM) and Epi (0.5 μg/ml) treated cells. The sizes of the specific RT-PCR products are 200 bp for topo IIα gene and 621 bp for β-actin gene. The cell line was treated at (a) 24 h and at (b) 48 h intervals.

Table 3
Effect of Epi and/or Tam on the expression of topo IIα gene in MCF-7 and NCI-adr cells, using β-actin gene as an endogenous control

Groups	MCF-7 cell line		NCI-adr cell line	
	24 h[a]	48 h	24 h	48 h
Control	0.68 ± 0.255	0.54 ± 0.115	0.52 ± 0.06	0.72 ± 0.225
Epi (0.5 μg/ml)	0.88 ± 0.025	0.89 ± 0.01	0.89 ± 0.18	0.86 ± 0.12
Tam (10 μM)	0.97 ± 0.27	0.76 ± 0.075	0.99 ± 0.085	0.86 ± 0.13
Epi + Tam	0.79 ± 0.16	0.66 ± 0.005	0.75 ± 0.205	0.92 ± 0.15

[a] Drug treatment was carried out for 24 and 48 h.

times more resistant to the cytotoxic effect of Epi, when compared to MCF-7 cell line. Pre-treatment of cells with Tam was observed to enhance Epi cytotoxicity by 4.3- and 6.5-folds in the MCF-7 and NCI-adr cells, respectively. The interaction between Epi and Tam was additive in MCF-7 cell line and synergistic in the NCI-adr cell line. So, Tam was found to induce more pronounced sensitizing effect in NCI-adr cell line than its effect in MCF-7 cell line.

To gain further insight into the interaction mechanism between Tam and Epi, flowcytometric DNA ploidy analysis was performed and revealed that Epi treatment induced preferential cell arrest at G_2/M phase. Tam pre-treatment enhanced the blocking activity of low dose of Epi in MCF-7 and induced nearly two-fold increase in the percentage of S phase in NCI-adr cells. Indeed, cells in the S and G_2/M phases are the most sensitive to the effect of Epi. It has been justified that the anthracyclines are most active on proliferating cells in the S and G_2 phases due to the maximal expression of their target enzyme topo II at these phases [16,17]. Therefore, from the flowcytometric study we can conclude that the modulatory effect of Tam on Epi cytotoxicity can be explained by the increase in the accumulation of cells in G_2/M phase (for MCF-7 cell line) and S phase cells (for NCI-adr cell line), since these phases are the most sensitive phases.

To determine if the modulatory effect of Tam on Epi cytotoxicity was mediated by changing its cellular accumulation, Epi level was detected and revealed that Tam preserved the intracellular Epi level significantly higher in resistant NCI-adr cells after 48 h. So, from the results of uptake studies and cytotoxicity assays, we can find a good connection between increased Epi accumulation and reversal of MDR by Tam in NCI-adr cell line. Our results are confirmed by an earlier study of Kang and Perry [18], which demonstrated that pre-treatment of chinese hamster ovary cells with Tam resulted in an increased doxorubicin accumulation. These observations suggested that the reversal effect of Tam on MDR is likely to be, at least partially, mediated by enhanced intracellular accumulation of the anthracyclines.

Accordingly, we tried to explore if the increase in intracellular Epi accumulation is related to a decrease in the expression of P-gp function, which is the most consistent morphological finding in the MDR phenotype. As expected, the NCI-adr cell line was found to exhibit significantly higher percentage of P-gp efflux when compared to MCF-7 cell line, 40% for the former and 16% for the later. There are two important points should be discussed. Firstly, in NCI-adr cells, Tam failed to alter significantly the percentage of efflux of tracer as compared to cyclosporine A, a classical P-gp blocking agent. So, in the present study, the connection between the enhancement of Epi cytotoxicity and the increase in its accumulation in the resistant NCI-adr cells can not be explained on the basis of decreasing P-gp activity. Indeed, there is discrepancy between previous studies about Tam as a P-gp modulator. De Vincenzo et al. [12] reported that although Tam potentiated doxorubicin cytotoxicity in P-gp positive cells, it was found to be not effective on modulating P-gp activity. On the other hand, other studies showed that Tam is a P-gp modulator [6,19]. The reasons underlying these discrepancies are still unknown. It is likely that differences in the assay systems, such as the model used, the time scales over which the experiment was conducted and the concentrations of the agent tested, may be responsible.

Beyond the effect of Tam as P-gp modulator, another explanation for the enhancement effect of Tam on Epi accumulation can be postulated according to the study of Ramu et al. [20]. They suggested that Tam may interact with the membrane phospholipids domain in such a manner as to alter the lipid packing density and thereby the increase in the diffusion rate of certain drugs. However, the exact nature of this interaction with the cellular membrane has not been clarified. Thus, taken together, data from literature and our present results we can suggest that the ability of Tam to potentiate Epi cytotoxicity does not solely reflect a direct modulation of P-gp function, but is likely to involve other additional mechanisms that require further investigations.

The second important point; treatment of sensitive MCF-7 cells with either Epi and/or Tam was found to induce a significant increase in the percentage efflux of tracer as compared to the control cells. These results can be explained on the following basis: MDR can be acquired after initial exposure to cytotoxic agents [21]. In addition, several studies have found that P-gp antagonists such as verapamil and cyclosporine A can induce P-gp expression in colon carcinoma cells [22]. It is important to note that the time needed for expression and inhibition of P-gp by their antagonists is controversial. So, if a similar modulation of the MDR phenotype may occur in vivo then, the

duration of treatment with Tam becomes important to design appropriate clinical trials.

Since, topo II is the target site for anthracyclines and the development of resistance may occur by decreasing topo II expression [23], we assessed the expression of topo IIα and found that, in both cell lines, topo IIα gene was expressed positively. However, there was non-significant difference in its expression between sensitive and resistant cell line. In addition, treatment of both MCF-7 and NCI-adr cells by Epi and/or Tam resulted in a non-significant change in the expression level of topo IIα gene mRNA as compared to the control cells. Although the present results showed unexpected lack of correlation between topo II expression and cell resistance to Epi, these results are in accordance with few previous studies. Schneider et al. [24] examined the mRNA levels of topo IIα by quantitive PCR and found that its level of expression was essentially the same in both MCF-7/WT and its etoposide resistant counterpart MCF-7/VP-16 cells. Also, our results are further emphasized by a most recent study conducted by Petit et al. [25] who described that topo IIα expression was not a predictive marker of either the clinical or radiological tumor response to anthracyclines based chemotherapy in breast cancer.

In conclusion, Tam, an anti-estrogenic drug, potentiates Epi cytotoxicity in sensitive and resistant breast cancer cell lines. The postulated mechanisms underlying this interaction include an enhancement of accumulation of cells in S and G_2/M phases, at which the cells are most sensitive to the cytotoxic effect of Epi as well as an increase in the intracellular level of Epi in resistant cell line. Other postulated mechanisms for cell resistance including modulation of P-gp function and topo IIα gene expression were not found to have a role in the present study. Because of the diverse biological effects of Tam and the complex mechanisms whereby it enhances Epi accumulation and cytotoxicity, further studies are needed to clarify other mechanisms by which Tam can enhance Epi cytotoxicity.

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