

Cross-resistance to Vincristin and Etoposide in a sub line of the human breast cancer T47D cells selected for Adriamycin-resistance

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Abstract

Breast cancer is one of the most common malignancies among women. Although chemotherapy remains a major therapeutic approach to treat cancers, drug therapy often fails for several reasons, particularly the drug resistance. Resistance to multiple chemotherapeutic agents is one of the most important problems in the treatment of different types of cancers. Therefore, in this study a resistant sub line of the human breast cancer T47D cells was isolated *in vitro* by stepwise exposure to increasing concentrations of Adriamycin (ADR) to compare the characteristics of parent and resistant cells. We also evaluated the phenomenon of cross-resistance to some other chemotherapeutic drugs. A significant increase in doubling time of resistant cells, named T47D/ADR, (94 h) was observed when compared to the parental T47D cells (50 h) that indicates a relatively slow growth rate pattern of these cells. T47D/ADR cells were 4 fold resistant to adriamycin and also showed cross-resistance to vincristin (VCR, 3.5 fold) and to etoposide (VP-16, 5.5 fold) when compared to parent cells. Therefore, our results indicate that T47D/ADR cells are also cross-resistant to structurally and functionally different chemotherapeutic agents and can be used as a model for studying molecular changes of drug resistance.

Keywords: Breast cancer; T47D cells; Adriamycin; Etoposide; Vincristin; Drug resistance.

Introduction

In recent years, the incidence and mortality of breast cancer among women worldwide have become the most important medical issues. With progress in understanding of the pathobiology of breast cancer, diagnosis and treatments have improved (1, 2, 3).

Unfortunately, resistance to multiple chemotherapeutic agents is a common problem in the treatment of different types of cancers including breast cancer. This resistance termed multidrug resistance (MDR) (4, 5), may be intrinsic or acquired by tumor cells during or after treatment. Several drugs including Adriamycin (ADR), Vincristin (VCR), and Etoposide (VP16) with different structures and

mechanisms of antitumor actions fail to be effective due to MDR phenomenon (6-9).

Adriamycin, an anthracycline antitumor drug, is clinically active against many human malignancies including breast cancer (10-12). Several mechanisms explain the antitumor activity of ADR including DNA intercalation, inhibition of topoisomerase II α (TOPO II α), interaction with membrane, and generation of oxygen free radicals (13, 14). Vincristin is a vinca alkaloid antitumor agent, which exerts its effects by binding to tubulin and therefore inhibiting microtubule formation during mitosis (6). Etoposide is a derivative of podophyllotoxin, which inhibits TOPO II α and is believed to cause breakdown of DNA (15).

Therefore, this study was conducted to further elucidate the changes that occur in drug resistant cells compared to parent cells. We

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isolated an Adriamycin-resistant sub line of the human breast cancer T47D cells *in vitro* by gradual exposure to increasing concentrations of ADR. This sub line named T47D/ADR was then compared to the parental cells with respect to the growth characteristics and cross-resistance to VCR, and VP16.

Experimental

Methods

Cell line and culture conditions

The human breast cancer T47D cell line (ATCC HTB-133, USA) was obtained from Pasteur Institute Cell Bank of IRAN (Tehran, IRAN). Cells were maintained in RPMI-1640 (Gibco, USA) culture medium supplemented with 10% fetal bovine serum (Gibco, USA) and 100 U/ml of penicillin and 100 ng/ml of streptomycin (Sigma, UK) at 37°C in 5% CO₂ incubator.

Establishment of an Adriamycin-resistant sub line

An Adriamycin-resistant sub line was isolated by continuous exposure of T47D cells to ADR (Adriablastina, Italy) at concentrations starting from 1×10^{-9} M and increasing in a stepwise manner to 2.5×10^{-8} M within 9 months. Cells that were capable of sustained growth in

medium containing 1×10^{-8} M of ADR were considered to be resistant to Adriamycin and are referred to T47D/ADR cells here after. Cell viability was determined after each step using trypan blue dye exclusion method.

Determination of growth characteristics of T47D and T47D/ADR Cells

T47D and T47D/ADR cells were seeded in 24 well plates at 6×10^4 cells/well in 1 ml growth medium and incubated in the presence or absence of ADR at 37°C in 5% CO₂ incubator. After washing with PBS, the cells were trypsinized and then counted using trypan blue dye exclusion method every 48 h for 11 days. The doubling time for each cell population was then determined from its growth curve, in which each point was the average determination of triplicate wells in three independent experiments.

Cytotoxicity and cross-resistance assay

The MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) based assay was performed by seeding 2000 cells in 100 μ l growth medium in the presence of increasing concentrations of chemotherapeutic drugs (ADR: 0.1 to 100 nM, VCR: 0.01 to 100 nM, VP16: 0.1 to 1000 nM) into 96-well plates and incubated at 37°C in 5% CO₂ incubator for 96 hours. The cells were then incubated with 25 μ l MTT (5 mg/ml) at 37°C for 4 hours. After dissolving the formazan crystals in 0.04 N HCl in isopropanol, plates were read in a micro plate reader (Dynatech Lab Inc, USA) at 570 nm. This experiment was performed in triplicate determination and repeated three times.

Statistical analysis

SIGMASTAT™ (Jandel Software, San Raphael, CA) was used to perform statistical analysis of data. The students t-test was used to examine the differences among treatments. Mean differences with P values less than 0.05 were considered to be significant.

Results and Discussion

Growth characteristics of T47D and T47D/ADR Cells

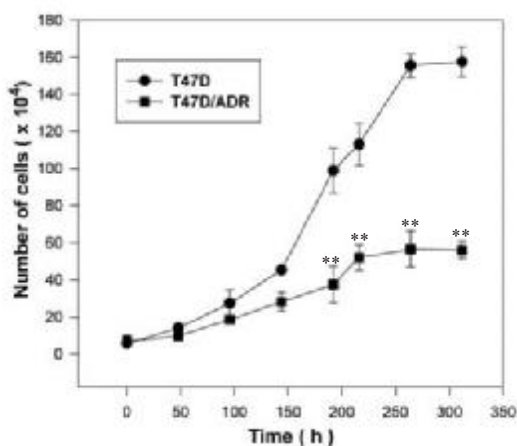


Figure 1. Growth characteristics of T47D and T47D/ADR Cells.

Cells were seeded in 24 well plates at 6×10^4 cells/well in the complete RPMI 1640 culture medium and then counted using trypan blue dye exclusion method every 48h for 11days. Triplicate determinations were used for each point and data presented as Mean \pm SE of three independent assays (N=3). ** indicates significant difference between parent and resistant cells ($P < 0.001$).

The doubling time of the T47D/ADR cells (94 h) increased significantly as compared with that of the parental T47D cells (50 h). This indicates the slow growth rate pattern of isolated resistant cells (Figure 1). The viability assay also showed more than %95 viable cells in all steps of experiments.

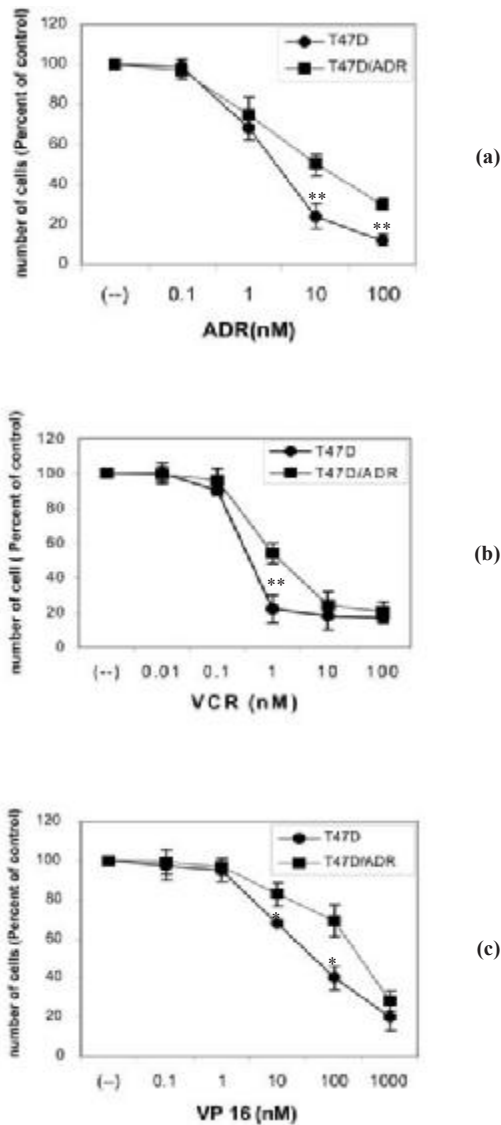


Figure 2. Cytotoxicity of ADR, VCR, and VP16 on T47D and T47D/ADR cells. Cells were seeded in 96 well plates at 2000 cells/well. After 96 hours of exposure to different concentrations of drugs, MTT based assay was performed. **a)** ADR (conc. 0.1 to 100 nM), **b)** VCR (conc. 0.01 to 100 nM), and **c)** VP16 (conc. 0.1 to 1000 nM). Triplicate determinations were used for each point and data presented as Mean +/- SE of three independent assays (N=3). * indicates significant difference between parent and resistant cells (P < 0.05). ** indicates significant difference between parent and resistant cells (P < 0.01).

Table 1. IC₅₀ values of ADR, VCR, and VP16 for T47D and T47D/ADR cells.

The T47D cells and its ADR-resistant subline were treated with or without ADR, VCR, and VP16 for 96 h in 96 well plates to determine their effects as indicated in the experimental section.

Cell Line	IC ₅₀ [M] (fold resistance)		
	ADR	VCR	VP16
T47D	2.5 x 10 ⁻⁹	3.7 x 10 ⁻¹⁰	4 x 10 ⁻⁸
T47D/ADR	1 x 10 ⁻⁸ (4)	1.3 x 10 ⁻⁹ (3.5)	2.2 x 10 ⁻⁷ (5.5)

Cytotoxicity of ADR, VCR, and VP16 on T47D and T47D/ADR cells

The anti-proliferative effects of ADR, VCR and VP16 on T47D cells and its resistant sub line measured by MTT based assay indicate the cross-resistant properties of T47D/ADR cells to VCR and also VP16 (Figure 2). The IC₅₀ value for each drug determined from corresponding survival curves was used to calculate the fold-resistance to each drug by T47D/ADR compared to the parental T47D cells (Table 1).

Many adriamycin-resistant cells exhibited a high level of cross-resistance to other structurally unrelated drugs that is called multidrug resistance (MDR) (4, 16, 17). Son et al. showed that an ADR resistant human stomach-adenocarcinoma cell line (MKN/ADR) had a high level of cross-resistance to topoII-targeted drugs such as mitoxantron and etoposide but showed no cross resistance to other chemotherapeutic agents such as cisplatin, carboplatin and 5-FU. The doubling time of the MKN/ADR cells (2.1 days) was more than the parent MKN cells. They suggested that a quantitative reduction in topo II α may contribute to the resistance of MKN cells to ADR and other topoII-targeted drugs. It has also been indicated that the differences in topo II α expression was not the reason for the difference in growth rate (10, 18).

In fact, one possible explanation for the reduced proliferation rate and drug resistance would be a reduction in expression of topo II α which is required for DNA replication and has been correlated with the cell proliferation (19-22). Reduced expression of topo II α has been implicated as a mechanism of resistance to topo II inhibitors such as ADR, VP16, m-amsacrine and mitoxantron (23, 24). Wosikowski et al. indicated that the doubling time of resistant cells increased significantly compared with parental

cancer cells. In contrast to other studies, they failed to show correlation between topo II α expression and proliferation rate of resistant cells (25).

In other study, an etoposide-resistant breast cancer cell line (MCF7/VP16) was 28, 21 and 9 fold resistant to VP16, Vm26 and doxorubicin, respectively. MCF7/VP16 cells also exhibited 2.8 and 5 fold resistance to mitoxantron and vincristin but no cross-resistance to camptothecin, an inhibitor of topo I. Their results showed that resistance to epipodophyllotoxines in MCF7/VP16 cells is multi-factorial involving reduction in intracellular drug concentration, possibly MRP over expression, and also altered topo II α drug sensitivity (15).

Another study showed a strong correlation between the degree of P-gp expression and *in vitro* resistance to taxol and adriamycin (26). Overexpression of P-gp or MRP levels may reduce ADR and VCR accumulation and increase drug efflux. On the other hand, reduction in the topo II α protein levels diminishes the main intracellular target of ADR and VP16 and other topo II α poisons. These changes may confer cross-resistance to these agents (6,17).

Therefore, observed resistance in the T47D/ADR cells apparently involves multiple mechanisms including topo II α , MDR1, and other genetic and epigenetic alterations. Finally, it cannot be excluded that the cells selected for resistance to chemotherapeutic drugs, in our study as well as other similar studies, compose of a heterogeneous population of cells each with its own distinct characteristics and mechanism(s) of developing resistance to tested chemotherapeutic drugs that needs to be further elucidated.

Acknowledgement

The authors are thankful to the office of vice-chancellor for research of Tehran University of Medical Sciences (TUMS) for the financial support of this project.

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