Drug Resistant MCF-7 Cell Lines Also Developed Cross-Resistance to Structurally Unrelated Anticancer Agents

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ABSTRACT

The cells developing resistance to an applied drug may also present cross-resistance to other anticancer drugs which are not applied. In this study, the development of cross-resistance in paclitaxel (MCF-7/Pac), docetaxel (MCF-7/Doc), vincristine (MCF-7/Vinc) and doxorubicin (MCF-7/Dox) resistant MCF-7 cells to selective anticancer drugs, tamoxifen and all trans-retinoic acid (ATRA) were investigated. Combined antiproliferative effects of these drugs in different combinations were also evaluated by checkerboard combination assay. MCF-7/Pac and MCF-7/Doc cells developed cross-resistance to vincristine (13- and 12-folds, respectively) and tamoxifen (3- and 2-folds, respectively). MCF-7/Dox cells developed cross-resistance to paclitaxel (109-fold), docetaxel (10-fold), tamoxifen (2-fold) and ATRA (3-fold). MCF-7/Vinc cells developed cross-resistance to paclitaxel (48-fold), doxorubicin (6-fold) and tamoxifen (2-fold). Combinations of paclitaxel and docetaxel with doxorubicin exerted synergic antiproliferative effect. Tamoxifen had synergic effect with doxorubicin and vincristine. ATRA had indifferent effect with paclitaxel, docetaxel and doxorubicin where it had antagonistic effect with vincristine. The data presented here may provide an insight to assess response of breast tumors to anticancer drug combinations.

Key Words: Multiple drug resistence, MCF-7, Cross-resistance, Chemotherapy

ÖZET

Dirençli MCF-7 Hücre Hatlarında Yapısal Olarak Farklı Antikanser İlaçlara Karşı Çapraz Direnç Gelişimi

Kemoterapi sırasında uygulanan belli bir ilaca direnç geliştiren hücreler, diğer ilaçlara da çapraz direnç geliştirebilmektedir. Bu çalışmada amaç, paklitaksel (MCF-7/Pac), dosetaksel (MCF-7/Doc), vinkristin (MCF-7/Vinc) ve doksorubisine (MCF-7/Dox) dirençli MCF-7 hücre hatlarında paklitaksel, dosetaksel, vinkristin, doksorubisin, tamoksifen ve all-trans retinoik asit çapraz direnç gelişiminin ve dirençli hücrlerde farklı ilaç kombinasyonlarının antiproliferatif etkilerinin "checkerboard combination assay" yöntemi ile incelenmesidir. Sonuçlar değerlendirildiğinde, MCF-7/Pac ve MCF-7/Doc hücrelerinde vinkristin (sırasıyla 13 ve 12 kat) ve tamoksifene (sırasıyla 3 ve 2 kat) karşı çapraz direnç geliştiği görülmüştür. Bunun yanısıra, MCF-7/Dox hücrelerinin 109 kat paklitaksele, 10 kat dosetaksele, 2 kat tamoksifene ve 3 kat ATRA'ya, MCF-7/Vinc hücrelerinin ise 48 kat paklitaksele, 6 kat doksorubisine ve 2 kat tamoksifene çapraz direnç geliştirdiği saptanmıştır. Kombine ilaç uygulamalarında, paklitaksel ve dosetaksel doksorubisin ile, tamoksifen ise doksorubisin ve vinkristin ile beraber sinerjik antiproliferatif etki göstermiştir. ATRA'nın paklitaksel, dosetaksel ve doksorubisin ile indiferant (birbirinden bağımsız) vinkristin ile beraber uygulandığında ise antagonist etkileri olduğu saptanmıştır. Bulgular, meme tümörlerinde uygulanan antikanser ilaç etkilerinin değerlendirilmesi açısından önem taşımaktadır.

Anahtar Kelimeler: Çoklu ilaç direnci, MCF-7, Çapraz direnç, Kemoterapi

INTRODUCTION

Most patients with metastatic breast cancer typically respond to initial chemotherapy, but many have disease recurence that is a serious limitation to the effective chemotherapeutic treatment. One of the causes of disease reoccurence is multidrug resistance[1], a complex phenotype whose predominant feature is resistance to wide range of structurally unrelated anticancer agents[2]. There are several mechanisms by which cancer cells develop resistance to cytotoxic agents. Increased drug efflux that results from up-regulation of ATP binding cassette (ABC) transporters[3,4], mutations in genes encoding drug target proteins[5], differential expressions of drug target proteins and anti/proapoptotic proteins[6] are some common mechanisms of drug resistance.

Combination chemotherapy is a common practice in the treatment of breast cancer, in particular, tumors acquiring resistance to first-line chemotherapy. However, combination effect of two chemotherapeutics is not addition of antitumor activities of two drugs. Drug interactions may result in antagonism as well as synergistic, additve or indifferent effect.[7] The interactions of administrated drugs are related to effects of these drugs on pharmacokinetic (absorption, distribution or metabolism) and/or pharmacodynamic (cellular targets or cell cycle) parameters.[8] Athough a proper combination therapy has increased efficacy, multidrug resistance mechanisms developed against an agent may also effect the cytyotoxicity of another agent. Therefore, tumor drug responsiveness in second-line chemotherapy may be related to differences in the capacities of the drugs to induce cross-resistance to each other and possibly to other drugs.

Antimicrotubule agents including paclitaxel, docetaxel and vincristine are widely used in the treatment of variety of tumors including metastatic breast cancer.[9,10] Taxoid drugs, paclitaxel and its semisynthetic derivative docetaxel bind to ß-tubulin of microtubules and stabilize microtubules against depolymerization.[11,12] The vinca alkaloid vincristine also binds to beta-tubulin, however prevents polymerization of microtubules.[13] Another anticancer agent, doxorubicin, has been effectively used for the treatment of breast cancer. It disrupts the uncoiling of DNA by topoisomerase II [14,15], intercalates between DNA strands[16] and induces G2-M cell arrest.[17] Tamoxifen is a synthetic non-steroidal antiestrogen that is used in the treatment of estrogen receptor-positive breast cancer patients.[18] Retinoids, alone or in combination, have been promising agents for treatment of some cancers including breast cancer. All-trans-retinoic acid (ATRA) is a natural ligand of nuclear retinoic acid receptors (RARs) that activate RA-responsive genes which have effects on cell proliferation, differentiation and apoptosis.[19]

In the present study, paclitaxel, docetaxel, vincristine and doxorubicin resistant MCF-7 breast carcinoma cells were selected as previously described[20] and served as in-vitro models for acquired multidrug resistance. The selective anticancer drugs (paclitaxel, docetaxel, doxorubicin and vincristine) tamoxifen, and ATRA were tested for development of cross-resistance in the resistant cell lines. The combined applications of the anticancer agents were also studied.

MATERIALS AND METHODS

Chemicals

Paclitaxel, tamoxifen, all-trans retinoic acid (ATRA) (Sigma, St. Louis, MO, USA) and docetaxel (Fluka, St. Gallen, Switzerland) were dissolved in dimethyl sulfoxide (DMSO) to prepare stock solutions. Vincristine and doxorubicin were diluted in deionized water. Cell Proliferation Kit (Biological Industries, Israel) was used in cyototoxicity tests.

Cell Lines

The drug resistant MCF-7 cell lines, which were models for drug resistant human mammary carcinoma, were used. Parental MCF-7 cell line was donated by ŞAP Institute, Ankara-Turkey and resistant sublines were developed from parental MCF-7 cells. The features and growth conditions of the parental and resistant sublines were described previously by Kars et al.[20] The sublines resistant to 400 nM paclitaxel (MCF-7/Pac), 120 nM docetaxel (MCF-7/Doc), 120 nM vincristine (MCF-7/Vinc) and 1000 nM doxorubicin (MCF-7/Dox) were used to test the antiproliferative effects of anticancer agents.

Assays for Cell Proliferation and Cross Resistance

The effects of the chemotherapeutic agents and their combinations on the proliferation of sensitive and resistant MCF-7 cell lines were tested in 96-well microtiter plates. Antiproliferative effects of anticancer agents on parental MCF-7 cells and drug selected sublines were evaluated by means of Cell Proliferation Kit (Biological Industries, Israel). Assay is a colorimetric test based on the tetrazolium salt (XTT). In brief, cells were seeded to 96-well microtiter plates (5 x 10^3 cells/well) and incubated for 72 h in medium containing horizontal dilutions of anticancer agents (except for the control wells). Dilutions of 0.4-200 μM for docetaxel, doxorubucin, tamoxifen, ATRA; 0.2-100 μM for paclitaxel; and 0.1-80 μM for vincristine were applied horizontally. XTT reagent was applied to form a soluble dye which was measured at 500 nm with a Spectromax 340 (Molecular Devices, Sunnyvale, CA, USA). Control wells were set as 100% cell viability. Average of viable cell numbers at different drug concentrations were expressed as percentage of the control and percent cell proliferation versus log (anticancer drug concentration) curves were constructed. For the assessment of antiproliferative effect of the drugs on cells, inhibitory concentrations (IC50), drug concentrations at which 50% of cells were viable, were calculated from the logarithmic trendlines of the proliferation graphs. Then, resistance indices of each cell line to anticancer agents were calculated to determine the degree of acquired resistance or cross-resistance of each cell line to anticancer drugs. The resistance indices (R = IC50 resistant cell line / IC50 sensitive cell line) were evaluated.

Combination Test by Checkerboard Microplate Method

Checkerboard combination assay was applied to study the effects of drug interactions between two anticancer drugs on resistant MCF-7 cell lines as previously described.[21] In brief, the dilutions of anticancer drugs (A) were made in horizontal direction and the dilutions of second drug (B) vertically in microtiter plate in 100 μL. The cells were distributed to each well in 50 μL containing 5 x 10^3 cells and incubated for 72h at 37°C in CO_2 incubator. The cell growth was determined after XTT staining and intensity of colored formazan crystals was analysed on ELISA reader. Drug interaction was evaluated according to the following expressions:

FICA= IC50A in combination / IC50A alone [Equation 1]

FICB= IC50B in combination / IC50B alone [Equation 2]

where FIC is fractional inhibitory concentration.

Fractional inhibitory index, FIX = FICA + FICB [Equation 3] demonstrates the effect of combination of anticancer drugs. It is accepted that if FIX value is between 0.51 and 1, it indicates an additive effect; if FIX value is less than 0.5 the effect is synergism. FIX value in between 1 and 2 is considered an indifferent effect while the value greater than 2 indicates antagonism.[22]

Statistical Analysis

The results of XTT cytotoxicity assays were subjected to two-tailed t-test by using SPSS Software (SPSS Inc., Illinois, USA) to determine significant difference between means of groups (α= 0.05).

RESULTS

Cell Proliferation and Cross Resistance

The results obtained from cytotoxicity tests and resistance indices (Table 1) represent that MCF-7/Pac, MCF-7/Doc, MCF-7/Vinc and MCF-7/Dox cell lines developed 150, 47, 30 and 160 fold resistance to the anticancer drugs that select the cells. DMSO was used as solvent of docetaxel and paclitaxel and it did not exert any antiproliferative efffect when relevant solvent concentrations were tested. Moreover, it was ineffective in development of resistance when applied individually. Development of cross-resistance to different anticancer agents (other than their selective agents) was also investigated for each resistant subline. Interestingly, degree of cross-resistance developed by MCF-7/Pac and MCF-7/Doc to vincristine and tamoxifen were similar. The resistance levels were 13 folds and around 2 folds, respectively. However, these two sublines did not develop statistically significant cross-resistance to doxorubicin and ATRA. MCF-7/Vinc

Table 1. Antiproliferative effects of anticancer drugs on sensitive and resistant MCF-7 cell lines and resistance indices

Cell Line	Anticancer Drug	IC50 (μM) ± SEM[γ]	Resistance Index
MCF-7/S	Paclitaxel	2.12 ± 0.23	-
	Docetaxel	3.49 ± 1.55	-
	Vincristine	5.45 ± 0.66	-
	Doxorubicin	1.14 ± 0.38	-
	Tamoxifen	6.02 ± 1.30	-
	ATRA	40.78±9.42	-
MCF-7/Pac	Paclitaxel	317.94± 0.20	**149.98***
	Vincristine	71.64 ± 11.59	13.14*
	Doxorubicin	1.64 ± 0.33	1.44
	Tamoxifen	15.06 ± 0.75	2.5*
	ATRA	36.76 ± 9.05	0.90
MCF-7/Doc	Docetaxel	163.21 ± 11.19	**46.77***
	Vincristine	66.21 ± 9.4	12.15*
	Doxorubicin	1.55 ± 0.44	1.40
	Tamoxifen	12.09 ± 1.48	2.01*
	ATRA	45.80 ± 11.50	1.12
MCF-7/Vinc	Vincristine	162.29± 2.19	**29.78***
	Paclitaxel	101.99 ± 13.85	48.11*
	Doxorubicin	6.98 ± 1.64	6.12*
	Tamoxifen	12.24 ± 0.75	2.03*
	ATRA	54.82 ± 8.14	1.34
MCF-7/Dox	Doxorubicin	183.11 ± 23.63	**160.62***
	Paclitaxel	231.15 ± 49.15	109.03*
	Docetaxel	33.61 ± 7.42	9.63*
	Tamoxifen	14.18 ± 0.50	2.36*
	ATRA	103.40 ± 20.11	2.54*

[γ] SEM (standard error of means) values were derived from the IC50 values of three independent experiments.

* Represents statistically significant difference between the groups (MCF-7/Pac, MCF-7/Doc, MCF-7/Vinc, MCF-7/Dox vs MCF-7/S) with $p<0.05$.

cell line developed 48 fold cross resistance to paclitaxel which is higher than the resistance against vincristine (30 fold) itself. This cell line was also cross-resistant to doxorubicin and tamoxifen about 6 and 2 folds significantly; however did not develope cross-resistance to ATRA. MCF-7/Dox cell line developed about 109, 10, 2 and 3 folds cross-resistance to paclitaxel, docetaxel, tamoxifen and ATRA respectively. The resistance indices were significantly different and lower than the resistance developed by MCF-7/Dox to doxorubicin.

Table 2. Effects of anticancer drug interactions on MCF-7 sublines

Cell Line	Anticancer Drug	FIX ± SEM[γ]	Interaction
MCF-7/Pac	Doxorubicin	0.43 ± 0.07	Synergistic
	Vincristine	0.92 ± 0.04	Additive
	Tamoxifen	0.84 ± 0.14	Additive
	ATRA	1.55 ± 0.39	Indifferent
MCF-7/Doc	Vincristine	1.19 ± 0.21	Indifferent
	Doxorubicin	0.43 ± 0.10	Synergistic
	Tamoxifen	0.87 ± 0.09	Additive
	ATRA	1.43 ± 0.20	Indifferent
MCF-7/Vinc	Paclitaxel	0.70 ± 0.18	Additive
	Doxorubicin	1.60 ± 0.51	Indifferent
	Tamoxifen	0.44 ± 0.03	Synergistic
	ATRA	3.56 ± 0.64	Antagonistic
MCF-7/Dox	Paclitaxel	0.22 ± 0.07	Synergistic
	Docetaxel	0.43 ± 0.05	Synergistic
	Tamoxifen	0.15 ± 0.06	Synergistic
	ATRA	1.14 ± 0.18	Indifferent

[γ] SEM were derived from at least three FIX values

Anticancer Drug Combinations

According to fractional inhibitory indices (Table 2), combinations of paclitaxel (FIX: 0.22 ± 0.07 < 0.5) and docetaxel (FIX: 0.43 ± 0.05< 0.5) with doxorubicin exerted synergic antiproliferative effects on the resistant sublines. However, doxorubicin combination with vincristine did not interact effectively on the resistant sublines. Tamoxifen had synergic effect in combination with either doxorubicin (FIX: 0.15 ± 0.06 < 0.5) or vincristine (FIX: 0.44 ± 0.03 < 0.5). It also had additive antiproliferative effects when applied with paclitaxel and docetaxel. According to results (Table 2), paclitaxel, docetaxel and doxorubicin have shown indifferent interactions with retinoic acid while vincristine was antagonistic. Vincristine and doxorubicin combination also did not exert effective antiproliferative effect on the resistant subline.

DISCUSSION

The results presented here suggested that the resistant sublines developed varying degree of cross-resistance to different anticancer agents. Similar findings were reported in clinical studies during development of multidrug resistance.[23,24] The results are consistent with literature that the breast cancer cells developing resistance to an anticancer drug may also develop cross-resistance to another agent.[25] In clinic, development of cross-resistance affect the success of chemotherapy and some patients become refractory to treatment.[26,27]

Several studies previously showed that paclitaxel, docetaxel, doxorubicin and vincristine cause the development of acquired drug resistance in various tissue types during clinical chemotherapy.[28,29,30] In resistant model cell lines, development of cross-resistance to another anticancer agent is a frequently observed situation in-vitro.[31] McDonald et al. previously showed that docetaxel resistant mammary carcinoma cell lines MCF-7 and MDA-MB exhibited cross-resistance to paclitaxel and vincristine[32], which is concordant to our findings. According to the findings in this study paclitaxel and docetaxel resistant sublines display similar cross-resistance indices for the same anticancer agents. This similar

trend may be due to the similarity of selective agents in their chemical structures and mechanism of action. All the resistant sublines express multidrug resistance 1 (MDR1) and multidrug resistance-associated protein 1 (MRP1) genes[20] so it is natural for the sublines to develop cross-resistance to MDR1/P-gp and MRP1 pump substrates (paclitaxel, docetaxel, doxorubicin and vincristine). On the other hand, vincristine and doxorubicin resistant subline did not develop same level of cross-resistance to the same anticancer agents.

Senstive MCF-7 cell line may have become resistant to different drugs through diverse mechanisms, so that the sublines may have responded differently. However tamoxifen and ATRA are not the substrates of efflux pumps so this fact may be the major cause for the sublines developing no or very low level of cross resistance to these agents.

Retinoids are known to suppress carcinogenesis in various epithelial tissues. Among them, ATRA is recognized as active retinoid.[33] Despite its anticarcinogenic activity, development of acute retinoid resistance limits its clinical application. Drug resistance to tamoxifen is another significant clinical problem but the mechanism through which this occurs was not well understood.[34] Approximately 30% of estrogen receptor alpha-positive breast cancers do not respond to tamoxifen treatment. In addition, the majority of tumors that initially respond to treatment develop resistance over time.[31,34]

Modern medicine uses defined ingredients or compositions, optimizes treatment schedules and combination proportions, refines routes of drug delivery, and combines different modalities of treatments.[35] The development of combination chemotherapy strategies is a very promising way to overcome the difficulties in chemotherapy. Cancer therapy is a combination approach, and may be easily acceptable for clinicians under controlled conditions. The results obtained from resistant cell lines show that tamoxifen can be used in combination with paclitaxel, docetaxel, vincristine and doxorubicin. Paclitaxel and tamoxifen were previously applied in combination in-vivo.[36] Also effective combinations of paclitaxel with doxorubin or vincrsitine and docetaxel combination with doxorubicin in-vitro may also be good models for in-vivo combined chemotherapy.

Different sensitivities of the sublines to the anticancer agents and to their combined applications can be explained by cellular differences in the resistance mechanisms and differences in the expression levels of the resistance related genes in the sublines. Effective antiproliferative effects of paclitaxel-doxorubicin, paclitaxel-vincristine, docetaxel-doxorubicin, paclitaxel-tamoxifen, docetaxel-tamoxifen, vincristine-tamoxifen and doxorubicin-tamoxifen pairs may also be related to the independent effects of anticancer agents on different pathways in the resistant sublines and their combined cytotoxic effects.

Although the data presented here provides a valuable information about feasibility of combined anticancer applications in in vitro models, it should be further supported by clinical trials prior to routine clinical applications.

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