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Paclitaxel Resistance in MCF-7/Pac Cell Line is Reversed Successfully by Saikosaponin A and Saikosaponin D

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ABSTRACT

Cancer cells demonstrate multiple drug resistance phenotype frequently after chemotherapy. The resistance of cancer cells to various chemotherapeutic agents is defined as multiple drug resistance. The purpose of this study is to investigate the potential reversal effects of active agents, that are found high amount in plants, on resistant MCF-7 cell lines. The effects of potential MDR modulators combined with anticancer drugs were also evaluated. Flow cytometry, fluorescence microscopy and checkerboard combination assays were performed to study the reversal of drug resistance and for investigation of the antiproliferative effects of the combination of anticancer drugs with the modulators. Paclitaxel and potential MDR modulators (verapamil, saikosaponin A, D and isoquercitrin) were applied to the sublines in combination. Fluorescence accumulation levels and fractional inhibitory indices show that saikosaponin A and D are effective MDR reversal agents that may be used together with paclitaxel in drug resistant mammary carcinoma subline. In conclusion this report represents saikosaponin A and D from natural resources are valuable reagents that may improve the success of chemotherapy.

Keywords: MDR, MCF-7, MDR reversal, Saikosaponins, Checkerboard microplate method

ÖZET

Saikosaponin A ve Saikosaponin D ile MCF-7/Pac Hücre Hattında Paklitaksel Dirençliliğinin Engellenmesi

Kanser hücreleri kemoterapi sonrasında sıklıkla çoklu ilaç dirençliliği fenotipini geliştirirler. Kanser hücrelerinin çeşitli kemoterapötiklere dirençliliği çoklu ilaç dirençliliği (ÇİD) olarak tanımlanır. Bu çalışmanın amacı, bitkilerde yüksek miktarda bulunan aktif bileşiklerin dirençli MCF-7 hücreleri üzerinde potansiyel dirençliliği geri çevirme etkilerini araştırmaktır. Bu potansiyel MDR modülatörlerinin antikanser ilaçlarla kombine uygulandıkları zaman etkileşimleri de belirlenmiştir. Akım sitometri, floresan mikroskopi ve checkerboard kombinasyon testi dirençliliği geri çevirme etkileri ve ilaç-modülatör etkileşimlerini belirlemek amacıyla uygulanmıştır. Paklitaksel ve MDR modulator adayları (verapamil, saikosaponin A, D ve isoquercetin) dirençli hücre hattına kombine uygulanmıştır. Floresans birikim seviyeleri ve fraksiyonel inhibisyon indisleri, saikosaponin A ve saikosaponin D'nin paklitaksel ile birlikte birlikte uygulanabilecek etkili MDR modülatörleri olduklarını göstermektedir. Sonuç olarak, doğal kaynaklardan elde edilen saikosaponin A ve saikosaponin D, kemoterapinin başarısını artıracak değerli bileşikler oldukları söylenebilir.

Anahtar Kelimeler: Çoklu ilaç dirençliliği, MCF-7, ÇİD geri çevirilmesi, Saikosaponinler, Checkerboard mikroplaka yöntemi

INTRODUCTION

The resistance of cancer cells to multiple chemotherapeutic agents prevents the success of therapy.[1] A variety of specific changes have been identified in cancer cells which protect them against chemotherapeutic agents.[2] According to in vitro studies cancer cells might acquire resistance to chemotherapeutics through increased drug efflux that results from up-regulation of ATP binding cassette (ABC) transporters such as multidrug resistance protein 1 (MDR1, P-gp).[3-6] Investigators intensively make research to overcome MDR and to suppress MDR mechanisms by inhibiting ABC transporters.[7] The agents that reverse resistance against anticancer drugs are called MDR modulators.[8,9] Various synthetic and natural compounds were studied on various tumor cells to identify the most effective resistance modifiers.[10,11] Verapamil is a known P-gp modulator and acts as a direct calcium channel blocker.[12] In addition to antiproliferative effects, many natural compounds obtained from plants and their modified forms have been investigated in terms of their drug resistance reversal activity.[8, 11,13] Therefore, a natural substance which has low toxicity may potentially be used as a drug resistance reversal agent. Quercetin and isoquercitrin are plant phenolic compounds which have antioxidant and chemopreventive activities.[14] Saponins are plant glycosides with aglycan and glycan parts.[15] Saponins have been recently reported to possess anticancer activities by making complex with cholesterols in cell membrane and producing pores that induce apoptosis.[16]

This study represents the results of investigation of MDR reversal by saikosaponin A, saikosaponin D and isoquercitrin on paclitaxel and vinctistine resistant MCF-7 cell lines. The so called compounds were found high in amount in a natural Turkish endemic source, roots of *Bupleurum* species in our previous study.[17] The effect of the combined application of anticancer drugs and effective reversal agents were also tested.

MATERIALS AND METHODS

Chemicals

XTT reagent, 3-[4, 5- Dimethylthiazol-2-yl] 1-2, 5-diphenyltetrazolium bromide (Biological Industries) was used for cytotoxicity tests. Verapamil (injection form) was used as positive control that inhibits P-gp. The accumulation of the fluorescent anticancer agent doxorubicin was measured to determine drug efflux through P-gp. The potential reversal agents (saikosaponin A, saikosaponin D and isoquercitrin (Sigma)) were dissolved in DMSO.

Cell lines

The drug resistant MCF-7 cell lines, as models for drug resistant human breast cancer, were used. The features of the parental and resistant cell lines were described previously by Kars et al.[18] The sublines resistant to 400 nM paclitaxel (MCF-7/Pac) and 120 nM vincristine (MCF-7/Vinc) were used to test the MDR modulator effects of reversal agents. MCF-7/Pac is 150 fold and MCF-7/Vinc is 30 fold resistant with respect to sensitive cell line MCF-7/S.

Assay for the reversal of MDR in MCF-7 cell lines

Parental and resistant cell concentration was adjusted to $2x10^6$ cells/mL, then the cells were suspended in serum free RPMI 1640 medium and distributed in 0.5 mL aliquots into centrifuge tubes. Compounds to be tested (verapamil, saikosaponin A, saikosaponin D and isoquercitrin) were added (40 μg/mL) and samples were incubated for 10 min at 25^0C. Doxorubicin (P-gp substrate) as fluorescent indicator was added (10 μM final concentration) to samples and cells were incubated for 20 min at 37^0C. The cells were centrifuged at 800 rpm for 5 minutes, washed twice in 0.5 mL PBS and finally resuspended in 0.5 mL PBS. The fluorescence of the cell population was measured using flow cytometry (BD FACS Calibur). Verapamil was used as positive control in the doxorubicin exclusion assays. The fluorescent activities for the treated MCF-7 cell lines were calculated by comparing them with the fluorescent activities of the untreated cells. The ratio was calculated using the following formula[19] taking account of the measured fluorescent intensities (F) from histograms. Fluorescent activity ratio (FAR) was calculated from FAR= F MDR treated / F MDR control.

The term "MDR treated" stands for the resistant cell line treated with MDR modifying agent, "MDR control" stands for the untreated drug resistant cells. Compounds were judged to be active modulators if the ratios (fluorescence activity ratio, FAR) were greater than that of verapamil.[20]

Fluorescence microscopy

Cells were trypsinized and pelleted. Viable cell count was performed in a Thoma counter under light microscope using tryphan blue (Sigma). MCF-7/S, MCF-7/Vinc, and MCF-7/Pac cells were seeded on to cover slips as 6 x 10^5 cells /coverslip and they were allowed to attach in complete medium in incubator. Cells were washed with PBS for three times and incubated with verapamil, saikosaponin A, saikosaponin D and isoquercitrin for 30 min. A control of untreated cells was also prepared. Treated and untreated cells were incubated with 3 μM doxorubicin for 1 h. Preparations were observed under a Olympus BX51 fluorescent microscope, 100X objective with green filter.

Assay for antiproliferative effect / Checkerboard microplate method

The effects of the potential MDR modulators with anticancer agents on the proliferation of sensitive and resistant MCF-7 cell lines were tested in 96-well plates as previously described.[18,21] The compounds were diluted from high to low concentrations horizontally in the plates. Cells were seeded to each well ($1x10^4$) with the exception of medium control wells. The plates were incubated at 37°C for 72 h, and then XTT solution was added to each well. After incubation at 37°C for 4 h, inhibition of cell proliferation was determined by measuring the optical density of the chromogenic product with an ELISA reader (Biotek). The inhibition of cell proliferation and IC50 values were determined for each cell line. A checkerboard micro plate method was applied to study the effects of drug interactions between resistance modifiers and anticancer drugs on resistant MCF-7 cell lines. The dilutions of anticancer drugs (paclitaxel, vincristine) (A) were made horizontally and modulators were diluted (verapamil, saikosaponin A, saikosaponin D and isoquercitrin) (B) vertically in a 96 well plate. Modulator- drug interaction was evaluated according to the following expressions:

FICA= IC_{50A} in combination / IC_{50A} alone

FICB= IC_{50B} in combination / IC_{50B} alone

FIC stands for the fractional inhibitory concentration. The fractional inhibitory index, FIX = FICA + FICB demonstrates the effect of the combination of anticancer drug and resistance modifier. When FIX value is 0.51-1.00, it is an additive effect; if it is less than 0.50 the effect is a synergistic, while a value greater than 2 is an antagonistic effect.[22]

Statistics

The results of cytotoxicity tests and reversal assays were analyzed by two-tailed t-test by using SPSS Software to determine significant difference between groups ($\alpha = 0.05$).

RESULTS

Reversal of MDR in resistant MCF-7 sublines

Reversal of drug resistance was evaluated by the application of resistant cells by modulator agents. The flow cytometry results demonstrate that the inhibition of P-gp resulted in cellular accumulation of doxorubicin. The fluorescent activity ratios obtained in both resistant cell lines indicate that verapamil is effective in modulating P-gp on both cell lines (Table 1). Additionally saikosaponin A and saikosaponin D are good reversal agents for inhibiting P-gp in MCF-7/Pac but not in MCF-7/Vinc cells. Fluorescent microscope images also are in concordance with flow cytometry results (Figure 1). According to the observations, doxorubicin was accumulated in sensitive cell line but doxorubicin was effluxed out in MCF-7/Pac effectively. Also it was observed that saikosaponin treatment resulted in re-accumulation of doxorubicin in MCF-7/Pac as if in MCF-7/S case.

Combination of reversal agents and anti-cancer drugs

Checkerboard microplate method was performed to test the antiproliferative effects of the effective reversal agents when combined with paclitaxel and

Table 1. Fluorescent activity ratio (FAR) after application of compounds

Compound	FAR ± SEM	
	MCF-7/Pac	MCF-7/Vinc
Verapamil	1.05 ± 0.01	1.12 ± 0.03
Saikosaponin A	1.23 ± 0.02	1.06 ± 0.01
Saikosaponin D	1.31 ± 0.08	1.02 ± 0.07
Isoquercitrin	1.07 ± 0.03	1.09 ± 0.05

SEM: standart error of means (P<0.05).

vincristine. Verapamil, saikosaponin A, saikosaponin D and isoquercitrin were applied with paclitaxel and vincristine in combination and the effects on sublines were studied separately. The results show that combined effects of paclitaxel-verapamil, paclitaxel-saikosaponin A, paclitaxel-saikosaponin D pairs exerted additive antiproliferative effects when applied to respective MCF-7/Pac (0.50<FIX<1.00). However combined applications of isoquercitrin with paclitaxel and vincristine and combinations of vincristine- saikosaponin A, vincristine- saikosaponin D pairs did not enhance antiproliferative effects (FIX> 1.00) to the resistant cells (Table 2).

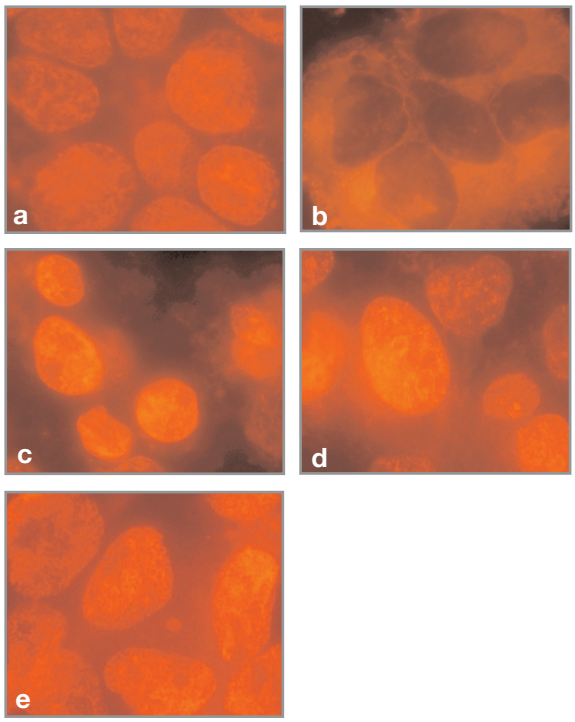

Figure 1. Fluorescence microscope images (100X, green filter) of doxorubicin accumulation
a) Doxorubicin applied MCF-7/S
b) Doxorubicin applied MCF-7/Pac
c) Verapamil-doxorubicin applied MCF-7/Pac
d) Saikosaponin A- doxorubicin applied MCF-7/Pac
e) Saikosaponin D – doxorubicin applied MCF-7/Pac cells is presented.

DISCUSSION

Development of new anticancer agents from natural sources is an important research area. Saikosaponins and isoquercitrin are active ingredients of roots of endemic Bupleurum species.[17] Previously we found that saikosaponin A and saikosaponin D exerted 1.5 - 2 fold more antiproliferative effect on MCF-7/S and MCF-7/Vinc cells than on MCF-7/Pac cells. Also we found that isoquercitrin is not toxic to MCF-7 cells. Hsu et al. reported that saikosaponin D affected the proliferation of A549 lung cancer cells with IC_{50} value of 10 μM 23 . In the so called previous study we found that saikosaponin D affected MCF-7 cells in a similar manner.

The development of pharmacological agents that reverse resistance to anticancer drugs can be reversed by a variety of resistance modulating agents in vitro. The modulators interact with ABC transporter proteins and may also with the lipid bilayers of the cellular membrane. So, membrane lipids may be one of the targets for modulators. Most of the reversing compounds are soluble in lipids and they may influence the physical state of lipid bilayer and membrane integrated proteins.[24,25,26] The fluorescent activity ratios indicate that saikosaponin A and saikosaponin D were effective as P-gp

Table 2. Drug-compound interactions

Cell	Application	FIX ±SEM	Comment
MCF-7/Pac	Paclitaxel (+)		
	Verapamil	0.13 ± 0.04	Synergism
	Saikosaponin A	0.95 ± 0.03	Additive
	Saikosaponin D	0.81 ± 0.16	Additive
	Isoquercitrin	7.50 ± 0.02	Antagonism
MCF-7/Vinc	Vincristin (+)		
	Verapamil	0.62 ± 0.14	Additive
	Saikosaponin A	2.86 ± 0.36	Antagonism
	Saikosaponin D	6.86 ± 0.95	Antagonism
	Isoquercitrin	8.65± 0.04	Antagonism

SEM (standart error of means) values were derived from the standard errors of the means of at least three FIX values.

modulators when compared to verapamil. However isoquercitrin did not act as a good reversal agent. The different degrees of modulator effects of these compounds in individual sublines may be due to differences in expression level of P-glycoprotein in the membrane and also due to other resistance mechanisms acquired in the sublines at variable levels. Saponins induce apoptosis by making pores on cell membrane as they produce complex with cholesterol.[27] Saponins obtained from Phytolacca species reversed drug resistance in ovary cancer cells 28 . Saikosaponin A and D were previously demonstrated to alter cell membrane fluidity by Li et al.[27] Many known MDR modulators alter membrane fluidity.[24,25] Changes in cell membrane may alter the active functional conformation of P-gp.[26] This information supports our flow cytometry and fluorescent microscopy results exhibiting saikosaponin A and D may be used as MDR resersal agents in paclitaxel resistance. We previously found out that endemic *Bupleurum* species root extracts contain high amounts of saikosaponin A, saikosaponin D and isoquercitrin.[17] It is known that plant phenolics have chemopreventive property and antioxidant activity.[14] Phenolic compounds produce phenolate with hydroxyl grups of proteins that change three dimensional structure of proteins. It was reported that such interactions may change the activity of P-gp.[29] However our results present that isoquercitrin did not contribute to the accumulation of doxorubicin in drug resistant MCF-7 cells.

Combined therapy by application of anticancer drugs with reversal agents may be acceptable if the interaction is synergistic and additive. So accordingly, combinations of paclitaxel with saikosaponin A and saikosaponin D make them candidates as MDR reversal agents in combination therapy of paclitaxel resistant breast cancer.

To conclude, here we declare for the first time that, saikosaponin A and saikosaponin D extracted from roots of endemic *Bupleurum* species are effective reversal agents to be used in combination with paclitaxel for treatment of paclitaxel resistant breast cancer.

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