

## Doxorubicin Cytotoxicity in Combination with Soy Isoflavone Daidzein on MCF-7 Breast Cancer Cells

Farjadian S<sup>1</sup>, Khajoei Nejad L<sup>2</sup>, Fazeli M<sup>2</sup>, Askari Firouzjaei H<sup>3</sup> & Zaeri S<sup>4</sup>

<sup>1</sup> Department of Immunology, Shiraz University of Medical Sciences, Shiraz, Iran

<sup>2</sup> Department of Pharmacology, Shiraz University, Shiraz, Iran

<sup>3</sup> Department of Immunology, Tarbiat Modares University, Tehran, Iran

<sup>4</sup> Department of Pharmacology, Shiraz University of Medical Sciences, Shiraz, Iran

### ABSTRACT

**Introduction:** Combination chemotherapy regimens offer a promising approach to the prevention of recurrence, metastasis and drug resistance during breast cancer management. Combined tumor therapy using natural substances is highly suggested. Daidzein is one of the major isoflavones in soy beans with anti-tumor activity but its effect in combination with common chemotherapeutic agents is still unclear. This study was designed to investigate whether daidzein increases the antitumor activity of doxorubicin against MCF-7 human breast cancer cells. **Methods:** The cytotoxic activity of doxorubicin, daidzein and a combination of the two drugs was determined at different concentrations using LDH release assay. The average values of each experiment were adjusted to the values determined from untreated controls and 50% inhibitory concentration (IC<sub>50</sub>) value for each drug was calculated by CompuSyn. *In vitro* interaction was also calculated using different combinations of doxorubicin and daidzein. Combination indices (CI) were calculated and combination index plot was constructed using the same software. **Results:** Analysis of the dose-effect curve showed that the treatment of MCF-7 cells with doxorubicin or daidzein for 24 h led to 50% cytotoxicity at 5.4 nM and 146.5 μM, respectively. **Conclusion:** The Combination index plot showed CI >1 for all combinations used in this study which indicates antagonistic interactions between daidzein and doxorubicin. This study results have implications for patients with breast cancer under treatment with doxorubicin if they are taking daidzein as a dietary supplement

**Key words:** Breast cancer, daidzein, doxorubicin, MCF-7, soy isoflavone

### INTRODUCTION

There is an increasing trend in the incidence and mortality of breast cancer worldwide (Porter, 2009; Bray, McCarron & Parkin, 2004). It is known that the frequency of breast cancer increases with age, mostly occurring in post-menopausal women (Benson & Jatoi, 2012). Doxorubicin

(Adriamycin), belongs to the anthracycline family, and is one of the most widely used anti-tumor antibiotics isolated from *Streptomyces* species. Its broad anti-tumor activity means doxorubicin is used in the treatment of a variety of tumors (Minotti *et al.*, 2004). Although anthracyclines are key drugs in the treatment of breast cancer, one

of the major adverse effects is cardiotoxicity (Lao *et al.*, 2013). Treatment with increasing doses of drugs is administered to avoid gradual occurrence of drug resistance to chemotherapeutic agents, which in turn is associated with more severe side effects in patients. The side effects of chemotherapy can be reduced by the synergistic effect of compounds which enhance the efficacy of the desired drug at below toxic concentrations (Bontenbal *et al.*, 2005). Moreover, a combination chemotherapy regimen remains a promising approach in terms of the prevention of recurrence, metastasis and drug resistance during breast cancer management (Marquette & Nabell, 2012). Combinational tumor therapy using agents with distinct molecular mechanisms, particularly natural substances, is highly suggested (Sarkar & Li, 2006). In recent years, dietary supplements of soy isoflavones have been widely used to improve menopausal symptoms (Newton *et al.*, 2009). Although soy isoflavones might have cancer chemopreventive activity and reduce the risk of breast cancer, the beneficial effect of these components in the treatment of breast cancer is still controversial (Trock, Hilakivi-Clarke & Clarke, 2006; Messina, McCaskill-Stevens & Lampe, 2006).

Daidzein is the second major isoflavone in soy beans after genistein; a substance that preferentially binds to estrogen receptor- $\beta$  and is classified as selective estrogen receptor modulator (Setchell, 2001). Daidzein has been found to have both weak estrogenic and weak anti-estrogenic effects (Mueller *et al.*, 2004). The dose-dependent anti-proliferative effect of genistein has previously been reported to have elicited MCF-7 cell proliferation at low concentrations (1 to 5  $\mu\text{M}$ ) and to have induced apoptosis at high concentrations (25  $\mu\text{M}$ ) (Lavigne *et al.*, 2008). Like genistein, daidzein also exerts its effect in a concentration-dependent manner. While tumor cell growth was observed to be stimulated at physiological concentrations

(Ju *et al.*, 2006), the anti-tumor activity of daidzein was reported to be mediated by cell cycle arrest or the induction of apoptosis at high concentrations (Choi & Kim, 2008; Jin *et al.*, 2010).

Accordingly, common anti-tumor agents combined with natural dietary compounds may potentiate chemotherapeutic effects through synergic action and reduce adverse reactions. Although there are some reports indicating the increased antitumor activity of cisplatin, docetaxel, doxorubicin, and gemcitabine by genistein in different human cancers (Li *et al.*, 2005; Banerjee *et al.*, 2005), the effect of daidzein in combination with common chemotherapeutic agents is still unclear. There are a small number of reports of therapeutic responses to daidzein derivative in combination with epirubicin against human colon cancer (Lo, 2012) and daidzein conjugate with daunomycin against ovarian cancer (Somjen *et al.*, 2008). The present study was therefore designed to investigate whether the antitumor activity of doxorubicin against a human breast cancer cell line, MCF-7, is increased by the administration of daidzein.

## METHODS

A stock solution of 1 M daidzein (LC Laboratories, Woburn, MA, USA) was prepared in dimethyl sulfoxide (DMSO) and further dilutions (0.1, 1, 2, 20, 50, 75, 100, 125, 150, 200, 300, 400, and 500  $\mu\text{M}$ ) were prepared in RPMI 1640 containing 1% fetal bovine serum. Doxorubicin (Ebewe Pharma Ges., Unterach, Austria) working dilutions (0.1, 1, 5, 10, 100 and 1000 nM) were also prepared in the same culture media. Estrogen-dependent MCF-7 breast cancer cells were cultured to logarithmic phase and then seeded in 96-well flat bottom plates at a density of  $2 \times 10^4$  cells per well and incubated for 24 h with freshly prepared working dilutions of daidzein or doxorubicin and different combinations of both drugs. The cytotoxic activity of certain

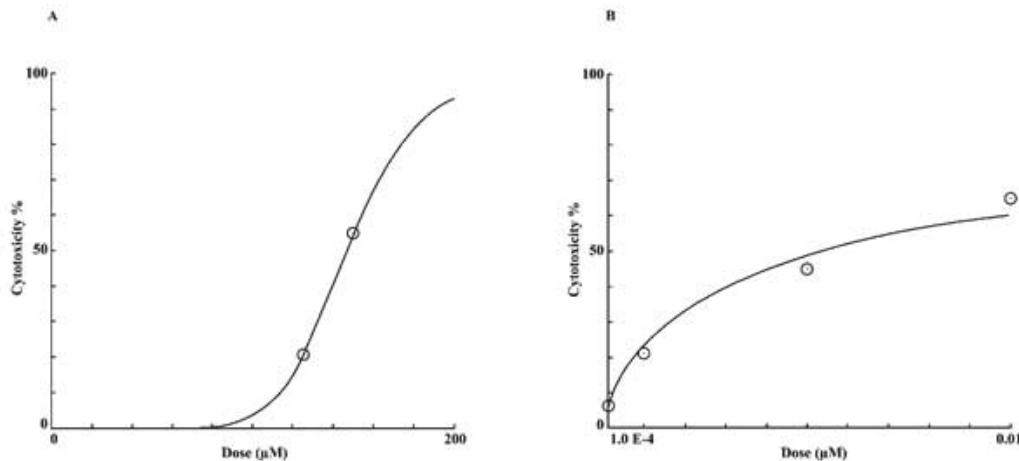


Figure 1. Dose-dependent cytotoxicity effects of daidzein (A) and doxorubicin (B) on MCF-7 cells.

concentrations of each drug was measured with lactate dehydrogenase (LDH) release assay by a commercial cytotoxicity detection kit (Roche Applied Science, Bavaria, Germany). Each experiment was performed in triplicate and repeated three times independently. Background values from wells without cells were subtracted and average values for the triplicates were calculated. Cytotoxicity was then calculated according to the following equation:

$$\text{Cytotoxicity \%} = \frac{(\text{experimental value} - \text{low control})}{(\text{high control} - \text{low control})} \times 100$$

where high control corresponds to maximum LDH released from cells after lysis by 1% Triton X-100 and low control represents spontaneous LDH released from untreated cells in the presence of 0.1% DMSO in the culture medium.

The average values of each experiment were adjusted to the values determined from untreated controls and the 50% inhibitory concentration ( $IC_{50}$ ) value for each drug was calculated by CompuSyn version 3.0.1 (ComboSyn, Inc., Paramus, NJ, USA 2007). *In vitro* interaction was also calculated using 14 dose combinations

of doxorubicin (1-10 nM) and daidzein (50-200 µM). Combination indices (CI), combination index plot and the normalised isobologram for combination treatments were calculated and plotted using the same software.  $CI < 1$ ,  $= 1$ , and  $> 1$  were considered representative of synergism, additive effect, and antagonism, respectively (Chou, 2006).

## RESULTS AND DISCUSSION

### Cytotoxicity effects of daidzein or doxorubicin on MCF-7 cells

To test the effect of daidzein and doxorubicin on MCF-7 breast cancer cells, the cells were treated with increasing concentrations of daidzein (0.1 to 500 µM) or doxorubicin (0.1 to 1000 nM) for 24 h. Figure 1 shows that dose-dependent cytotoxicity effects were observed for both compounds. The  $IC_{50}$  of daidzein was 146.5 µM and showed no cytotoxicity at low concentrations ( $\leq 100$  µM); however, it killed all target cells at high concentrations ( $\geq 200$  µM). The  $IC_{50}$  of doxorubicin was 5.4 nM and had slight cytotoxic effect on MCF-7 cells at concentration of 0.1 nM but total cell cytotoxicity was observed in concentrations  $\geq 100$  nM.

**Table 1.** Cytotoxicity and combination index values for doxorubicin and daidzein at different dose combinations.

Doxorubicin (nM)	Daidzein ( $\mu$ M)	Cytotoxicity (%)	Combination index (CI)
1.0	200.0	62	1.37994
1.0	150.0	2	2.55402
1.0	125.0	21	2.23241
1.0	100.0	2	2.15167
1.0	75.0	235	1.59233
1.0	50.0	19	1.88081
5.0	200.0	81	1.26561
5.0	150.0	42	2.53646
5.0	125.0	45	2.11054
5.0	100.0	425	2.13765
5.0	75.0	46	1.68930
5.0	50.0	475	1.41656
10.0	150.0	74	1.32035
10.0	50.0	65	1.08352

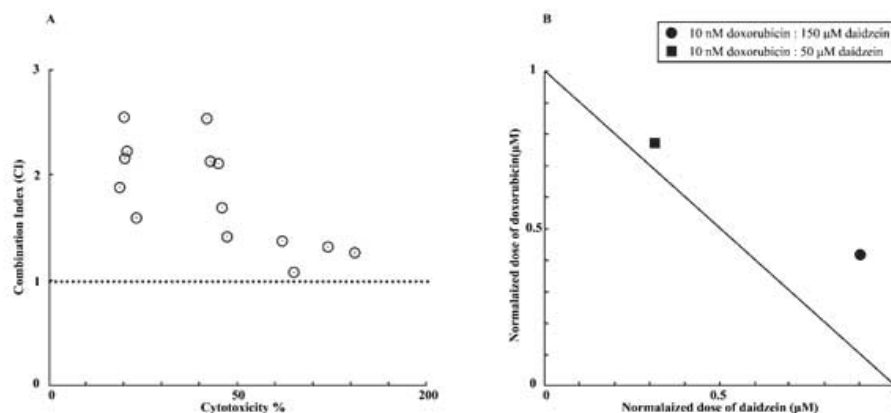
#### Combination effects of doxorubicin and daidzein on MCF-7 cells

The results of this study showed that daidzein had no cytotoxic effect on MCF-7 cells at IC<sub>50</sub> concentrations of  $\leq 100 \mu\text{M}$  while all the target cells were killed at concentrations of  $\geq 200 \mu\text{M}$ . Cytotoxicity and combination index values for doxorubicin and daidzein at different dose combinations are summarised in Table 1. The combination index plot and normalised isobologram for combination treatments are shown in Figure 2 where all of the combination points have CI values  $>1$  and fall on the upper-right of the hypotenuse.

Herbal extracts in the form of phytochemicals have recently been considered as complementary treatment in combination with conventional chemotherapy drugs in cancer prevention and tumor therapy (González-Vallinas *et al.*, 2013; Yin *et al.*, 2013). Phytoestrogens are non-steroidal phytochemicals that exert weak estrogen-like effects on the body (Usui, 2006). Isoflavones are one of the main biologically active phytoestrogens found especially in soy products (Wahajuddin *et*

*al.*, 2013). It has been demonstrated that soy isoflavones inhibit tumor cell growth *in vitro* and *in vivo* without toxicity to normal cells (Messina & Loprinzi, 2001). Therefore, soy isoflavones can be considered as candidates for combination therapy in conjunction with chemotherapeutic agents that have toxic effects, such as doxorubicin. Our results demonstrate that daidzein induces cytotoxic effects in breast cancer cells at high concentrations, whereas low concentrations of daidzein stimulate the growth of these cells. The dose-dependent antitumor effects of soy isoflavones have previously been reported by others (Lavigne *et al.*, 2008; Choi *et al.*, 2008; Jin *et al.*, 2010).

The increased anticancer efficacy of various chemotherapeutic compounds in combination treatment with genistein or an isoflavone mixture containing genistein and daidzein has also previously been reported (Li *et al.*, 2005; Banerjee *et al.*, 2005). Despite the identified antitumor activity of daidzein, only a small number of reports mention the effects of daidzein in combination therapy for the treatment of tumors. Constantinou *et*



**Figure 2.** Combination index plot (A) and normalised isobologram for combination treatments; all of the combination points have  $CI > 1$  and fall on the upper-right of the isobologram hypotenuse. Because of limitations in graphical presentation, only two combination doses are shown on the normalised isobologram.

*al.* (2005) showed an increased effect for tamoxifen in combination with daidzein in the prevention of breast cancer whereas Shiau *et al.* (2010) reported that daidzein combined with trichostatin A had no significant growth-inhibitory effect in adenocarcinomic human alveolar basal epithelial cells.

Mousavi *et al.* (2009) reported that breast cancer is the most common form of cancer among Iranian women and patients with breast cancer are often treated with doxorubicin used either alone or in conjunction with other chemotherapeutic agents (Loa *et al.*, 2013; Bontenbal *et al.*, 2005). The present study was undertaken to investigate whether daidzein could improve the cytotoxic effect of doxorubicin in the treatment of breast cancer. Analysis of the dose-effect curve showed that the treatment of MCF-7 cells separately with either doxorubicin or daidzein for 24 h led to 50% cytotoxicity at 5.4 nM and 146.5 µM, respectively (Figure 1). Our results showed that a combination of doxorubicin with daidzein could not improve the growth inhibition of MCF-7 cells in the studied combinations as all the values for CI were

$> 1$  and all the datapoints in isobologram were on the upper-right zone of the hypotenuse. Thus, the results indicate an antagonistic interaction between daidzein and doxorubicin (Figure 2). Analysis of the combination effects in broader ratios would be helpful in establishing whether these drugs are antagonistic in other combinations as well.

In their experiments, Li *et al.* (2005) showed the pretreatment of cancer cells with 15 to 30 µM of genistein for 24 h in combination with 50 nM of doxorubicin could increase inhibition of cell growth after 48 to 72 h, but despite the structural similarity between daidzein and genistein, their mechanisms of action may differ. In this study we investigated the effects of the two drugs simultaneously on tumor cells, but pretreatment of the cells with daidzein prior to exposure to doxorubicin may have a different outcome. The effects of drugs on cells are both dose and time dependent. In this study we analysed the combination effects after 24 h but it could be helpful to extend follow-up over a longer period of time.

## CONCLUSION

Our data showed combination index values  $>1$  for all combinations of daidzein and doxorubicin used in this study which indicates an antagonistic interaction between these two compounds.

If future research demonstrates the antagonistic effects of daidzein on doxorubicin, patients with breast cancer under treatment with doxorubicin should be advised to avoid taking daidzein as a supplement and perhaps even to exclude soy from their diet.

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