Isoflavones augment the effect of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) on prostate cancer cells

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KEY WORDS

prostate isoflavones ◗ TRAIL ◗ prostate cancer ◗ chemoprevention

ABSTRACT

Introduction. Isoflavones are the subclass of flavonoids with chemopreventive and anticancer activities. Isoflavones induce cytotoxicity and apoptosis in prostate cancer cells. TRAIL plays an important role in immune surveillance and the defense mechanism against tumor cells. However, not all tumor cells are sensitive to TRAIL. TRAIL-resistant cancer cells can be sensitized to TRAIL-induced apoptosis by flavonoids.
We investigated the effect of TRAIL in combination with isoflavones on prostate cancer cells.
Materials and methods. The LNCaP human prostate cancer cells were incubated with TRAIL and/or isoflavones (daidzein, puerarin, ipriflavone, genistein, neobavaisoflavone, and biochanin-A). Cytotoxicity was determined by MTT and LDH assays.
Results. Our study confirmed that LNCaP prostate cancer cells were resistant to TRAIL. We therefore examined the cytotoxic effect of TRAIL in combination with isoflavones on LNCaP cells. We showed for the first time that tested isoflavones markedly augmented TRAIL mediated cytotoxicity against prostate cancer cells. The strongest cytotoxic effect in combination with TRAIL was exhibited by neobavaisoflavone and biochanin-A. Co-treatment of prostate cancer cells with TRAIL and isoflavones, especially neobavaisoflavone and biochanin-A significantly sensitized LNCaP cells to TRAIL induced cytotoxicity.
Conclusion. The tested isoflavones augmented the cytotoxic effect of TRAIL on LNCaP cells. The obtained results suggest that isoflavones supported TRAIL-induced cytotoxicity in prostate cancer cells and thus they might be a promising chemopreventive strategy in prostate cancer.

INTRODUCTION

Prostate cancer is the most frequent male malignancy and the second most common cause of cancer related death in men in many European countries and the USA. In recent years a gradual increase in the incidence of prostate cancer has been observed. It is estimated that it constitutes 12% of freshly diagnosed cases of malignancies in the European Union and 29% in the USA [1, 2].

It has been shown that a diet rich in flavonoids has been associated with the reduced risk of several types of tumors [3]. Worldwide disparities exist between geographic regions with regard to prostate cancer incidence and mortality. Countries in East Asia have lower rates of prostate cancer compared with Western countries. The dietary differences between the two geographic regions, particularly the higher amount of isoflavones consumed in East Asia, is responsible for the difference in prostate cancer incidence [4-7].

Isoflavones are the subclass of flavonoids with immunomodulatory, antioxidant, estrogenic, anticancer, and chemopreventive activities. These compounds are distributed in a wide variety of plants and commonly consumed by humans. The isoflavones are identified in soy, red clover (*Trifolium pretense*), *Psoralea corylifolia*, and *Pueraria mirifica* [6-8].

Prevention is an important strategy for limiting prostate cancer morbidity and mortality [9]. The term "chemoprevention" was first introduced by Dr Michael Sporn, when he referred to the prevention of cancer development by natural forms of vitamin A and by its synthetic analogs [10]. Cancer chemoprevention is a mean of cancer control in which malignancy is prevented or reversed by nutritional or pharmacological intervention with natural or synthetic substances [11, 12]. The beneficial health effects of isoflavones have been known for a long time. The epidemiological studies and clinical trials have shown a significant cancer protection and decreased risk of cardiovascular diseases [13]. Isoflavone rich food and food supplements have gained increasing popularity also in the Western world. The *in vitro* tests showed that isoflavones induce apoptosis and cell cycle arrest, inhibit cancer growth, and reduce angiogenesis and tumor progression in prostate cancer [7, 9, 11, 13]. Prostate cancer represents an ideal disease for chemopreventive intervention due to its long latency, late age of onset, relatively slower rate of growth and progression, high incidence, tumor marker availability, identifiable pre neoplastic lesions, and risk group [11, 13, 14].

Tumor necrosis factor related apoptosis inducing ligand (TRAIL) is one of the several members of the TNF superfamily. TRAIL induces cell death in a wide variety of tumor cell lines and xenografts without causing toxicity to normal cells [15-17]. Soluble or expressed on immune cells (lymphocytes T, NK cells, neuthrophils, monocytes, and macrophages), molecules of TRAIL play an important role in immune surveillance and defense mechanisms against tumor cells [18]. The natural occurring TRAIL mediates apoptosis following binding to the two death receptors, TRAIL-R1 (DR4) and TRAIL-R2 (DR5), on cancer cell surfaces [19]. However, some tumor cells are resistant to TRAIL-mediated cytotoxicity. The decreased expression of TRAIL death receptors and proapoptotic proteins or increased expression of anti-apoptotic proteins in cancer cells are involved in TRAIL resistance [19, 20]. We and others have shown that TRAIL resistant prostate cancer cells can be sensitized by chemotherapeutic agents, ionizing radiation, or dietary flavonoids [21-27].

In this study we investigated the cytotoxic effect of TRAIL in combination with isoflavones (daidzein, puerarin, ipriflavone, genistein, neobavaisoflavone, and biochanin-A) on LNCaP prostate cancer cells. Figure 1 presents the structures of isoflavones used in

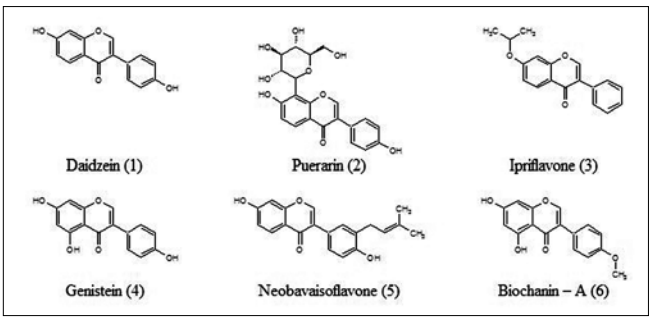


Fig. 1. Chemical structures of the tested isoflavones.

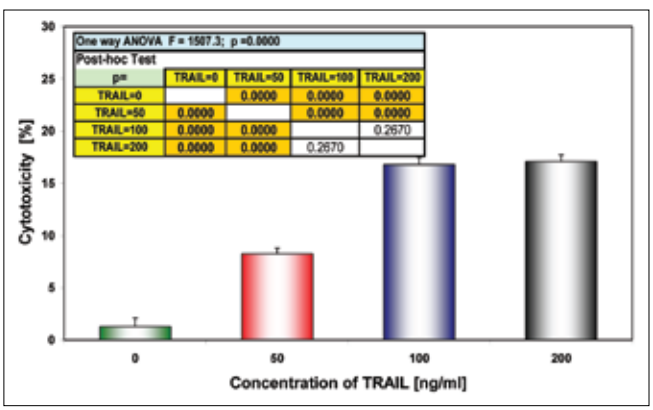


Fig. 2. Cytotoxic effect of TRAIL at the concentrations of 50-200 ng/ml on LNCaP prostate cancer cells.

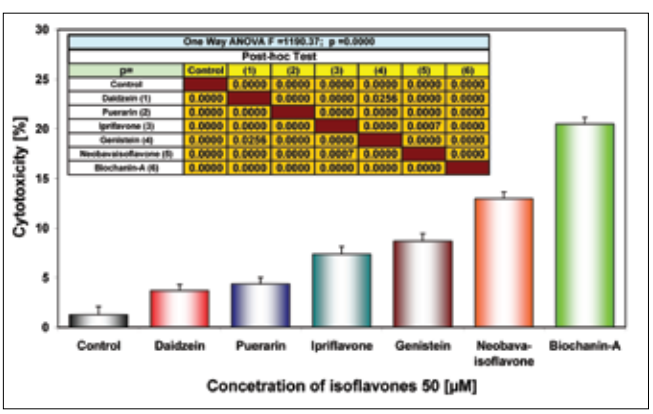


Fig. 3. Cytotoxic effect of isoflavones at the concentration of 50 µM on LNCaP prostate cancer cells.

this study. Our work is the first, preliminary communication that describes the synergistic effect of TRAIL with isoflavones on prostate cancer cells.

MATERIALS AND METHODS

Prostate cancer cells

The tests were performed on LNCaP cell line catalogue number ACC-256. The human hormone sensitive prostate cancer cells were obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH - German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and derived from supraclavicular lymph node metastasis of prostate cancer in a 55-year-old patient [24-26].

The prostate cancer cells were grown in monolayer cultures in plastic bottles of 25 cm^2 and 70 ml and 500 ml (Nunc A/S Roskilde, Denmark) in RPMI 1640 (90%) culture medium with the addition of heat inactivated bovine fetal serum (10%). The cells were

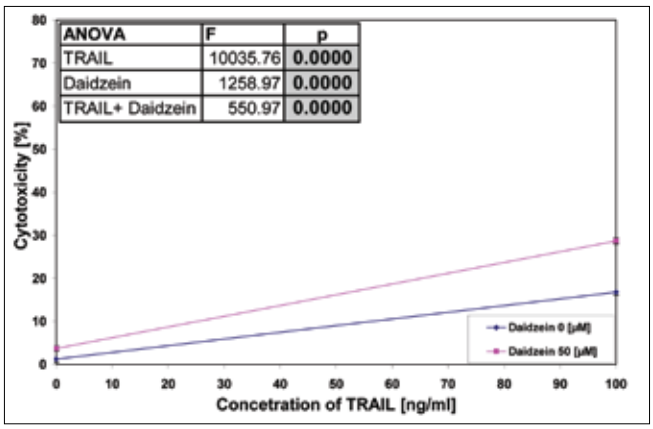


Fig. 4. Cytotoxic effect of TRAIL at the concentration of 100 ng/ml in combination with daidzein at the concetration of 50 µM on LNCaP prostate cancer.

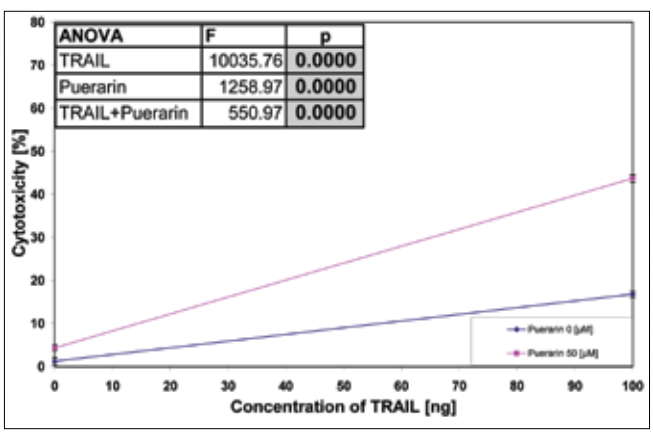


Fig. 5. Cytotoxic effect of TRAIL at the concentration of 100 ng/ml in combination with puerarin at the concentration of 50 µM on LNCaP prostate cancer cells.

cultivated constantly at 37°C, atmosphere with 5% CO_2, in an incubator with 100% relative humidity, and passaged three times a week. Reagents for the cell culture were purchased at PAA, The Cell Culture Company (Pasching, Austria). The cancer cells adhering to the container bottom were trypsinized and suspensions were prepared, which were used during subsequent experiments [26]. The number of LNCaP cells tested in each experiment was $2.5x10^5$ per 1 ml of the medium.

TRAIL

Soluble, human recombinant TRAIL called "SuperKillerTRAIL" [rhsTRAIL (CC-mutant)] and dilution buffer "KillerTRAIL Storage and Dilution Buffer" were purchased from Alexis (San Diego, CA, USA).

Isoflavones

The six isoflavones: daidzein (1), puerarin (2), ipriflavone (3), genistein (4), neobavaisoflavone (5), and biochanin-A (6) were purchased from Alexis Biochemicals (San Diego, CA, USA) and Sigma Chemical Company (St. Louis, MO, USA).

Cytotoxicity assays

Mitochondrial dehydrogenase activity (MTT test)

The cytotoxic effects of TRAIL and/or isoflavones on prostate cancer cells were assessed with a MTT test (bromo-3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium) involving the measurement of mitochondrial dehydrogenase activity [28-30]. The reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA).

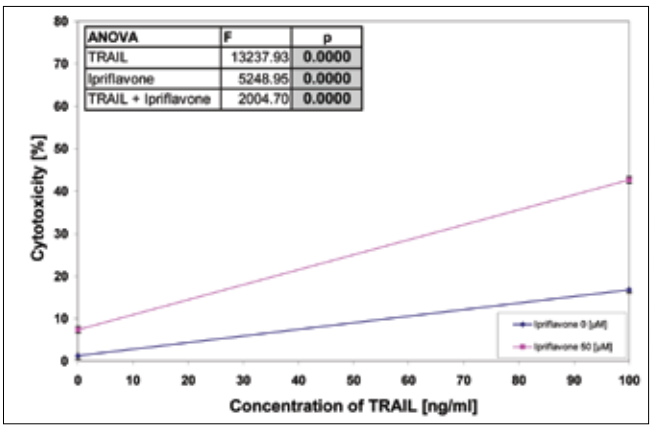


Fig. 6. Cytotoxic effect of TRAIL at the concentration of 100 ng/ml in combination with ipriflavone at the concentration of 50 μM on LNCaP prostate cancer cells.

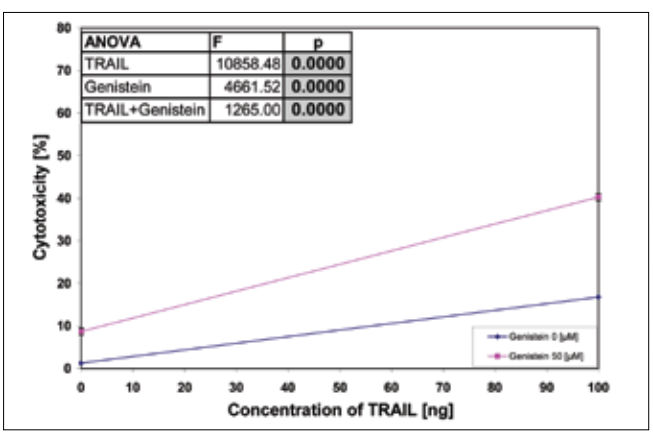


Fig. 7. Cytotoxic effect of TRAIL at the concentration of 100 ng/ml in combination with genistein at the concentration of 50 μM on LNCaP prostate cancer cells.

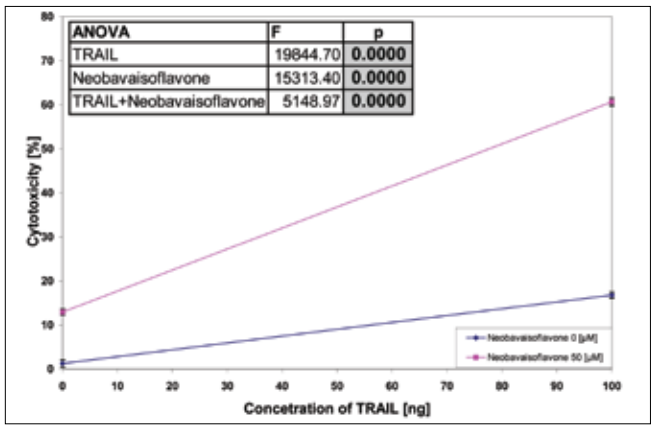


Fig. 8. Cytotoxic effect of TRAIL at the concentration of 100 ng/ml in combination with neobavaisoflavone at the concentration of 50 μM on LNCaP prostate cancer cells.

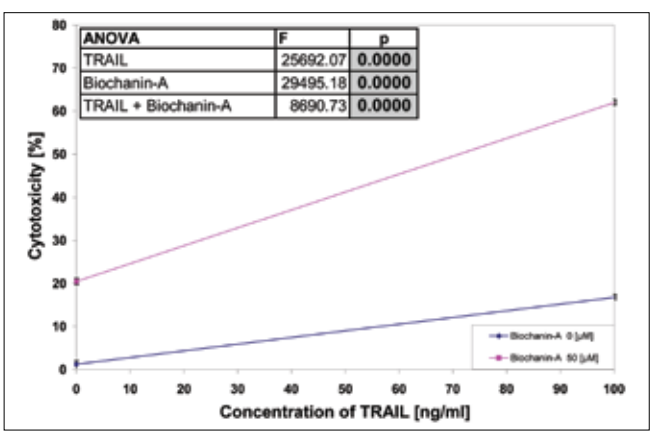


Fig. 9. Cytotoxic effect of TRAIL at the concentration of 100 ng/ml in combination with biochanin-A at the concentration of 50 μM on LNCaP prostate cancer cells.

Lactate dehydrogenase activity (LDH test)

The cytotoxic effects of studied agents on prostate cancer cells were assessed by measuring lactate dehydrogenase (LDH) activity. The LDH test was obtained from Roche Molecular Biochemicals (Mannheim, Germany) [28-30]. Lactate dehydrogenase is released from the cytoplasm into the culture medium as a result of cell membrane damage and cell lysis. The LDH activity increase in cell culture supernatants correlates with the rate of the damaged cells (necrotic cells).

Statistical analysis of results

The tests were obtained in the same experimental conditions. The results are expressed as means ±S.D. obtained from three independent experiments performed in quadruplicate (n = 12). Statistical significance was evaluated using Levene's test and analysis of variance (ANOVA). A p-value <0.05 was considered significant.

RESULTS

Cytotoxic effect of TRAIL on prostate cancer cells

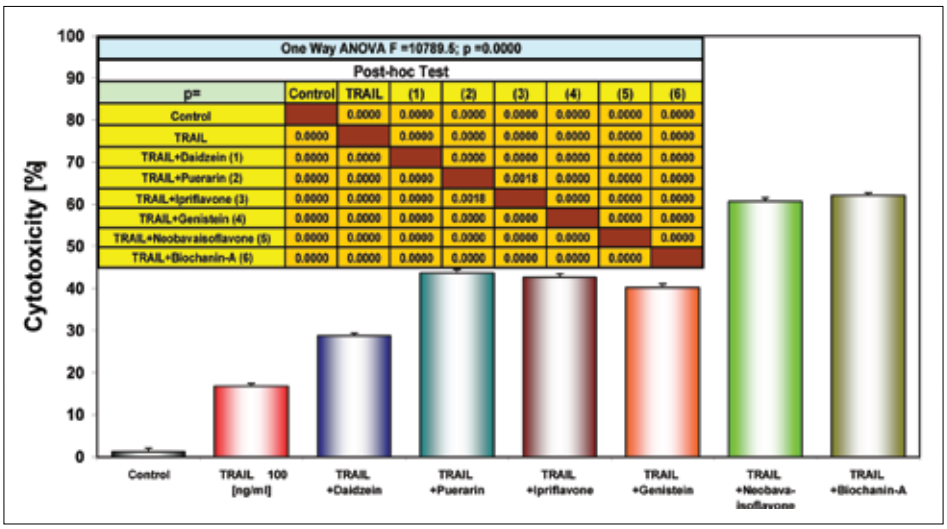
The LNCaP cells were incubated with 50-200 ng/ml TRAIL for 24 hours. TRAIL induced cytotoxic effects on tested cells in a dose dependent manner (Fig. 2). The cytotoxicity of TRAIL at the concentration of 100 ng/ml on prostate cancer cells was 16.78 ±0.69%. The obtained results indicated that LNCaP cells were resistant to TRAIL mediated death. The ligand concentration higher than 100 ng/ml had no significant influence on its cytotoxicity increase with relation to tested prostate cancer cells. In further stages TRAIL at the concentration of 100 ng/ml was used. Ligand at the concentrations of 50-200 ng/ml did not induce the lysis of cancer cells determined in the LDH test.

Cytotoxic effect of isoflavones on prostate cancer cells

The prostate cancer cells were incubated with six isoflavones at the concentration of 50 μM for 24 hours. The compounds induced cytotoxicity in LNCaP cells (Fig. 3). The cytotoxic effect of the tested isoflavones on cancer cells was 3.70 ±0.61% cell death for daidzein, 4.37 ±0.67% cell death for puerarin, 7.38 ±0.75% cell death for ipriflavone, 8.66 ±0.78% cell death for genistein, 12.94 ±0.70% cell death for neobavaisoflavone, and 20.47 ±0.67% cell death for biochanin-A. The strongest anticancer activity was exhibited by biochanin-A. Isoflavones did not induce the lysis of cancer cells determined in the LDH test.

Cytotoxic effect of TRAIL in combination with isoflavones on prostate cancer cells

Next, we investigated the effect of TRAIL in combination with isoflavones on prostate cancer cells (Figures 4-9). The LNCaP cells were incubated with TRAIL at the concentration of 100 ng/ml and isoflavones at the concentration of 50 μM for 24 hours. The co-treatment of prostate cancer cells with TRAIL and isoflavones increased the percentage of cell death to 28.70 ±0.67% for daidzein, to 43.69 ±0.84% for puerarin, to 42.66 ±0.79% for ipriflavone, to 40.25 ±0.84% for genistein, to 60.66 ±0.88% for neobavaisoflavone, and to 62.10 ±0.64% for biochanin-A. All tested isoflavones



p=	Control	TRAIL	(1)	(2)	(3)	(4)	(5)	(6)
Control		0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
TRAIL	0.0000		0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
TRAIL+Daidzein (1)	0.0000	0.0000		0.0000	0.0000	0.0000	0.0000	0.0000
TRAIL+Puerarin (2)	0.0000	0.0000	0.0000		0.0018	0.0000	0.0000	0.0000
TRAIL+Ipriflavone (3)	0.0000	0.0000	0.0000	0.0018		0.0000	0.0000	0.0000
TRAIL+Genistein (4)	0.0000	0.0000	0.0000	0.0000	0.0000		0.0000	0.0000
TRAIL+Neobavaisoflavone (5)	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000		0.0000
TRAIL+Biochanin-A (6)	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	

Fig. 10. Cytotoxic effect of TRAIL at the concentration of 100 ng/ml in combination with isoflavones at the concentration of 50 μM on LNCaP prostate cancer cells.

augmented TRAIL-induced cytotoxicity in cancer cells. The strongest cytotoxic activity in combination with TRAIL was demonstrated by neobavaisoflavone and biochanin-A against LNCaP cells. The isoflavones, especially neobavaisoflavone and biochanin-A, sensitized TRAIL resistant LNCaP cell to TRAIL mediated cytotoxicity. The cytotoxic effect of TRAIL in combination with all studied isoflavones on prostate cancer cells are demonstrated in Figure 10. TRAIL with isoflavones did not induce the lysis of cancer cells determined in the LDH test, that excludes the necrotic cell death.

DISCUSSION

Epidemiological evidence suggests a geographical basis for the incidence of prostate cancer and dietary factors, including isoflavones consumption, which may be linked to this phenomenon [3-8]. Chemoprevention is a rapidly growing area of urooncology, which focuses on prevention of malignant disease using natural occurring or synthetic agents [9, 10]. The 5-alpha-reductase inhibitors, selenium, vitamins E and D, lycopene, soy isoflavones, and green tea flavanols have been considered for potential application in prostate cancer prevention [9, 11, 13, 14]. The use of naturally occurring agents is becoming increasingly appreciated as an effective strategy of cancer chemoprevention [10, 13]. Epidemiological findings together with preclinical data from animal and *in vitro* studies strongly support a correlation between isoflavones consumption and protection against prostate cancer [7-9]. It has led to an increased emphasis on cancer prevention strategies in which plant isoflavones will be used as dietary components or supplements [6, 11].

The tested isoflavones exhibited cytotoxic activity against LNCaP cells. In previous *in vitro* and *in vivo* studies the authors demonstrated the mechanism of action of isoflavones on prostate cancer. Daidzein, genistein, and biochanin-A affected the cell cycle, induced apoptosis and inhibited tumor growth, establishing these compounds as a cytotoxic agents for prostate cancer [7, 9, 11, 13, 31-33]. There is no evidence of cytotoxicity of puerarin, ipriflavone, and neobavaisoflavone against prostate cancer cells.

The isoflavones alone induce a low anticancer activity, thereby they are often tested in combination with other agents, such as radiation or chemotherapeutic drugs [34, 35]. The goal of our present study was to investigate the synergistic cytotoxic effect of TRAIL and isoflavones on prostate cancer cells.

Tumor necrosis factor related apoptosis inducing ligand plays an important role in maintenance of immune homeostasis, host tumor surveillance and defense against cancer cells [18, 20]. However, some tumor cells are resistant to TRAIL-mediated cytotoxicity [19, 20]. We investigated the cytotoxic effect of TRAIL in combination with isoflavones in prostate cancer cells. Recombinant human TRAIL used in this study is a soluble protein based on a natural ligand [24, 26]. We and others demonstrated that LNCaP prostate cancer cells were resistant to TRAIL [23, 25, 26]. For the first time our results demonstrated that isoflavones, such as daidzein, puerarin, ipriflavone, genistein, neobavaisoflavone, and biochanin-A augmented TRAIL induced cytotoxicity in LNCaP prostate cancer cells. The strongest cooperation with TRAIL was exhibited by neobavaisoflavone and biochanin-A. There are no reports describing the effect of TRAIL in combination with isoflavones on prostate cancer cells. The similar findings in other cancer cell line models also demonstrated that these compounds overcome TRAIL resistance [29, 36-42]. Nozawa et al. for the first time showed the effective result of TRAIL and genistein combination by inhibiting pancreatic cancer growth [37]. Jin et al. demonstrated the sensitization of TRAIL resistant human gastric cancer by genistein through activation of caspase-3 [38]. Siegelin et al. explained the enhancement of TRAIL induced apoptosis by genistein and daidzein in glioma cells. The authors confirmed that these isoflavones decreased the expression of antiapoptotic proteins cFLIP or Bcl-2 [39, 40]. Jin et al. again described the overcome of TRAIL resistance in hepatocellular cancer cells by genistein *via* inhibition of p38-beta mitogen-activated protein kinase (MAPK) activation or increase of proapoptotic Bid (BH3-interacting domain death agonist) cleavage [41, 42]. The obtained data suggest the significant role of isoflavones in anticancer immune effectors mechanisms with TRAIL against cancer cells. Therefore, further *in vitro* and *in vivo* investigations will be required to explain the cellular and molecular mechanism by which isoflavones enhance the TRAIL induce death in prostate cancer.

CONCLUSIONS

Isoflavones possess anticancer and chemopreventive properties. Our study showed that isoflavones augment TRAIL induced cytotoxicity in prostate cancer cells and confirmed the importance of isoflavones in chemoprevention of prostate cancer.

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