Vol. 179, No. 1, 1991
August 30, 1991

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 661-667

GENISTEIN INHIBITION OF THE GROWTH OF HUMAN BREAST CANCER CELLS: INDEPENDENCE FROM ESTROGEN RECEPTORS AND THE MULTI-DRUG RESISTANCE GENE

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Received July 25, 1991

The effect of isoflavones on the growth of the human breast carcinoma cell lines, MDA-468 (estrogen receptor negative), and MCF-7 and MCF-7-D-40 (estrogen receptor positive), has been examined. Genistein is a potent inhibitor of the growth of each cell line (IC_{50} values from 6.5 to 12.0 μg/ml), whereas biochanin A and daidzein are weaker growth inhibitors (IC_{50} values from 20 to 34 μg/ml). The isoflavone β-glucosides, genistin and daidzin, have little effect on growth (IC_{50} values >100 μg/ml). The presence of the estrogen receptor is not required for the isoflavones to inhibit tumor cell growth (MDA-468 vs MCF-7 cells). In addition, the effects of genistein and biochanin A are not attenuated by overexpression of the multi-drug resistance gene product (MCF-7-D40 vs MCF-7 cells).

Rats consuming a soy-based diet develop a lower number of mammary tumors following administration of the carcinogens N-methylnitrosourea and 7,12-dimethylbenz[a]anthracene than rats on isonitrogenous and isocaloric diets without soy (1). We have speculated (1) that the aglucones of the isoflavones in soy, genistein (5,7,4'-trihydroxyisoflavone) and daidzein (7,4'-

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Abbreviations: ER, estrogen receptor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MDR, multi-drug resistance; TPK, tyrosine protein kinase; EGF-R, epidermal growth factor receptor; HPLC, high performance liquid chromotography; gp 170, a 170,000 Da glycoprotein, a product of the multidrug resistance gene.

0006-291X/91 $1.50

dihydroxyisoflavone), may have properties similar to the antiestrogen drug tamoxifen, which competes with estrogen for occupancy of the estrogen receptor (ER), thereby inhibiting the metastatic growth of breast cancer. On the other hand, it has been shown that isoflavones, particularly genistein, are potent inhibitors of the tyrosine protein kinase (TPK) activity of growth factor receptors, such as epidermal growth factor receptor (EGF-R) (2), and several oncogenes which may be associated with tumor cell growth and tumor recurrence, such Ha-ras (3) and $pp56^{lck}$, a src-family kinase (4). Since many recurrent breast cancers are ER-independent, a drug or dietary agent that inhibits the growth of both ER^+ and ER^- tumors would be of great interest.

In addition, it is important to determine whether such compounds are substrates of the multi-drug resistance (MDR) gene product, P-glycoprotein. This 170 KDa cell membrane protein (gp 170) confers resistance to a wide range of chemotherapeutic agents by acting as a drug efflux pump, thereby reducing the concentration of the drug in the cytoplasm of the tumor cell (5).

In this study, we have examined whether: (1) soy isoflavones inhibit the growth of human breast cancer cells in culture, (2) whether inhibition is dependent on the expression of the ER and (3) whether inhibition is attenuated by expression of gp 170.

MATERIALS AND METHODS

Materials: Soy molasses was a gift of the Archer Daniels Midland Co. (Decatur, IL). Fetal bovine serum and antibiotics were obtained from Gibco (Gaithersburg, MD). Tissue culture supplies were from Costar (Charlotte, NC). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), doxorubicin, α- and β-glucosidases and biochanin A (4'-methoxygenistein) were from Sigma Chem. Co. (St. Louis, MO). Microtiter plates, Sephadex G-25 and CNBr-activated Sepharose were purchased from Pharmacia (Piscataway, NJ). Aquapore C_8 columns were from Brownlee Labs (Santa Clara, CA).

Cell Culture: MCF-7 and MCF-7 D-40, and MDA-468 human breast cancer cells lines were gifts of Dr. William Dalton (University of Arizona) and Dr. Jeff Kudlow (Division of Endocrinology, University of Alabama at Birmingham), respectively. MCF-7 and MCF-7 D-40 cells were maintained in RPMI 1640 medium supplemented with 7% (v/v) fetal-bovine serum and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin); MCF-7-D-40 cells also received 10^{-8} M doxorubicin to maintain the MDR phenotype; MDA-468 cells were maintained on Dulbecco's Modified Eagles medium low glucose, with 10% (v/v) fetal bovine serum and antibiotics (as above). Cells were cultured as

monolayers (passed every 7-8 days) in a 95% air: 5% CO_2, water-saturated atmosphere.

Isoflavone Preparation: Genistin and daidzin were isolated from soy molasses by fractional crystallization (6) and by adsorption chromatography (7), respectively. Their aglucones, genistein and daidzein, were prepared by hydrolysis in methanol: 1 M HCl (1:1 v/v).

Sample Preparation: Isoflavone samples were prepared from 10, 5, or 2.5 mg/ml stock solutions in DMSO. Aliquots were then taken to prepare the various samples (final concentrations from 1 to 100 μg/ml). DMSO was added as necessary to give a final DMSO concentration of 1% (v/v) in each well.

HPLC Analysis: The purity of the stock solutions and the stability of the isoflavones in the tissue culture media during incubation with the cells were determined by reversed-phase HPLC on a 30 x 0.45 cm Aquapore C_8 column using gradient elution with a mobile phase consisting of 0-45% acetonitrile in 0.1% (v/v) aqueous trifluoroacetic acid. Eluting substances were detected by their absorbance at 262 nm.

Viability Assay: Cytoxicity of the isoflavones was determined by a modification of the MTT assay (8), which is based on the reduction of MTT by the mitochondrial dehydrogenases of viable cells. Cells were plated into 96-well tissue culture clusters at densities of 2 x 10^3 cells/well (MCF-7), 10^4 cells/well (MCF-7-D40) and 2.5 x 10^3 cells/well (MDA-468) in 198 μl of media (optimal numbers of cells for each well were previously determined by ^3H-thymidine uptake). After plating, the cells were allowed to attach for 2 days. Isoflavones were then added (2 μl volumes as described) and incubation continued for 4 days; control wells received 2 μl DMSO. After 4 days, 50 μl of 2 mg/ml MTT was added to each well and the plates incubated for 4 h at 37° C. Media and unreacted MTT were then removed by gentle aspiration. One row of cells had the media removed for HPLC analysis. DMSO (100 μl) was added to each well and the plates were gently shaken for 5 min at room temperature. The optical density at 540 nm was immediately determined using a MAXLINE plate reader (Molecular Devices, Menlo Park, CA). Absorbance at 690 nm was also measured to compensate for interfering effects of cell debris and the plate itself. The percent survival was determined by comparing the absorbance for treated cells to that obtained for control cells. Each experiment consisted of 3 plates, and the results given are the mean and standard error of three separate experiments.

RESULTS AND DISCUSSION

Genistein was a potent growth inhibitor in both MCF-7 cells (IC_{50} 10.5 μg/ml) and MDA-468 cells (IC_{50} 6.5 μg/ml) (Fig. 1A). Biochanin A had weaker inhibitory effects on the growth of MCF-7 and MDA-468 cells (IC_{50} values of 22 μg/ml and 30 μg/ml, respectively) (Fig 1B). Daidzein, also had weak effects on cell growth, with IC_{50} values of 28 μg/ml for MCF-7 cells and 34 μg/ml for MDA-468 cells (Fig 1C). The isoflavone β-glucosides, genistin and daidzin, were not effective

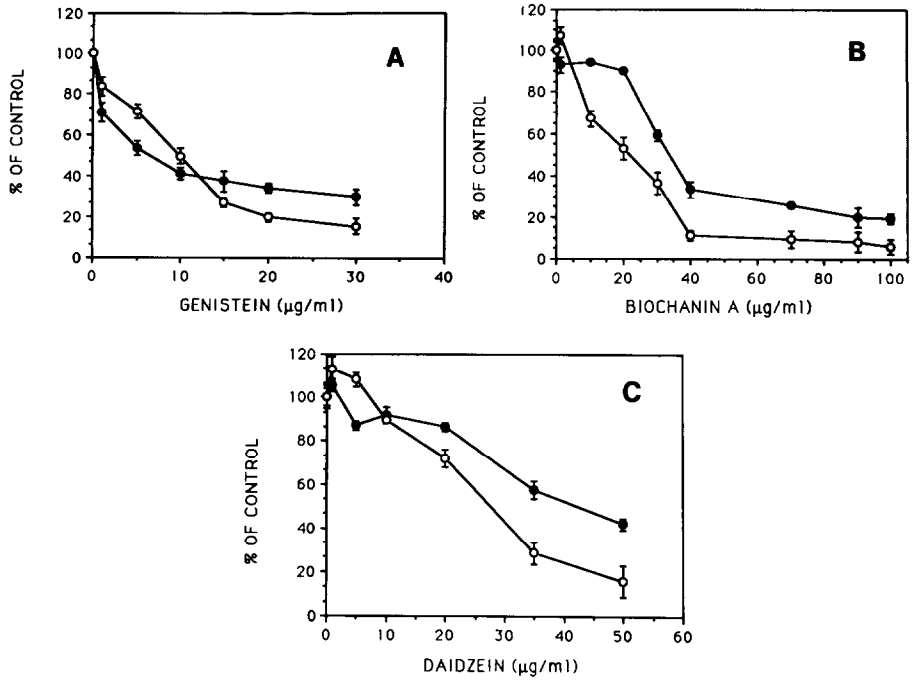

Figure 1. Inhibition of growth relative to controls of MCF-7 (0) and MDA-468 (●) cells by the isoflavones genistein (panel A), biochanin A (panel B) and daidzein (panel C).

in inhibiting cell proliferation, with IC_{50} values above 100 µg/ml (Fig. 2A and B). The weak growth inhibition observed at higher concentrations of genistin in MDA-468 cells appeared to be due to tumor-cell induced hydrolysis of genistin to genistein (data not shown). This hydrolysis did not occur in MCF-7 cells.

There was no significant difference in the potency of growth inhibition of MDA-468 and MCF-7 cells by each isoflavone. These data suggest that the

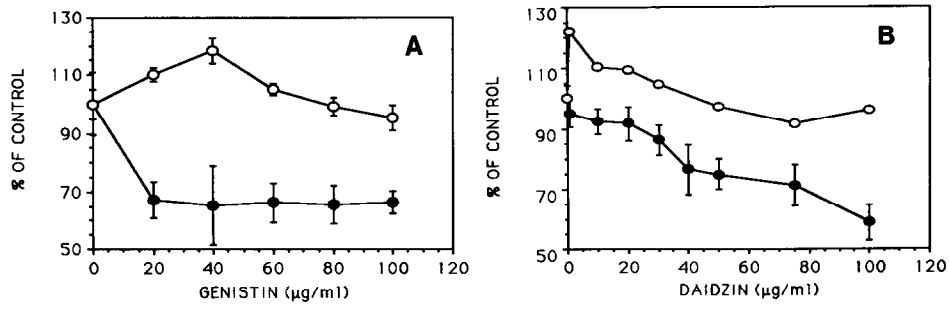

Figure 2. Inhibition of growth relative to controls of MCF-7 (O) and MDA-468 (●) cells by the isoflavones genistin (panel A) and daidzin (panel B).

isoflavones can act via an ER-independent pathway. This does not, however, rule out the involvement of ER in isoflavone action in ER^+ cells. Recent evidence has shown that increased phosphorylation of the estrogen and progesterone receptors can alter the activity of these receptors (9,10). The isoflavones could, therefore, in part, exert their effect by interfering with their phosphorylation state.

The precise mechanism of action of genistein, and of isoflavones in general, on tumor cell proliferation is at present unknown. The effect of genistein is not, however, non-specific; although the growth of ras-transformed NIH-3T3 cells was inhibited by genistein, the growth of non-transformed cells at the same genistein concentration was unaffected (11). Genistein inhibits the intrinsic TPK activity of many growth factor receptors, including EGF-R (2,3) and platelet-derived growth factor receptor (12). The isoflavones could also inhibit targets downstream of the activated receptor such as phospholipase C-γ, phosphatidylinositol kinases, or MAP kinase, all of which show increased tyrosine phosphorylation in response to EGF treatment (13,14,15,16). In support of this view, the isoflavone psi-tectorigenin (8-methoxygenistein) has been shown to inhibit cellular phosphatidylinositol turnover without inhibiting EGF-R TPK activity in A431 fibroblasts (17). Also, genistein can cause cytostatic effects on cell growth without inhibiting the EGF-R TPK activity in NIH-3T3 cells, possibly due to its preferential inhibition of ribosomal S6 phosphorylation (18), which is thought to occur via MAP kinase (19). However, Ogawara et al. (3) found no close correlation between inhibition of EGF-R tyrosine kinase activity in vitro and the reduction in the growth of Ha-ras transformed NIH-3T3 cells.

An alternative mechanism for the action of isoflavones is their inhibition of DNA topoisomerases. Genistein has been shown to inhibit mammalian DNA topoisomerase II in L-1210 cells (12). Also, a chinese hamster ovary cell line with altered DNA topoisomerase activity has been isolated that is more resistant to genistein than the parental cell line (20).

A derivative of the MCF-7 cell line, MCF-7-D40, which overexpresses gp 170, is resistant to the potent anticancer drug doxorubicin (Fig. 3A) (6). However, the

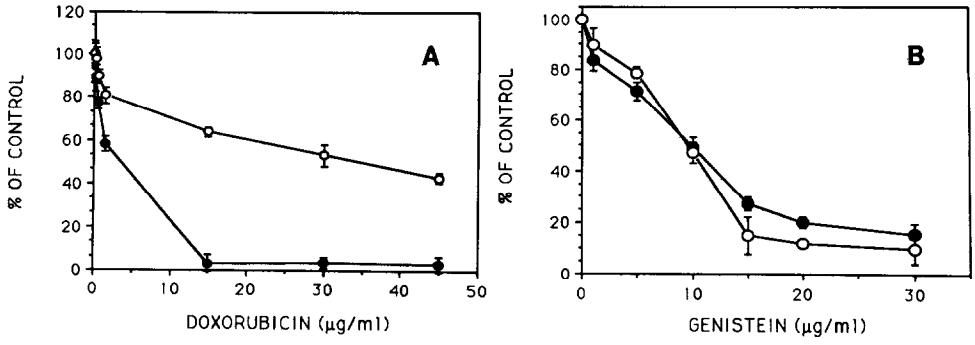

Figure 3. Inhibition of growth of MCF-7 wild type (●) and MCF-7-D40 (○) cells by doxorubicin (panel A) and genistein (panel B).

IC_{50} for genistein was the same for the MCF-7 and MCF-7-D40 cell lines (Fig. 3B). In addition, the IC_{50} for biochanin A was lower in the MCF-7-D40 cells than in the MCF-7 cells (data not shown). These results show that neither genistein nor biochanin A are adversely effected by overexpression of gp 170, and suggest that the isoflavones, in general, may be immune to the multidrug resistance phenomena. In support of this observation, Honma et al. have shown that genistein induces differentiation of a multi-drug resistant K562 (human myelogenous leukemia) cell line as effectively as in its parental cell line (21).

The data obtained in this study support the notion that the isoflavones genistein and daidzein are active anti-cancer agents in soy. Thus soy, a significant part of the diet of many Orientals, may be an important factor which accounts for the low rate of breast cancer in Oriental women (22).

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