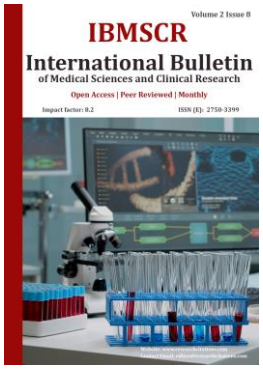

EFFECTS OF DIETARY DAIDZEIN AND ITS METABOLITE, EQUOL, AT PHYSIOLOGICAL CONCENTRATIONS ON THE GROWTH OF ESTROGEN-DEPENDENT HUMAN BREAST CANCER (MCF-7) TUMORS IMPLANTED IN OVARIECTOMIZED ATHYMIC MICE

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Abstract
Genistein and daidzein are the main isoflavones in legumes. Equol is an intestinal bacterial metabolite of daidzein. In this study, we evaluated the estrogenic potential of daidzein and synthetic (±)-equol to stimulate growth of estrogen-dependent breast cancer (MCF-7) *in vitro* and *in vivo*. We hypothesize that estrogenic effects of daidzein and (±)-equol could modulate the growth of MCF-7 cells both *in vitro* and also once implanted into ovariectomized athymic mice. At concentrations between 0.001 and 50 μM, daidzein and (±)-equol stimulated the growth of MCF-7 cells with maximal stimulation at 1 μM *in vitro*. To evaluate their effects on the growth of MCF-7 cells implanted in ovariectomized athymic mice, two dietary dose–response studies [daidzein (125, 250, 500 and 1000 p.p.m.) and (±)-equol (250, 500 and 1000 p.p.m.)] were conducted. Tumor size and body weight were monitored weekly during the study. At completion of the study, we analyzed cellular proliferation of tumors using immunohistochemical staining (ki-67), pS2 expression in tumors using a real time quantitative reverse transcription–polymerase chain reaction (qRT–PCR), and total daidzein and (±)-equol levels in plasma using liquid chromatography–electrospray tandem mass spectrometry (LC–ES/MS/MS). Dietary daidzein had a slight but significant stimulatory effect on MCF-7 tumor growth in mice. No significant induction of pS2 mRNA (an estrogen-responsive marker) in tumors by dietary daidzein was observed. Total plasma daidzein concentrations in plasma were between 0.25 and 1.52 μM. Dietary equol treatment (for 37 weeks) did not stimulate MCF-7 tumor growth. There were no statistical differences in tumor size, proliferation and pS2 expression among any treatment groups. Total equol concentrations in plasma were 2.10–3.21 μM. In conclusion, daidzein and (±)-equol have proliferative effects on MCF-7 cell growth *in vitro* within the concentration range tested. Dietary daidzein had a slight but significant stimulatory effect on tumor growth, whereas (±)-equol did not stimulate the growth of estrogen-dependent breast tumor growth in athymic mice, increase the cell proliferation in tumors, or induce an estrogen-responsive pS2 expression. Total daidzein or (±)-equol plasma levels in mice fed the isoflavones were in the range that stimulated MCF-7 cell growth *in vitro*. These results suggest that pharmacokinetic and/or metabolic factors attenuate the estrogenic effects of daidzein and equol *in vivo*.

Key words: AIN93G, American Institute of Nutrition 93 growth semi-purified diet, BCS, bovine calf serum, CD-BCS, charcoal-dextran stripped BCS, E_2, 17β-estradiol, ER, estrogen receptor, LC-ES/MS–MS, liquid chromatography–electrospray/tandem mass spectrometry,

MCF-7, Michigan Cancer Foundation-7, PBS, phosphate buffered saline, MEM, minimal essential media, MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, OD, optical density, qRT–PCR, real time quantitative reverse transcription polymerase chain reaction, SEM, standard error of mean. Previous SectionNext Section

Introduction

Dietary isoflavones are abundant in legumes, particularly in soy. Genistein and daidzein, the principal soy isoflavones, have attracted attention because of their putative value as natural alternatives to hormone replacement therapy (HRT) for relieving post-menopausal symptoms (1). Although many studies have been conducted to understand the effects of soy isoflavones on breast cancer during various stages of life, the results are inconsistent and the mechanism of action is still unclear. Our previous studies in animals have shown the possibility that genistein stimulates estrogen-dependent human breast tumor growth (2–4) and negates the effectiveness of tamoxifen treatment (5).

Daidzein is the second-most prominent isoflavone in soy products. After ingestion, daidzein is converted by the gut microflora into dihydrodaidzein, S-(−)-equol (70%) and *O*-desmethylangolensin (5–20%) (6–10). Equol was first isolated from the urine of pregnant mares in 1932 (11) and was subsequently identified in the plasma of sheep (derived from formononetin in red clover) (12) and in the urine of human (from daidzein) (13). Equol is consistently observed in high amounts in blood from experimental animals, including rodents and monkeys. However, equol is observed in only ~35% of humans, presumably because of differences in the composition of gut microflora (14–18). It has been shown that the amount of urinary equol excretion is correlated to the consumption of soy products (14,19,20) and a reduced risk of breast cancer (21–23).

Equol is a chiral molecule that can exist as the R-(+)- and S-(−)-enantiomers and previous work suggested that both enantiomers were more estrogenic *in vitro* than daidzein (24–26). Daidzein acts as an agonist for both ERα and ERβ and induces ER-mediated estrogenic responses both *in vitro* (27) and *in vivo* (28–32). Daidzein has much lower affinity than genistein for binding to and transactivation through ERα and ERβ (26). In mice, genistein and equol are uterotrophic, but daidzein is not (33–35). The use of soy products and isoflavone supplements by women with breast cancer is increasing to treat cancer-related symptoms as well as to relieve menopausal symptoms, and to enhance general well-being based on their estrogenic properties (36). Isoflavone levels in dietary supplements vary and can contain over 100 mg per serving. Major isoflavone components in dietary supplements are biochanin A, genistin, and genistein, and formononetin, daidzin and daidzein (17). The dietary effect of equol has not been studied apart from the other isoflavones, principally because there is no commercial source for sufficient quantities. The significant binding affinity and effective transactivation activity of synthetic racemic (±)-equol on the ER subtypes and the association of the excretion of equol with effects on breast cancer risk prompted the current study. In this study, we tested the hypothesis that daidzein and equol have biological effects on the growth of estrogen-dependent human breast cancer cells *in vitro* and after implantation into athymic mice.

Materials and methods

Materials

Daidzein was purchased from Indofine (Somerville, NJ) and Plantech (Reading, UK) supplied dihydrodaidzein. Racemic equol (purity ~98% as determined by ^{1}H-NMR and LC/MS, data not

shown) was prepared as described by Muthyala *et al*. (37). Minimal Essential Medium (MEM, without gentamicin, with glutamine) and phenol red-free MEM was purchased from the Media Facility at University of Illinois at Urbana-Champaign. Bovine calf serum (BCS) was purchased from Hyclone (Logan, UT). Penicillin/streptomycin and trypsin/EDTA were purchased from Invitrogen (Houston, TX). Laboratory animal diet and dietary components were purchased from Dyets (Bethlehem, PA). Reagents for qRT–PCR were purchased from PE Applied Biosystems (Foster City, CA), Synthegen (Houston, TX) and Invitrogen (Carlsbad, CA).

Human breast cancer cell maintenance

MCF-7 cells are estrogen-dependent tumor cells isolated from a post-menopausal woman (38). MCF-7 cells were maintained in MEM supplemented with 5% BCS, 1% penicillin (100 U/ml)/streptomycin (100 µg/ml) and 1 nM 17β-estradiol (E_2). MCF-7 cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air as a monolayer culture in plastic culture plates (100 mm diameter). One week before the cell proliferation assay or the injection of MCF-7 cells into athymic mice, the medium was switched to phenol red-free MEM containing 5% charcoal dextran stripped (CD)-BCS (2) and 1% penicillin/strepomycin.

Cell proliferation

To compare the effects of physiological and phamacological dosage of isoflavones on MCF-7 cell proliferation, a modified colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (39) was used. Mitochondrial dehydrogenase activity was measured by MTT. MCF-7 cells were grown in estrogen-free culture media (phenol red-free MEM containing 5% CD–BCS and 1% penicillin/streptomycin) for 2 weeks prior to the treatment. For the growth experiments, MCF-7 cells (1.5×10^4) were inoculated in triplicate in 1 ml of estrogen-free culture media in a 24-well polystyrene culture plate. After 24 h, the MCF-7 cells in each well were washed with 1 ml of phosphate-buffered saline (150 mM PBS, pH 7.4, without Ca^{2+} and Mg^{2+}) and treated with various concentrations (0.001, 0.01, 0.1, 1, 5, 10 and 50 µM) of daidzein or (±)-equol every 48 h. After the 5 day treatment, the cells were treated with MTT for 5 h and treated with 10% SDS (in 0.01 N HCl) for 12–18 h. Optical density (OD) was measured at 570 nm. OD values were normalized to number of cells based on a standard curve. Daidzein and (±)-equol were also evaluated in the presence of ICI 182 780 (100 nM).

Athymic nude mice

Female athymic BALB/c (nude) mice were purchased from Charles River Laboratories (Wilmington, MA) and acclimated for a week. The xenograft model used in this study, ovariectomized athymic mice implanted with MCF-7 cells, is an appropriate model of post-menopausal women with estrogen-dependent breast cancer. Mice were ovariectomized at 21 -day of age by the vendor and the allowed to recover for 7 day. During the study, the mice were maintained under the standard light/dark cycle (12 h light, 12 h dark).

E_2 pellet preparation

An E_2 pellet contained 2 mg of E_2 and 18 mg of cholesterol. An E_2 pellet was placed subcutaneously under the skin of each mouse before MCF-7 cells were injected into the mice (40).

Diet formulation

American Institute of Nutrition 93 growth diet (AIN93G) semi-purified diet (Dyets, Bethlehem, PA) was selected as a base diet for control mice as it has been established as meeting all of the nutritional requirements of mice (41). Soy oil was removed from all diets

and corn oil added in order to eliminate any additional components of soy being added to the diets. Treatment animals were fed AIN93G diet plus different dosages of daidzein (125, 250, 500 or 1000 p.p.m.) or (±)-equol (250, 500 or 1000 p.p.m.).

Tumor growth analysis

Study I: daidzein

One week after the E_2 pellets were inserted into mice, MCF-7 cells [1×10^5 cells/40 µl of Matrigel® (Collaborative Biomedical Products, Bedford, MA] were injected at 40 µl/site into each of the four sites on the backs of the athymic mice. All mice were on AIN93G diet. Tumors were grown until their average cross-sectional area reached 35 mm^2. Mice were divided into six treatment groups: MCF-7 control, E_2 control, 125, 250, 500 and 1000 p.p.m. of daidzein (10–11 mice/group). E_2 pellets were removed from all mice, except the E_2 group, and dietary daidzein treatment was started. Tumor growth and body weight were measured weekly for 21 week and cross-sectional area was determined using the formula [length/2 × width/2 × π] (42). Owing to a skin rash in all treatment as the attending veterinarian clinician recommended the study be terminated. All mice were killed at week 21 and tumors and blood were collected for analysis. Food intake was measured throughout the study. At the end of the study, uterine weight was measured. Tumors and blood samples were collected for analysis.

Study II: equol

When the mean tumor surface area reached 37 mm^2, mice were divided into five treatment groups: MCF-7 control, E_2 control, 250, 500 and 1000 p.p.m. (±)-equol (11–12 mice/group). Tumor growth was monitored for 37 weeks. All the other experimental steps were the same as described for Study I.

Ki-67 Expression (a measure of proliferation)

Cell proliferation in tumors was determined using immunohistochemical analysis. Tumors in 10% formalin were embedded in paraffin blocks, cut into 5 µm sections and placed on microscope slides. Slides were deparaffinized by immersing in xylene twice for 12 min each and hydrated by immersing in a series of 100% ethanol, 95% ethanol and three times in dH_2O for 5 min each. To block endogenous peroxidase, slides were immersed in 0.3% H_2O_2 for 20 min and then washed with dH_2O. Slides were placed in citrate buffer (pH 6), microwaved for 20 min, and then cooled at 24°C. Then, slides were washed in PBS (pH 7.4) for 5 min. Anti-human Ki-67 antibody (Pharmingen, San Diego, CA) (1 : 3000 dilution in 1% BSA/PBS) was added to the slides, which were then incubated for 1 h at 24°C in a humidity chamber. Slides were washed in PBS before the addition of biotinilated anti-mouse secondary antibody (VECTASTAIN Elite ABS reagent, Vector Laboratories, Inc. Burlingame, CA) was added to slides and incubated for 30 min at 24°C. Slides were then washed in PBS. One drop of 3,3′-diaminobenzidine tetrahydrachloride (DAB) prepared right before used was added to each slide. Slides were then washed twice in dH_2O and PBS and counterstained with 20% hematoxlin for 1 min. The slides were then dehydrated by submerging them in 80% ethanol for 5 min, 95% ethanol for 5 min and 100% ethanol for 5 min, followed by submerging slides in xylene four times for 5 min each. Slides were mounted and analyzed using a light microscope. Both positive and background stained cells were counted in a given area of tissue. A total of 25 fields from 5 tumors per treatment group were evaluated. The data were then presented as a percentage of cells proliferating in a given area of tumor.

RNA preparation and analysis of changes in gene expression using qRT–PCR

Expression of pS2 mRNA was analyzed using qRT–PCR. Tumors with areas similar to the mean tumor surface area of the each treatment group were used for mRNA analysis. RNA from frozen tumor (≤200 mg) was prepared as described by Ju *et al*. (5). cDNA were generated using 10 ng of RNA and TaqMan Reverse Transcription Reagents (PE Applied Biosystems, Foster City, CA). The pS2 primers and fluorescence (6-FAM)-labeled probes were designed using Primer and Probe Design Express (PE Applied Biosystems) (39). The human GAPDH primers and a fluorescent (6-FAM/TAMRA)-labeled probe (User Bulletin #2, PE Applied Biosystems) were used as a control. PCR and analysis of PCR products were performed using an ABI PRISM 7700 Sequence Detector (PE Applied Biosystems). Data were analyzed using a comparative threshold cycle (C_t) method (User Bulletin, PE Applied Biosystems). Amplicons were run as triplicates in separate tubes to permit quantification of target genes normalized to a control, GAPDH.

Plasma analysis

Blood samples were collected by cardiac puncture at sacrifice, placed into EDTA-containing tubes and centrifuged at 500 *g* for 5 min. Plasma samples were stored at −20°C until analyzed by liquid chromatography and isotope dilution electrospray tandem mass spectrometry (LC–ES/MS/MS) using a method previously validated (43). Levels of total daidzein, dihydrodaidzein and equol were determined in 25–50 µl plasma after treatment with β-glucuronidase/sulfatase for deconjugation and addition of deuterated daidzein (d3) and equol (d4) as internal standards. Deuterated daidzein was used as the internal standard for dihydrodaidzein. Five to eight plasma samples per treatment group were analyzed. The limits of quantification for the three analytes were ~0.01 µM, method accuracy was 88–98%, and the method precision was 3–13% (43).

Statistics

Data from tumor area at final week, cell proliferation, qRT–PCR and plasma analysis were analyzed using one-way or repeated-measures analysis of variance according to the characteristics of the dataset using the SAS program. If the overall treatment F-ratio was significant ($P < 0.05$), the differences between treatment means were tested with Fisher's least significant difference (LSD) test.

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Results

Effect of daidzein and (±)-equol on the growth of MCF-7 cells in vitro

MCF-7 cellular proliferation is expressed as mean number of MCF-7 cells ± SEM. At concentrations of daidzein and (±)-equol ≤1 µM, MCF-7 cell proliferation increased in a concentration-dependent manner to a maximum of 1 µM, which was 2.5-fold higher than the MCF-7 control for daidzein (gray bars, Figure 1A) and 2.3-fold higher for (±)-equol (gray bars, Figure 1B). The daidzein- and (±)-equol-induced MCF-7 cell growth was blocked by ICI 182,780 (100 nM), further confirming that the isoflavone-induced cell growth is ER-mediated (white bars, Figure 1A and 1B). At all concentrations of daidzein and (±)-equol, there were significant differences between isoflavone alone and isoflavone + ICI 182,780. At higher concentrations of daidzein and equol (5–50 µM), MCF-7 cell proliferation decreased from the maximum level.

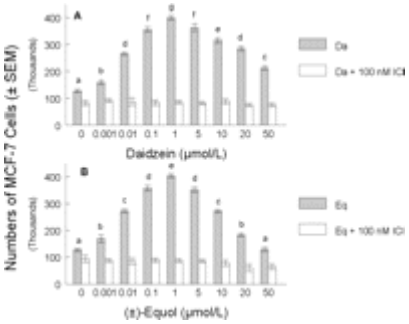

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Fig. 1.

Effects of daidzein and equol on the growth of MCF-7 cells. Daidzein (**A**) and (±)-equol (**B**) at concentrations between 1 nM and 50 μM in the presence of (white bars) and in the absence of ICI 182,780 (100 nM) (gray bars) were evaluated for their effects on MCF-7 cell growth using MTT assay. Values are means ± SEM (n = 9). Bars with different letters differ, $P < 0.05$.

Effect of daidzein and (±)-equol on pS2 mRNA expression in MCF-7 cells

MCF-7 cells were treated with daidzein or equol at a concentration of 1 μM, which induced highest cell proliferation for 24 h, and pS2 mRNA expression in MCF-7 cells was evaluated. Daidzein (1 μM) increased pS2 mRNA level by 4.18 ± 0.54 fold, and equol increased by 3.79 ± 0.20 fold over the MCF-7 control (Figure 2) ($P < 0.05$). Genistein induced similar levels of pS2 expression.

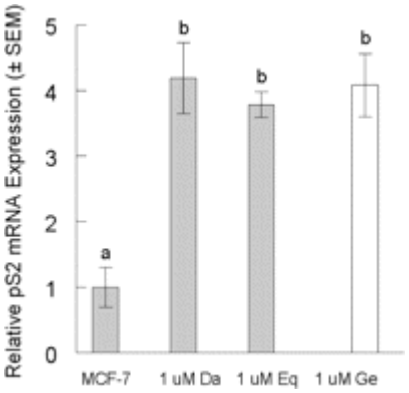

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Fig. 2.

Estrogen-responsive pS2 gene expression in tumors. MCF-7 cells were treated with 1 μM daidzein, equol or genistein for 24 h and analyzed using qRT–PCR. Numbers on the y-axis represent the relative mRNA level; bars ± SEM. Data points were obtained from three replicas. GAPDH was used as a standard. Bars with different letter are significantly different, $P < 0.05$.

Tumor growth

Study I: daidzein

Mean tumor surface area in the E_2 positive control group reached 138.0 mm^2 3 weeks after the study started and mice were terminated because of a high tumor burden. Tumors in the

rest of the treatment groups were monitored for 21 weeks (Figure 3). After removal of the E_2 pellets, tumors in the MCF-7 and daidzein treatment groups regressed. Tumors in the daidzein treatment groups regressed slower than ones in the MCF-7 control group. Tumors in the 125 and 250 p.p.m. daidzein groups showed steady regression during 21-weeks dietary treatment, but tumors in the 500 and 1000 p.p.m. groups regressed for 18 weeks and then appeared to grow slowly. At week 21, the mean tumor surface areas were 4.8 ± 0.7 mm^2, 14.3 ± 2.2 mm^2 (by 3.0× larger than tumors in the MCF-7 control group), 12.0 ± 1.5 mm^2 (by 2.5×), 20.8 ± 2.6 mm^2 (by 4.3×) and 30.9 ± 3.9 mm^2 (by 6.4×), for the MCF-7, 125, 250, 500 and 1000 p.p.m. daidzein groups, respectively. There were statistically significant differences in tumor area between the MCF-7 control and all the daidzein treatment groups ($P < 0.05$) (Figure 3). The average tumor size of the 1000 p.p.m. daidzein group was significantly than other daidzein treatment group ($P < 0.05$) (Figure 3). No statistically significant difference was observed between the 125 and 250 p.p.m. group ($P = 0.3857$) or between 250 and 500 p.p.m. ($P = 0.0652$).

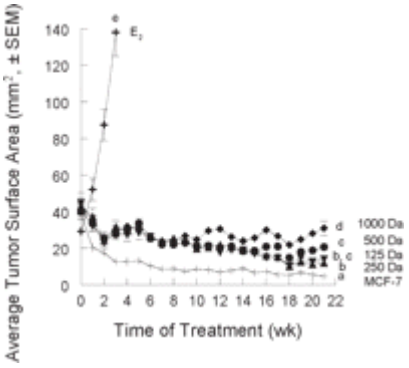

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Fig. 3.

Effect of dietary daidzein on MCF-7 tumor growth in athymic mice. Mice were assigned into six treatment groups: MCF-7 control (12 mice; 48 tumors at K 0 and 29 tumors at week 21), E_2 control (9 mice; 34 tumors at week 0 and 34 tumors at week 3), 125 p.p.m. daidzein (8 mice; 31 tumors at week 0 and 23 tumors at 21), 250 p.p.m. daidzein (9 mice; 35 week at week 0 and 26 tumors at week 0), 500 p.p.m. daidzein (9 mice; 35 tumors at week 0 and 27 tumors at week 21) and 1000 p.p.m. daidzein (9 mice; 34 tumors at week 0 and 2 tumors at week 21). Week 0 was the first day that animals were started on the experimental diets. Tumors were then measured weekly for 21 weeks. Data are expressed as average cross sectional tumor area (mm^2 ± SEM) for all tumors in each treatment.

Study II: equol

Tumors in the E_2 group reached 123.3 mm^2 3 weeks after the study started and mice were terminated because of a high tumor burden. Tumors in the rest of the treatment groups were monitored for 37 weeks (Figure 4). After removal of the E_2 pellets, tumors in all equol treatment groups regressed. At week 37, the mean tumor surface areas were 6.9 ± 1.1, 9.1 ± 3.1, 12.1 ± 2.4 mm^2, and 10.1 ± 2.4 mm^2, for MCF-7 control, 250, 500, and 1000 p.p.m. equol groups, respectively. No statistically significant differences were observed between the MCF-7 and equol treatment groups (Figure 4).

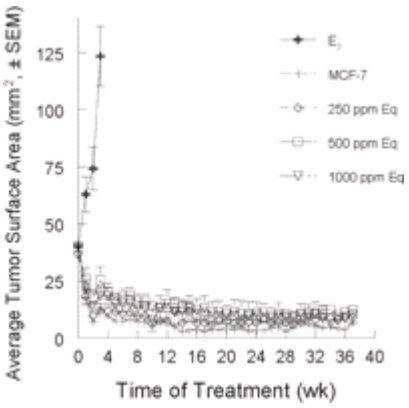

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Fig. 4.

Effect of dietary (±)-equol on MCF-7 tumor growth in athymic mice. Mice were assigned into six treatment groups: MCF-7 control (9 mice; 34 tumors at week 0 and 20 tumors at week 37), E_2 control (10 mice; 38 tumors at week 0 and 38 tumors at week 3), 250 p.p.m. equol (10 mice; 36 tumors at weeks 0 and 22 tumors at week 37), 500 p.p.m. equol (9 mice; 34 tumors at week 0 and 25 tumors at week 37) and 1000 p.p.m. equol (9 mice; 34 tumors at week 0 and 21 tumors at week 37). Week 0 was the first day that animals were started on the experimental diets. Tumors were then measured weekly for 37 weeks. Data are expressed as average cross sectional tumor area (mm^2 ± SEM) for all tumors in each treatment.

Cell proliferation in tumors

Percentages of Ki-67 expression were 2.4% for the MCF-7 the control, 3.6% for the 125 p.p.m. daidzein, 5.6% for the 250 p.p.m. daidzein, 5.9% for the 500 p.p.m. daidzein, 7.7% for the 1000 p.p.m. daidzein and 55.1% for the E_2 control group, respectively (Figure 5). There was significant difference between the MCF-7 and 1000 p.p.m. daidzein groups ($P < 0.05$) and between the MCF-7 and E_2 groups ($P < 0.05$) (Figure 5A). Ki-67 expression was unchanged for all equol groups but significantly increased for the E_2 group ($P < 0.05$) (Figure 5B).

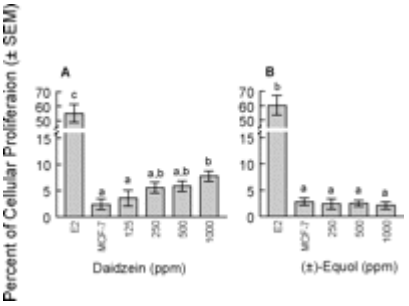

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Fig. 5.

Effect of dietary daidzein and (±)-equol on the cellular proliferation on MCF-7 tumors. Ki-67 expression was evaluated as a measure of proliferation. A total of 25 fields from 5 tumors per treatment group were evaluated. *y*-axis (cell proliferation) is presented as the percentage of

cells actively proliferating in a given area of tissue (± SEM). (**A**) Cell proliferation induced by Daidzein. (**B**) Cell proliferation induced by (±)-equol. Bars with different letters differ, $P < 0.05$.

Body weight, food intake, and uterine wet weight

Body weights were monitored weekly and no significant difference was observed among the treated and control groups (data not shown). No significant differences were observed in food intake among any of the treatment groups (data not shown). No significant difference was observed in uterine weight between the MCF-7 and daidzein/equol groups (data not shown).

pS2 expression in tumors

To evaluate the capability of daidzein and equol to modulate the expression of an estrogen-responsive gene, pS2, mRNA in tumors was analyzed using qRT–PCR (Figure 6). Expression of pS2 is presented as relative pS2 mRNA expression ± SEM. In the daidzein groups, pS2 expression was significantly increased by E_2 (15.0×), by 500 p.p.m. daidzein (3.1×) and by 1000 p.p.m. daidzein (4.1×), compared with the MCF-7 group ($P < 0.05$). In the equol groups, E_2 significantly enhanced pS2 expression over the MCF-7 group ($P < 0.05$). There were no statistically significant differences among the equol treatment groups.

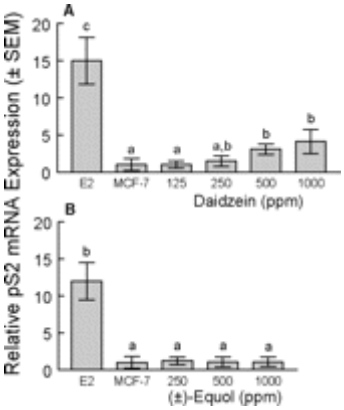

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Fig. 6.

Estrogen-responsive pS2 gene expression in tumors. Six to seven tumors per treatment group were analyzed using qRT–PCR. Numbers on the *y*-axis represent the relative mRNA level; bars ± SEM. GAPDH was used as a standard. (**A**) Dietary daidzein induced pS2 expression. (**B**) Dietary (±)-equol induced pS2 expression. There was no statistical difference among any treatment groups. Bars with different letter are significantly different, $P < 0.05$.

Isoflavone levels in plasma

While no daidzein or equol was detected in plasma from mice in the MCF-7 and E_2 groups (<0.01 μM), the concentration of total daidzein in plasma from daidzein-fed animals increased regularly as the dietary dose increased (125–1000 p.p.m.) with levels that ranged from 0.25 to 1.54 μM (Figure 7A, white bars). Plasma concentrations of daidzein measured in the 1000 p.p.m. daidzein group was significantly higher than that in 125 p.p.m. daidzein group ($P < 0.05$). Formation of equol in daidzein-fed mice was highly variable; in the 125 p.p.m. group, only two of the six mice formed equol, in the 250 p.p.m. group, one of the eight did, in the 500 p.p.m. group, one of the seven did, and in the 1000 p.p.m. group, none did. Equol levels in

these mice ranged from <LOD to 1.01 μM (Figure 7A, gray bars). In addition, dihydrodaidzein was observed in some but not all mice (Figure 7A, black bars); in the 125 p.p.m. group, only three of the six mice formed equol, in the 250 p.p.m. group, four of the eight did, in the 500 p.p.m. group, 4 of 7 did, and in the 1000 p.p.m. group, 1 of 8 did. No statistically significant correlations between the total equol or total dihydrodaidzein levels and daidzein dietary dosages were observed. Total equol in plasma from equol-fed mice ranged from 2.10 to 3.21 μM (Figure 7B) and there were no statistically significant differences between groups.

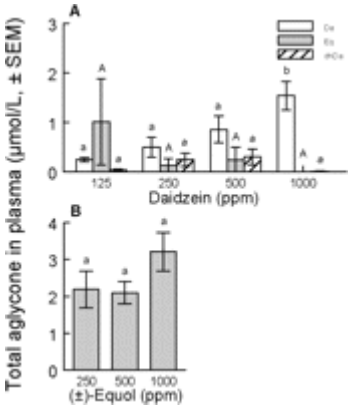

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Fig. 7.

(**A**) Total daidzein, equol and dihydrodaidzein (aglycone + conjugated) plasma levels in dietary daidzein dose–response study (Study 1). Total aglycone level on the y-axis represents μM ± SEM (total daidzein, white bars; total equol, gray bars; dihydrodaidzein, diagonal-patterned bar). (**B**) Total equol plasma levels in dietary equol dose-response study (Study 2). Total aglycone level on the *y*-axis represents μM ± SEM (total equol; gray bars). Bars with different letter are significantly different $P < 0.05$.

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Discussion

Based on *in vitro* studies (44–46) and computational models for ER binding affinities (47), it seemed likely that other dietary isoflavones and metabolites could produce effects similar to those observed for genistein *in vitro*. Genistein, daidzein and equol are agonists for both ERα and ERβ, with preferential binding to and transactivation through ERβ (37). However, the data presented here for daidzein and equol point out several critical differences *in vivo*. Similar to genistein, daidzein and equol showed proliferative effects on MCF-7 cell growth *in vitro* (Figure 1). Daidzein (0.001–50 μM) and (±)-equol (0.001–20 μM) stimulated growth with the maximal stimulation at 1 μM. The concentration dependence for effects by genistein (48), daidzein and equol (Figure 1) on MCF-7 cell growth *in vitro* are similar. Our previous studies have demonstrated that genistein stimulates MCF-7 cell growth *in vitro* at concentrations from 0.1 to 20 μM, with maximal stimulation at 1 μM, and induces expression of the estrogen-responsive gene pS2 (48,49). In addition, there is evidence for an anti-proliferative effect on cell growth apparent at concentrations above 10 μM (48,50,51). In this study, we also observed that daidzein and equol (at 1 μM) enhanced pS2 mRNA expression in MCF-7 cells *in vitro* (Figure 2), suggesting that the cell stimulatory effect observed in Figure 1

may involve an ER-regulated mechanism. Daidzein- and equol-stimulated cell growth was blocked by ICI 182 780 (Figure 1), confirming that the stimulatory effect on cell growth involves an ER-mediated activity. Administration of genistein to athymic mice in the diet between 125 and 1000 p.p.m. produced circulating total genistein concentrations of 0.4–3.4 μM, increased pS2 expression and markers of proliferation and stimulated growth of implanted MCF-7 tumors (2). Soy protein isolates containing increasing concentrations of genistein stimulated the growth of MCF-7 tumors in a dose-dependent manner (3). Consumption of genistein in pure or highly enriched forms (as in soy protein isolates) has a greater stimulatory effect on MCF-7 tumor growth than the same content of dietary genistein in soy flour. These results suggest that the matrix in soy flour alters the effects of dietary genistein on estrogen-dependent tumor growth (52). These estrogen agonistic actions of genistein, mediated through ERs (53), occur at circulating levels of total genistein that are quite similar to those observed in humans consuming soy foods (54–59) and nutritional supplements (60). Unlike genistein, equol, administered through the diet at levels comparable with those used previously for genistein (2), did not enhance growth of implanted MCF-7 tumors *in vivo* (Figure 4). And, dietary daidzein produced a modest, but significant, stimulatory effect (Figure 3). We observed a weak estrogenic effect of dietary daidzein at the concentration range tested (Figure 3), although it was not as potent as that of genistein (2). This mild estrogenic effect is likely due to enhanced cellular proliferation (Figure 5). Furthermore, evidence for ER activation (i.e. elevated pS2 mRNA expression) was not observed in mice fed equol and was significant but weak in mice fed daidzein at 250 ppm and above (Figure 6).

The absence of estrogenic effects in mice fed daidzein or equol is notable because the 'steady-state' plasma levels of total daidzein or equol (Figure 7) were quite similar to those previously measured for total genistein (2) and comparable with those that have estrogenic effects *in vitro* (Figure 1). These results are consistent with the conclusions of our recent study (61) that pharmacokinetic factors beyond bioavailability, possibly reflecting differences in the Phase II metabolism and distribution of active estrogenic isoflavone aglycones in the target tissue, are possible explanations for the observed *in vitro–in vivo* dichotomy between genistein and equol.

Isoflavone supplements have been consumed by post-menopausal women to relieve menopausal symptoms despite equivocal clinical evidence for efficacy (62,63). Furthermore, there is considerable controversy regarding whether isoflavones and their metabolites can produce adverse effects, including possibly stimulating the growth of estrogen-dependent breast tumors in women. Epidemiological evidence suggests that consumption of soy-rich diets can reduce breast cancer risk, particularly during the pre-pubertal period of life (64), and it is hypothesized that equol may be a contributor to the beneficial effects of soy (65). However, other epidemiological investigations show an increased odds ratio for breast cancer incidence for women with higher plasma and urinary phytoestrogen levels (66). Long-term dietary studies of equol, free from the confounding presence of other isoflavones, have not been possible in laboratory animal models until recently because synthetic material in sufficient quantities has not been available (37). The present study, which used a pre-clinical model for post-menopausal estrogen-dependent breast cancer, suggests that dietary daidzein and its metabolite equol have only minimal estrogenic effects. The clear difference in pharmacodynamic effects from genistein (61) suggests that it, and not daidzein or equol, is

the active soy isoflavone that affects breast cancer growth in the athymic mouse-MCF-7 xenograft model. This conclusion is consistent with previous research conducted using a pre-pubertal rat model of breast cancer (67). Lamartiniere *et al*. (67) reported that DMBA-induced mammary tumor multiplicity was not affected in rats fed daidzein-containing diets (250–1000 p.p.m.), despite the observation of high circulating levels of total daidzein (0.98–3.8 μM) and equol (0.16–1.0 μM). These internal exposures to daidzein and equol also failed to stimulate mammary gland differentiation. Those results were opposite to those previously observed by the same research group from identical pre-pubertal treatment with genistein, which produced significant reductions in tumor multiplicity and increased mammary gland differentiation (68). Ward *et al*. (69) reported that 200 p.p.m. daidzein intake for two weeks produced 0.18–0.36 μM daidzein and did not change uterine weight in four mouse strains [two inbred strains (C57BL/6 and C3H) and two outbred strains (CD-1 and Swiss–Webster)].

In summary, dietary daidzein and (±)-equol produce concentrations of total isoflavones in mouse plasma that are similar to those observed in humans. However, these internal exposures to equol did not stimulate growth of estrogen-dependent human breast tumor (MCF-7) growth or induce estrogen-responsive pS2 expression in tumors *in vivo*. Similarly, dietary daidzein had a very modest stimulatory effect on MCF-7 tumor growth and did not increase pS2 gene expression. Findings from this study and our previous studies (52,61) suggest that genistein is the major active estrogenic component from dietary soy isoflavone consumption and is likely responsible for the estrogenic effects on mammary and other estrogen-responsive tissues.

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