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(±)-Equol does not interact with genistein on estrogen-dependent breast tumor growth

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Abstract

Equol (EQ) is a prominent microbial metabolite of the soy isoflavone, daidzein, with estrogen-like properties. The major soy isoflavone, genistein (GEN), stimulated growth of estrogen-dependent breast cancer (EDBC) cells *in vitro* and tumor growth *in vivo* but EQ did not. To understand possible interactions of EQ and GEN on EDBC, EQ was used with GEN in combination *in vitro* and *in vivo*. Effects of EQ, GEN and EQ + GEN were evaluated using MCF-7 and T47D EDBC. Ovariectomized athymic mice were used as a model for *in vivo* tumor growth. Dietary EQ had no effect on MCF-7 tumor growth and the absence of effect was confirmed using a T47D EDBC *in vivo* model. EQ alone or in combination with GEN increased EDBC cell proliferation *in vitro*. EQ alone neither stimulated EDBC tumor growth *in vivo* at various doses nor suppressed tumor growth induced by dietary GEN. In summary, EQ has similar estrogenic effect as GEN *in vitro* but does not interact with GEN on EDBC tumor growth. Based on the evidence presented here, dietary EQ is unlikely to have estrogenic effects *in vivo*.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

Keywords

Equol; Genistein; MCF-7; T47D

1. Introduction

The isoflavone genistein (GEN) is the most well studied and understood of the phytoestrogens and it is an active component of soy foods (Doerge et al., 2016; Ju et al., 2001; Liu et al., 2015; Markiewicz et al., 1993; Setchell et al., 2003; Shutt and Cox, 1972; Yang et al., 2015; Zubik and Meydani, 2003). Dietary GEN is firmly established to have estrogen-like activity *in vivo* as evidenced by the enhanced tumor growth of estrogen-responsive tumors, MCF-7, in the absence of endogenous estrogens (ovariectomized, OVX, mice) (Allred et al., 2005; Andrade et al., 2010; Ju et al., 2001; Liu et al., 2015; Yang et al., 2015). However, GEN is only one of the phytoestrogen isoflavones found in soy foods. Daidzein (DZN) is another important isoflavone in soy and makes up approximately 30% of soy isoflavones while GEN makes up approximately 60% (Divi et al., 1997; Franke et al., 1995; Kim et al., 2005; Kimura et al., 1976). Isoflavone found in soy germ contains mostly DZN and glycitein and a lower percentage of genistein (Imhof et al., 2018). Like GEN, DZN also has structural similarity to E₂. EQ, a reductive microbial metabolite of DZN and is an estrogen receptor agonist *in vitro* (Setchell et al., 2003; Setchell and Clerici, 2010; Setchell et al., 2005). EQ is a chiral molecule, and both enantiomers are ER ligands (Muthyala et al., 2004), and both S-(–)-equol and (±)-equol are estrogenic *in vitro* in several cell types (Ju et al., 2006; Wang et al., 2014). Route of administration and the magnitude of dose impacts the effects of EQ seen in animals. EQ displays some estrogenic activity in mice injected SC with EQ. Specifically, uterine wet weight was increased in mice injected with EQ while there was no significant increase in uterine wet weight in EQ-fed mice. While uterine cell proliferation increased through both EQ treatment routes, the magnitude of the response differed between oral and injected with the latter showing a larger effect. However, similar increases in vaginal epithelial cell height were noted for both treatment routes (Selvaraj et al., 2004). Sprague-Dawley rats fed a diet containing 400 ppm EQ displayed some uterotrophic effects, reduced intra-abdominal fat accumulation, but no effect on bone measures compared to controls (Rachon et al., 2007a; Rachon et al., 2007b; Rubin et al., 2016). The mixed effects seen *in vivo*, including decreased abdominal fat in rats fed EQ, increased uterine weight and uterine epithelial proliferation in mice when injected with EQ and increased uterine epithelial proliferation in mice fed EQ, all lead to questions regarding EQ bioactivity in estrogen-responsive tissues.

BC is a heterogeneous disease and tumors may respond differently to dietary changes/challenges based on their receptor status and cancer class (luminal/basal/stromal). Previous studies from our laboratory demonstrated that GEN can stimulate E₂-dependent MCF-7 tumor growth in mice at dietary intake concentrations (250 ppm to 1000 ppm) that reflect human consumption levels resulting in 1 – 3 μM total GEN in serum (Ju et al., 2001; Setchell et al., 2003). Further, our laboratory investigated the effect of EQ on E₂-dependent MCF-7 tumor growth, which resulted in no estrogenic effects on tumor size or proliferation and no effect on uterine weight (Ju et al., 2006). The T47D BC cell line is an estrogen-

responsive, luminal type A, hypotriploid human BC. In the current study, T47D cells were injected into mammary fat pads of athymic Balb/c mice fed increasing levels of EQ and evaluated for effects on tumor growth and bone, the latter being a sensitive and important estrogen target tissue. This model of BC expands our knowledge of the potential E₂-like actions of EQ and its potential interactions with dietary genistein.

Postmenopausal women may be ingesting supplements containing both EQ and GEN and/or consuming soy foods while supplementing with EQ for a variety of reasons. The mixed effects of EQ and the known effects of GEN, when combined may lead to an additive effect and therefore may increase BC risk. The results of our prior work indicate that EQ is estrogenic in MCF-7 cells; however, EQ alone did not alter the stimulatory effect on *in vivo* MCF-7 tumor growth as observed with GEN at similar concentrations (Ju et al., 2001; Ju et al., 2006). The possible interactive effects of concurrent consumption of EQ with GEN have not been studied. In the current investigation, dietary GEN is combined with various levels of EQ in the MCF-7 tumor model, which has been tested for effects of both EQ and GEN individually. Non-tumor bearing mammary glands from these animals are evaluated for differences in ductal branching. The resulting data provide missing information on the potential risk of combined supplementation by women.

This investigation also evaluates the estrogenic effects of EQ using the T47D cell line *in vitro* and tumor model (*in vivo*). Further, it evaluates the effects of dietary EQ on another target tissue, bone, from the animals in the T47D model. Meanwhile, the potential for an interactive effect of GEN consumed concurrently with EQ is addressed in the MCF-7 tumor model, as well as *in vitro*. The results of the work presented here offer insight into the bioactivity of EQ in BC *in vivo* models as well as the potential interactive effects of EQ and GEN when these are present together in the diet.

2. Materials and Methods

2.1 Materials

Improved Minimal Essential Medium (IMEM, without gentamicin, with glutamine) and phenol red-free MEM were purchased from the Media Facility at the University of Illinois at Urban-Champaign. Bovine calf serum (BCS) was purchased from Hyclone (Logan, UT). Penicillin/streptomycin and trypsin/EDTA were purchased from Invitrogen (Carlsbad, CA). Laboratory animal diets and dietary components were purchased from Research Diets, Inc. (New Brunswick, NJ). 17 β -estradiol (E₂), 4-OH-Tamoxifen (TAM) and cholesterol were purchased from Sigma (St Louis, MO). MatriGel™ matrix was purchased from BD Biosciences, Inc. (Bedford, MA). GEN was purchased from Indofine Chemical Company, Inc. (Hillsborough, NJ). (±)-EQ was synthesized in our laboratory (Muthyala et al., 2004). The purity of both GEN and EQ was verified 1H-NMR and LC-MS/MS.

2.2 Study Design

Two *in vivo* tumor models were used, T47D and MCF-7. *In vitro* models of the same cells were also used. T47D cells were injected into OVX mice with primary tumor growth driven by an implanted E₂ pellet. Mice were treated with EQ (in the absence of the E₂ pellet) for 13

weeks with tumor measurements occurring weekly (Figure 1). Animals were sacrificed by CO₂. Plasma was collected at necropsy and stored at –80°C for isoflavone analysis. Half of the tumors and one femur from each animal were excised and fixed in 10% neutral buffered formalin for histological analysis. The remaining tumors were flash frozen and stored in liquid nitrogen. Additionally, cell culture experiments were performed to evaluate the T47D cell line and its response to E₂, EQ, GEN and TAM *in vitro*.

MCF-7 cells were injected into OVX mice with prior primary tumor growth driven by an implanted E₂ pellet for 13 weeks (Figure 1.). Dietary treatment for T47D tumor growth study occurred over the following 18 weeks (8 weeks for positive control). Animals were sacrificed by CO₂. Plasma was collected and stored at –80°C for isoflavone analysis. Half of the tumors from each animal were excised and fixed in 10% neutral buffered formalin. The remaining tumors were flash frozen and stored in liquid nitrogen. Non-tumor bearing mammary fat pad was excised and fixed in Carnoy's fixative. Animal study timeline is illustrated in Figure 1. MCF-7 *in vitro* analysis assessed the activity of EQ and GEN between 1 nM to 100 µM individually and then in combination.

2.3 Maintenance of Human Breast Cancer Cells

MCF-7 (Estrogen receptor positive) cell line and T47D (Estrogen receptor positive, androgen receptor positive, progesterone receptor positive, glucocorticoid receptor positive) cell line (ATCC, ATCC® Number: HTB-133™) were maintained in IMEM supplemented with 5% BCS, 1% penicillin (100 U/ml)/streptomycin (100 mg/ml) and 1 nM 17β-estradiol (E₂) at 37 °C in a 5% CO₂ humidified atmosphere. The cells were switched to phenol red-free IMEM containing 5% charcoal dextran stripped (CD)-BCS (2) and 1% penicillin/streptomycin without E₂ supplementation one week prior to cell proliferation assay or injection into athymic mice.

2.4 Cell proliferation assay

To measure cell growth during exposure to our compounds of interest, a modified colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used which measures mitochondrial dehydrogenase activity. During experiments, cells (5×10³/well) were inoculated in 0.1 ml of estrogen-free culture media in a 96-well polystyrene culture plate. After 24 hours, the media in each well was removed and the cells were washed with 1 ml of phosphate-buffered saline (150 mM PBS, pH 7.4, without Ca²⁺ and Mg²⁺) and then treated with 0.01–1 nM E₂, 0.1–10 nM TAM, 1–100 µM GEN or 1–100 µM EQ containing media and combinations thereof. After being treated for 72 hours, the cells were treated with 50 µl of MTT for 5 hours and then 50 µl of 10% SDS (in 0.01 N HCl) for 12–18 hours. Optical density (OD) was measured at 570 nm. All *in vitro* experiments were repeated at least 3 times.

2.5 Athymic nude mice

All animal studies were approved by the University of Illinois Urbana Champaign IACUC committee. One hundred twenty-one female Balb/c athymic-nude mice were purchased from Charles River Laboratories (Wilmington, MA). The mice were ovariectomized at 21-days of age by the vendor and arrived at the UIUC AALAC approved animal facility at 28-days of

age. Mice were acclimated for one day, then offered a modified AIN-93G control diet (corn oil instead of soybean oil). Mice were maintained under the standard 12:12 light/dark cycle throughout the study and offered food and water *ad libitum*.

2.6 Diets

Phytoestrogen-free diets were modified from the standard AIN-93G diet in which soybean oil was replaced with corn oil to eliminate any potential confounding effects of soy in the diet. The sequence of treatment with E₂ pellets and GEN and EQ outlined in Figure 1 (Schematic).

2.7 E₂ pellet preparation

Low dosage E₂ pellet for EQ dose response using T47D cell derived tumors contained 1 mg of E₂ and 19 mg of cholesterol. High dosage E₂ pellet for EQ and GEN interaction studies using MCF-7 cell derived tumors contained 2 mg E₂ and 18 mg cholesterol. A single E₂ pellet was implanted subcutaneously under the skin between the ears of each animal three days prior to BC cell injection.

2.8 Cell injection

Cells grown in phenol red-free IMEM media (containing 5% CD-BCS and 1% penicillin/streptomycin) were used for the cell injection. Matrigel™ was thawed on ice overnight at 2–8 °C. Cells were harvested using trypsin/EDTA and adjusted to 3.75×10⁶ cells/ml. A volume of 900 µl of Matrigel™ was added to 100 µl of cell suspension to deliver 1.5 × 10⁵ cells per 40 µl of Matrigel™ suspension into each injection site. Each mouse received four tumor cell injections containing MCF-7 cells, one in each dorsal quadrant.

2.9 Effects of Dietary EQ on T47D tumor growth and bone in OVX athymic nude mice

All mice were maintained on the AIN-93G modified control diet and the E₂ pellet until the average cross-sectional tumor size grew to 35 mm², reached at 13 weeks as determined by electronic caliper reading of tumor length and width to the nearest mm. E₂ pellets were then removed from animals assigned to the NC, 250 ppm EQ and 750 ppm EQ groups (NC and PC n = 10, 250 and 750 EQ n=14) and dietary intervention began. Animals were offered their assigned treatment diets *ad libitum* for 13 weeks, the termination of the study. Tumors were measured weekly from 2–9 weeks and at 12 and 13 weeks. Blood samples, femurs, and tumors were collected at necropsy for evaluation.

2.10 Micro-computed tomography of T47D femurs

Femurs were imaged using microcomputed tomography (µCT; µCT40 scanner, Scanco Medical AG, Basserdorf, Switzerland) at 55 kVp x-ray voltage, 145 µA intensity, and 200 ms integration time at 12 µm × 12 µm × 12 µm voxel size. Filtering parameters sigma and support were set to 0.8 and 1, respectively. All specimens were scanned immersed in 70% ethanol. Bone segmentation was conducted at a threshold of 245 (scale, 0–1000) determined empirically. Femora (cortical + cancellous bone) were evaluated for total femur bone volume (mm³). Femur length was measured as the distance between the proximal end of the femoral head and distal end of the femoral condyles. Cortical bone architecture was evaluated in a

0.12 mm (10 slices) region of the diaphysis that started 60% distal from the top of the femoral head. Cross-sectional volume (mm^3 , cortical bone volume + marrow volume) and cortical bone volume (mm^3) were measured. Cancellous bone architecture was evaluated in 120 slices (1.44 mm) in the femoral metaphysis in a region that began 45 slices (0.54 mm) proximal to the growth plate. Direct cancellous bone measurements included cancellous bone volume fraction (%), ratio of the segmented bone volume to the total volume of the region of interest), trabecular thickness (μm), trabecular number (mm^{-1} , number of trabeculae intercepted per unit length), and trabecular spacing (μm). All μCT data are reported using standard three dimensional nomenclature (Bouxsein et al., 2010)

2.11 The combination effect of EQ and GEN on MCF-7 tumor growth in OVX athymic mice

All mice were maintained on the AIN-93G modified control diet and E_2 pellet remained in all treatment groups until the average tumor size reached 35 mm^2 . E_2 pellets were removed from all animals assigned to the NC, 500GEN, 500GEN+250EQ, 500GEN+500EQ, 500GEN+1KEQ ($n=12$ per group, $n=13$ for NC group) and dietary intervention began. Tumor size was measured weekly for 18 weeks. However, all PC samples and tissues were collected 8 weeks post injection as the animals had become moribund due to significantly greater tumor growth than other groups. Treatment diets were offered ad libitum until the termination of study. Blood samples (to measure circulating GEN and EQ), mammary glands and tumors were collected for evaluation.

2.12 Mammary whole mount

Mammary gland whole mounts were used to assess estrogenic effects of EQ combined with GEN *in vivo*. Briefly, one pair of caudal mammary glands and surrounding fat were carefully dissected out to include the mammary lymph node and spread onto a slide. The slides were then fixed in Carnoy's fixative and stained with carmine alum prior to quantification. Gland development was quantified by counting the number of end buds present on ten ductal branches per animal by three individuals that were blinded to treatment. Values were averaged across each animal in each treatment.

2.13 Data Analysis

Statistical analysis was performed using SAS® (Cary, North Carolina). The treatment effects on tumor growth was determined by comparing the final tumor size using mixed regression model for repeated measures. The rates of growing tumors, which is defined as tumor size greater 35 mm^2 at the end of the *in vivo* study, for each group were compared using the Fisher's Exact Chi-Square tests. Effects of the treatments on mammary end bud number, cell proliferation, bone parameters and serum phytoestrogen levels were analyzed using one-way ANOVA and linear regression tests.

3. Results

3.1. Effect of GEN or EQ on cell proliferation of T47D cells in vitro (Figure 2)

The stimulatory effect of 0.1 nM E_2 on cell proliferation was repressed by TAM treatment in a concentration-dependent manner. Compared with the control (NC) group, single treatments with either 100 nM EQ or 100 nM GEN significantly stimulated T47D cell

proliferation. The stimulatory effect of EQ and GEN was significantly repressed by 1nM and 10nM TAM in a concentration –dependent manner (Figure 2). These experiments confirm that the T47D cells respond to E₂, EQ, and GEN and that their responses are blocked by TAM.

3.2 Effect of EQ on T47D tumor growth in vivo

The dietary intervention was effective in increasing the levels of EQ in circulation. The total EQ concentrations in plasma for 250 EQ and 750 EQ were 2.24±1.0 µM and 6.84±2.41 µM (mean, ±SEM), respectively.

Mean tumor size in the E₂ positive control group reached 51 mm² 13 weeks after initiation of dietary treatments. At week 13 of dietary treatment, the mean tumor cross-sectional areas were 9.4±2.1 mm², 11.9±1.5 mm² and 10.9±2.3 mm² for the 250 EQ, 750 EQ groups and the negative control group, respectively (Figure 3). No statistically significant differences were observed between 250 EQ and 750 EQ groups or the NC group at any point of the study. The PC group had tumors that were significantly larger than all other groups beginning at week 6 and continuing to the end of the study.

There were no significant differences in bone analyzed by micro CT among groups (Table 1) with the exception that E₂ resulted in a higher total femur bone volume. There were no significant differences on uteri weight between 250 EQ and 750 EQ group.

3.3 Effect of GEN and EQ on the growth of MCF-7 cells in vitro

The initial assessment of the E₂-like effects of EQ and GEN on MCF-7 cells was conducted using individual compounds. Linear regression tests show that either EQ or GEN significantly stimulated MCF-7 cell proliferation in a concentration dependent manner (from 1nM to 2 µM) (Figure 4A). GEN and EQ both stimulated MCF-7 cell proliferation similarly from 1nM – 2µM. There are no significant differences between the number of cells between the EQ treatment and the GEN treatment at any exposure level. High doses of GEN or EQ (100 µM) are cytotoxic *in vitro*.

The *in vitro* effects of GEN and EQ in combination was examined (Figure 4B). All treatment groups significantly stimulated cell proliferation than NC group. The stimulatory effect of treatment of 1 µM EQ + 1 µM GEN was statistically lower than all other treatment groups except treatment group of 1 µM GEN +2 µM EQ (p<0.05). The stimulatory effect for treatment group of 1 µM GEN +2 µM EQ was not different than all other treatment groups.

3.4 The interactive effect of EQ and GEN on MCF-7 tumor growth in OVX athymic nude mice

We have previously shown that MCF-7 tumors are responsive to GEN, but not to EQ when these compounds are fed individually (Ju et al., 2006). Since the MCF-7 tumor model is known to be responsive to GEN or EQ individually, it is reasonable to use it to assess the combined effects of GEN, EQ. Mice in the positive control group retained the E₂ pellet, and their mean tumor size reached 98 mm² 8 weeks after the initiation of dietary treatment. These mice were terminated due to a high tumor burden and samples for this group were

collected at 8 weeks. Tumor growth in the rest of the treatment groups was measured weekly for 18 weeks.

No significant combination effect on mean tumor size at the conclusion of the dietary intervention (18 weeks) was identified in any EQ plus GEN groups compared with the GEN alone group (Figure 5A). Approximately, 40% to 60% of total implanted tumors in the isoflavone groups were at least 50% larger than their initial size after 18 weeks of dietary intervention while there was approximately 90% growth of tumors from baseline until termination in the positive control group (8 weeks).

Dietary intervention of 500 ppm GEN increased the total GEN blood concentrations equally across all dietary groups containing GEN (Figure 5B). Because there were no statistically significant differences between any GEN exposed groups, presence of EQ did not alter GEN metabolism based on the total GEN blood concentration. Dietary intervention increased the total EQ blood concentration from $1.72 \pm 0.55 \mu\text{M}$ to $4.42 \pm 1.12 \mu\text{M}$ as the EQ dose increased from 250 ppm to 1000 ppm (Figure 5B).

Mammary gland development is driven by a combination of progesterone (ductal elongation) and estrogen (ductal branching). Therefore, an increase in mammary end bud number around the mammary lymph node indicates estrogenic effects of treatments. In this study, the mammary glands from PC animals and GEN-fed animals increased terminal end bud count compared with the negative control (Figure 6). The positive control group had significantly higher terminal end bud counts compared with all other treatments. The mammary terminal end bud number was equivalent across all EQ plus GEN treatments and the GEN alone treatment. There was no additional increase in mammary end bud formation when dietary EQ was increased.

4. Discussion

Interest in equol as a 'natural' hormonal replacement therapy initially developed because equol has a higher affinity for the estrogen receptors than its precursor DZN and the bioactive form of equol, aglycone equol, has the highest binding affinity for estrogen receptor of all of the phytoestrogens derived from soy (Ishiwata et al., 2009; Tousen et al., 2011). Recent interest in equol as a supplement has increased with the development of fermented products using soy germ containing significant quantiles of S-(–)equol. However, the synthesis of the S-(–)-equol enantiomer is still very expensive (Heemstra et al., 2006; Muthyala et al., 2004). Women at a high risk of developing BC may consume 'natural' estrogenic products to treat menopausal symptoms, which can contain equol. While dietary EQ does not impact the growth of MCF-7 tumors, the mixed effects of EQ in other models of EDBC lead to further questions about the actions of EQ in other tumor models and in combination with dietary GEN.

The T47D cell line used in this study was first validated for estrogen-responsiveness. Both EQ and GEN stimulated T47D cell proliferation similarly to E_2 at physiological concentrations for E_2 and levels of EQ and GEN found in women taking supplements. The stimulatory effects were repressed by TAM.

Results from the T47D *in vivo* EQ study concur with published investigations in which dietary EQ was fed to mice with MCF-7 tumors (Ju et al., 2006). No stimulation of MCF-7 tumor growth was observed with increasing amounts of dietary EQ. As was observed with MCF-7 tumors, T47D tumor growth was stimulated in response to implanted E₂ pellets (positive controls), whereas, mice without E₂ pellets fed the phytoestrogen-free diet (negative control) did not grow. In mice fed dietary EQ, the serum levels of total EQ (conjugated plus aglucone) were higher in the 1000 ppm group compared to the 250 ppm EQ and 750 ppm EQ groups. MicroCT analysis of the femurs of the T47D animals demonstrated that bone, an important estrogen-sensitive tissue, was unaffected by dietary equol. Therefore, there was no evidence of estrogen-like effects on either T47D tumor growth or on bone in mice fed the EQ-containing diets.

While EQ did not stimulate *in vivo* estrogen-dependent MCF-7 tumor growth (Ju et al., 2006), EQ was able to stimulate estrogen receptor dependent growth *in vitro*. No additive effect was observed when EQ and GEN were combined at various concentrations. Additionally, when EQ was added to 2 μ M GEN, the growth was not significantly different than GEN at 2 μ M alone. Based on these data, EQ does not act in an additive or antagonistic manner with GEN.

Tumor growth in animals fed the combinations of EQ with GEN did not differ from the growth of tumors in the GEN-alone treated animals. Additionally, the mammary gland development showed a similar response across all dose levels of EQ combined with GEN and the GEN-alone group. Tumor growth and mammary gland development were significantly higher in mice fed GEN and EQ than the phytoestrogen-free diet and significantly lower than the positive (estradiol implant) control group. Tumors in the positive control group grew rapidly and 8-weeks after the dietary intervention began were terminated. In summary, EQ, when combined with GEN in this long-term dietary pre-clinical study, did not act in an additive manner with dietary GEN to alter tumor growth or mammary gland development from animals fed a GEN-alone diet.

One possible reason as to why EQ enhances estrogen-responsive MCF-7 growth *in vitro* and not *in vivo* is because of *in vivo* metabolism. EQ has a very high conjugation (Phase II metabolism) rate *in vivo* due to the rapid addition of a glucuronide moiety by UDP-glucuronosyltransferase in the intestine and liver (Allred et al., 2005; Gu et al., 2006). Once EQ is conjugated through glucuronide or sulfate formation, the resulting conjugated product no longer binds to the ER and instead is subject to rapid elimination through urine and feces (Gu et al., 2006; Ju et al., 2006; Selvaraj et al., 2004). Information about the metabolism of EQ is derived from the analysis of serum from animals and humans that have consumed either whole soy or DZN, rather than direct consumption of EQ. DZN is metabolized by the gut microbiome through one of two pathways: DZN to O-desmethylanagolensin (O-DMA) or DZN to EQ (Valentin-Blasini et al., 2005; Zubik and Meydani, 2003). Because of the rapid metabolic conjugation of EQ, it is unlikely that dietary EQ will have a biologically significant effect *in vivo*, except at high doses or by parenteral administration.

The microbially mediated conversion of DZN to EQ differs considerably between species. Nearly all mice, rats, and monkeys convert DZN to EQ, while only 30–70% of humans

convert DZN to EQ (Doerge et al., 2016; Gu et al., 2006). This is because the enzymes that convert DZN to EQ are produced by specific gut bacterial populations that reside in the lower portion of the digestive tract. It is likely that endogenous EQ undergoes enterohepatic recirculation from the digestive tract and is converted to phase II metabolites (Doerge et al., 2016; Rafii, 2015; Setchell et al., 2005; Vazquez et al., 2017). This rapid and extensive metabolism results in inactivation of estrogenic properties from dietary EQ.

While there have been no studies of the absolute bioavailability of EQ ingested orally, there is strong evidence that the conjugation rate for EQ is very high. Doerge et al. (Doerge et al., 2016) evaluated the pharmacokinetics of both GEN and DZN in rhesus monkeys using both oral and IV administration. While total EQ was detectable in serum across age groups and routes of DZN administration, the aglycone form (bioactive) was below the detection limits of the assay (> 3 nM) at all time points assessed. Less than 1% of total EQ is present in blood in the aglycone form in rodents and more than 90% of conjugated EQ is in the glucuronide form (Andrade et al., 2010; Gu et al., 2006). Thus, dietary EQ is likely to have no biological effect in humans at relevant intake levels.

The average blood concentration of total EQ from mice in this study fed the 250-ppm to 1000 ppm diets ranged from 1.72 μ M to 4.42 μ M. By extrapolation of our data with the information on the conjugation rate seen in mice and rhesus monkeys, the circulating aglycone EQ levels in this study is in the low nanomolar range (less than 17.2 to 44.2 nM). The total GEN blood concentrations for all groups with 500 ppm GEN are approximately 2 μ M (1.72 μ M to 2.53 μ M), of which approximately 5% is in aglycone form (Gu et al., 2006). Using the same extrapolation as above, the concentration of circulating aglycone GEN is approximately 100 nM. The *in vitro* assays assess the effects of these compounds in aglycone form on the MCF-7 and T47D cells directly, and the proliferation rates in 100 nM treatments of either EQ or GEN were similar in both T47D and MCF-7 cells, indicating that aglycone EQ is of insufficient concentration to elicit an estrogenic effect. Our *in vivo* results presented here confirm that a dietary exposure would result in sufficient active EQ concentrations to increase tumor growth *in vivo* or result in noticeable biological effects in other estrogen-responsive tissues including bone and mammary gland.

5. Conclusion

In summary, dietary EQ intake of 250 ppm to 1000 ppm did not alter the estrogen-dependent tumor growth in either the T47D or the MCF-7 models in long-term studies. No inhibition or potentiation of action of GEN by presence of EQ were observed in MCF-7 tumors or mammary development. EQ did not affect bone in the mouse model. Comparative studies of soy protein isolate indicate that rodents and monkeys are more frequent producers of EQ than are women and pigs (Gu et al., 2006). Onoda et al. evaluated dietary S-(–)EQ for proliferative effects in MCF-7 cells and MCF-7 tumors in OVX athymic mice and found similar results to the studies presented here using $\pm$ (Equol) both *in vitro* and *in vivo* (Onoda et al., 2011). Based on the evidence presented here, dietary EQ is unlikely to have estrogenic effects *in vivo*.

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Abbreviations:

BC	breast cancer
BCS	Bovine calf serum
DZN	daidzein
EQ	(±)-Equol
EDBC	estrogen-dependent breast cancer
GEN	genistein
IMEM	Improved Minimal Essential Medium
OVX	Ovariectomized
TAM	4-OH-Tamoxifen

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Highlights

- EQ is estrogenic *in vitro* on estrogen-dependent breast cancer cells.
- GEN stimulates growth of MCF-7 or T47D BC tumor cells in mice.
- EQ does not stimulate estrogen-dependent breast cancer growth *in vivo*.
- EQ does not alter the effect of GEN *in vivo*.

Figure 1. Study Schematic

AIN-93G phytoestrogen-free diets were used in all the studies. GEN, (±)EQ, or a combination of the two were added to the phytoestrogen-free diets.

Figure 2. T47D cell growth in the presence of E₂, EQ, or GEN with or without TAM
TAM significantly blocks T47D cell growth stimulated by 0.1 nM E₂, 100 nM Gen and 100 nM EQ at 1 and 10 nM. E₂, GEN and EQ increased cell growth.

Figure 3. Effect of dietary EQ on T47D tumor growth in athymic mice

Tumors were of equivalent size in all groups until week 6. The tumor sizes of the PC group were significantly larger than all other groups from 6 weeks to 13 weeks of treatment. * indicates that the PC group was significantly different from the NC, 250 EQ and 750 EQ groups. There was no significant difference in tumor sizes among NC, 250EQ and 750EQ groups in the 13 weeks of the dietary treatment.

Figure 4. GEN and EQ stimulate MCF-7 proliferation *in vitro*

(A) GEN and EQ both stimulated MCF-7 cell proliferation similarly from 1 nM – 2 μ M. High doses of GEN and EQ (10 and 100 μ M) are cytotoxic *in vitro*. (B) GEN (1 and 2 μ M) and EQ (1 and 2 μ M) individually increased cell growth when compared to the negative control (NC) treatment. When GEN and EQ were combined at 1 μ M GEN plus 1 μ M EQ [1GEN:1EQ] and 2 μ M GEN plus 1 μ M EQ [2GEN:1EQ] MCF-7 cell growth was stimulated above NC. In the [1:1 (1GEN:1EQ)] MCF-7 cell growth was modestly reduced (10–20%) whereas the [1GEN:2EQ] treatment group was not different from the individual GEN and EQ treatment groups.

Figure 5. (A) Effect of dietary EQ plus GEN on MCF-7 tumor growth in athymic mice.

There was no significant difference on final tumor size among 500GEN, 500GEN+250EQ, 500GEN+500EQ and 500GEN+1KEQ groups over the 18 weeks of dietary treatment. The tumors of the PC group were significantly larger than all other groups. The NC group had significant smaller tumor size than other groups. (B) The final plasma concentrations of GEN and EQ reflect the dietary treatments. There was no significant difference on GEN plasma concentrations among all treatment groups containing 500 ppm GEN. Plasma EQ levels increased between 250 EQ and 1000 EQ

Figure 6. Estrogenic effects of EQ and GEN on mammary gland

(A) Mammary gland whole mounts showed weak estrogenic effect of GEN on mammary development. End buds (indicated by arrows) of ten duct terminals within 1 mm² of the lymph node were counted for each sample (6 samples for each group). EQ had no estrogenic effect on mammary gland development. (B) The PC group had more end buds than any other group, statistical grouping c. There were no significant differences among the GEN and GEN plus EQ groups, statistical grouping b. There were very few terminal end buds in the negative control group, statistical grouping c. $p < 0.05$

Table 1.

Effects of EQ on total femur bone volume and cortical and cancellous bone architecture in ovariectomized athymic nude mice.

Endpoint	PC (n=5)	NC (n=7)	250EQ (n=11)	750EQ (n=11)
Total femur (cortical + cancellous bone)				
Bone volume (mm ³)	30.48 ± 2.13	25.25 ± 0.84	27.36 ± 1.14	26.86 ± 0.61
Midshaft femur (cortical bone)				
Cross-sectional volume (mm ³)	0.21 ± 0.002	0.21 ± 0.004	0.21 ± 0.005	0.21 ± 0.006
Cortical volume (mm ³)	0.16 ± 0.014	0.13 ± 0.007	0.13 ± 0.006	0.13 ± 0.005
Cortical volume/Total volume (%)	74 ± 7	65 ± 4	61 ± 3	62 ± 2
Distal femur metaphysis (cancellous bone)				
Bone volume/Total volume (%)	10 ± 5	4 ± 2	2 ± 1	4 ± 1
Trabecular number (1/mm)	2.30 ± 1.07	4.86 ± 5.69	1.31 ± 0.42	2.04 ± 0.92
Trabecular thickness (μm)	0.07 ± 0.01	0.05 ± 0.01	0.07 ± 0.02	0.06 ± 0.01
Trabecular spacing (μm)	0.51 ± 0.20	0.52 ± 0.43	0.83 ± 0.25	0.58 ± 0.24

Data are mean ± SE

Total femur bone volume in NC compared to PC. Data are shown as Mean ± SEM