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REGULATORY RESEARCH PERSPECTIVES

Impact on Public Health

Studies on the Influence of Dietary Isoflavones, Daidzein and Genistein, in Chemical Mutagenesis and Tumor Development in Female Rats

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* *The views presented in this article do not necessarily reflect those of the Food and Drug Administration.*

Abstract: The use of hormone replacement therapy (HRT) is known to confer several benefits, including a reduction of hot flashes, but it is linked to increased risks for heart attack, stroke, and breast cancer. Many women are switching to alternative sources, such as isoflavone phytoestrogens, in the belief that naturally occurring substances may be safer than HRT. Despite the large body of compelling evidence on the health benefits of isoflavones, it is possible that these compounds may also be toxic in that they can alter endogenous hormones and thus influence the pathogenesis of hormone-dependent cancers. Because the Western lifestyle normally precludes the consumption of adequate soy foods to meet health needs, many women are currently taking dietary supplements of phytoestrogens on a regular basis to forestall the complications of menopause. Earlier studies indicated that daidzein (DZ) and genistein (GE), major components of soy isoflavones presumed to be responsible for health-promoting effects, are mutagenic indicating that isoflavones may play a role in the carcinogenesis process. Although mutation alone may not be able to directly explain the possible carcinogenic role of isoflavones, understanding the effect of isoflavones on chemical mutagenesis provides great potential for future risk assessment strategies. At the heart of current mutation *in vivo* assays is the capacity to measure mutations in every tissue of Big Blue (BB) transgenic mice or rats. Thus using BB rats, ovariectomized to model menopause and to exclude the effects of intrinsic sex hormones, we investigated the effects of feeding DZ, GE, or 17β-estradiol (E2) on the genotoxicity of the potent rodent carcinogen 7,12-dimethylbenz(*α*)anthracene (DMBA) in several estrogen-responsive tissues, including mammary, uterus, heart, and liver. Mammary glands and uterus were also analyzed for tumorigenicity.

Introduction

The undesirable symptoms of menopause and other disorders, such as cardiovascular disease and osteoporosis, are linked to decreased levels of estrogen (1-3). To alleviate these problems, hormone replacement therapy (HRT) is often administered to menopausal women. However, in light of recent studies that call into question the safety of HRT [1, 2], many women are switching to naturally occurring estrogens such as soy isoflavones believed to exhibit beneficial effects in the prevention of menopausal symptoms and other related disorders [3, 4]. Structurally related to steroidal estrogens (Figure 1), DZ and GE, the major components of soy isoflavones, exhibit similar properties for receptor affinity [5], but like tamoxifen appear to be selective estrogen modulators without untoward estrogenic side effects [6]. Thus, isoflavones have been proposed as effective chemopreventive agents for certain types of cancer, particularly breast and prostate cancers [6-8]. Evidence also points to the beneficial effects of isoflavones in preventing cardiovascular disease and osteoporosis [9, 10]. In addition, there are other constituents of soy, including lignans, protease inhibitors, saponins, phytosterols, coumestans, and phytates that might also possess health-promoting benefits [11].

Given the potential role of soy isoflavones in decreasing the risk of certain hormone-dependent cancers and ameliorating menopausal symptoms, it is not surprising that isoflavones are ingested on a daily basis as nutritional supplements or as constituents of other preparations, including tofu, soy milk, tempeh, and textured soy protein. The isoflavones can exert their effects by genomic mechanism involving estrogen receptors or through a variety of nongenomic mechanisms, including tyrosine kinase and topoisomerase inhibition [8, 12, 13]. Genistein is reported to be a potent inhibitor of topoisomerase II by stabilizing a cleavable complex that results in DNA strand breaks [14]. Thus it is conceivable that the biological effects of DZ or GE may also be associated with DNA damage and potentiate the carcinogenesis process [15]. It has been reported that isoflavones are clastogenic, both *in vitro* and *in vivo* [16-18], and are genotoxic *in vivo* [18, 19]. In addition, dietary genistein has been shown to enhance chemical carcinogenesis in the colon and in mammary glands of rodents [20-22]. Administration of an isoflavone mixture to a p53-deficient mouse that develops early spontaneous tumors, however, showed no effect on the incidence and types of tumors produced [18]. Although consumption of soy products in the Western countries is much less than that in Asian countries where soybean consumption is associated with beneficial effects in women [23-25], dietary supplements of isoflavones are on the rise in this country suggesting a need to evaluate their potential mutagenicity and carcinogenicity in detail.

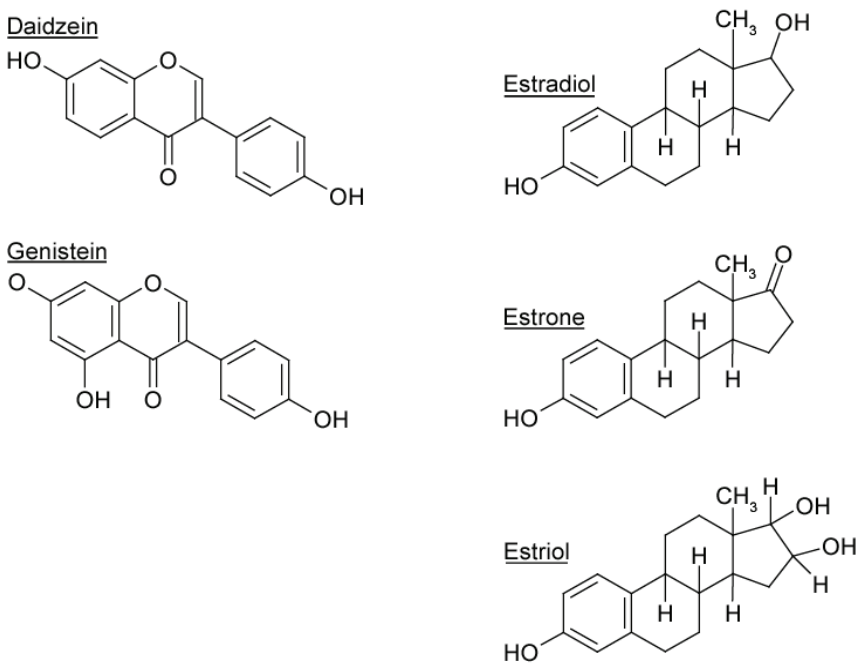

Figure 1. Structures of Estrogen and Isoflavone

Mutations, which are heritable alterations in the structure of a gene, are not only associated with physiological or biochemical changes in an organism, but are also involved in many diseases, including cancer. However, the assessment of mutation in certain tissues such as mammary or uterus has been hampered by a lack of relevant animal models. The use of the rat lymphocyte *Hprt* assay [26, 27] or the Big Blue® (BB) transgenic rat system, which harbors mutational target [28], has facilitated the measurement of *in vivo* mutations that can provide genetically useful information for chemical risk assessment. While the *Hprt* assay is useful for both animal and human studies, it is limited to circulating blood lymphocytes. On the other hand, the transgenic mutation assay can detect mutations in any tissue, including the mammary gland, heart, liver, and uterus. Thus, the BB transgenic assay readily permits direct comparison of cancer and mutation induction in the same tissues.

A significant advance in our understanding of chemical carcinogenesis in hormone-dependent tis-

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sues has come from the use of the model multi-organ carcinogen 7,12-dimethylbenz(*α*)anthracene (DMBA) [29-31]. DMBA is a synthetic polycyclic aromatic hydrocarbon (PAH) that has been used extensively as a prototype carcinogen in mutation and cancer research. Major target organs of this agent in rodents are the skin and mammary gland. Many other tissues are susceptible to DMBA insult [32]. Therefore, to better evaluate the genotoxic/carcinogenic effects of isoflavones, we treated transgenic BB rats with a single dose of DMBA, with or without dietary DZ, GE, or 17β-estradiol (E2) as positive control, and conducted mutagenesis and carcinogenesis experiments in the mammary and uterus. The heart and liver, which are estrogen-responsive tissues, were also included in the mutagenesis study; the lymphocytes were added as a surrogate tissue for evaluating responses in nonestrogenic tissue. Due to the existence of estrogen deficiency at menopause, both OVX and INT rats were used in the study.

Animals, diets, and carcinogen treatment

The experiments were conducted in female Big Blue® transgenic rats obtained from Taconic Farm (Germantown, N.Y.). Animals were acclimatized for two weeks at NCTR before treatment. The Institutional Animal Care and Use Committee at NCTR approved animal handling, maintenance, and the study protocol. Starting at five weeks of age, rats were fed *ad libitum* isoflavone-free diet (NIH-31C) and had free access to water. NIH-31C diet has the same basic formulation as standard NIH-31, except that the protein contributed by soy meal and alfalfa was replaced by casein and the soy oil by corn oil. The feed was analyzed by LC/MS and shown to be free of isoflavones at the detection limit of 0.5 µg. Beginning at 7 weeks of age, animals were fed *ad libitum* either NIH-31C or NIH-31C containing either 0.25 or 1.0 g/kg of the diet isoflavones: DZ and GE, respectively, or a mixture (DZG) containing 1.0 g/kg of diet DZ and 1.0 g/kg of diet GE. Other rats received 0.005 g/kg E2 as positive control in some experiments. The doses of the isoflavones used were biologically active, as evidenced by an increase in uterine cell proliferation as determined by proliferating cell nuclear antigen (PCNA) immunohistochemistry in the OVX rats. Also, in a similar experiment, 0.25 g/kg intake of GE increased the expression of Bcl_2 gene while 1.0 g/kg decreased this response in the pancreatic endo-

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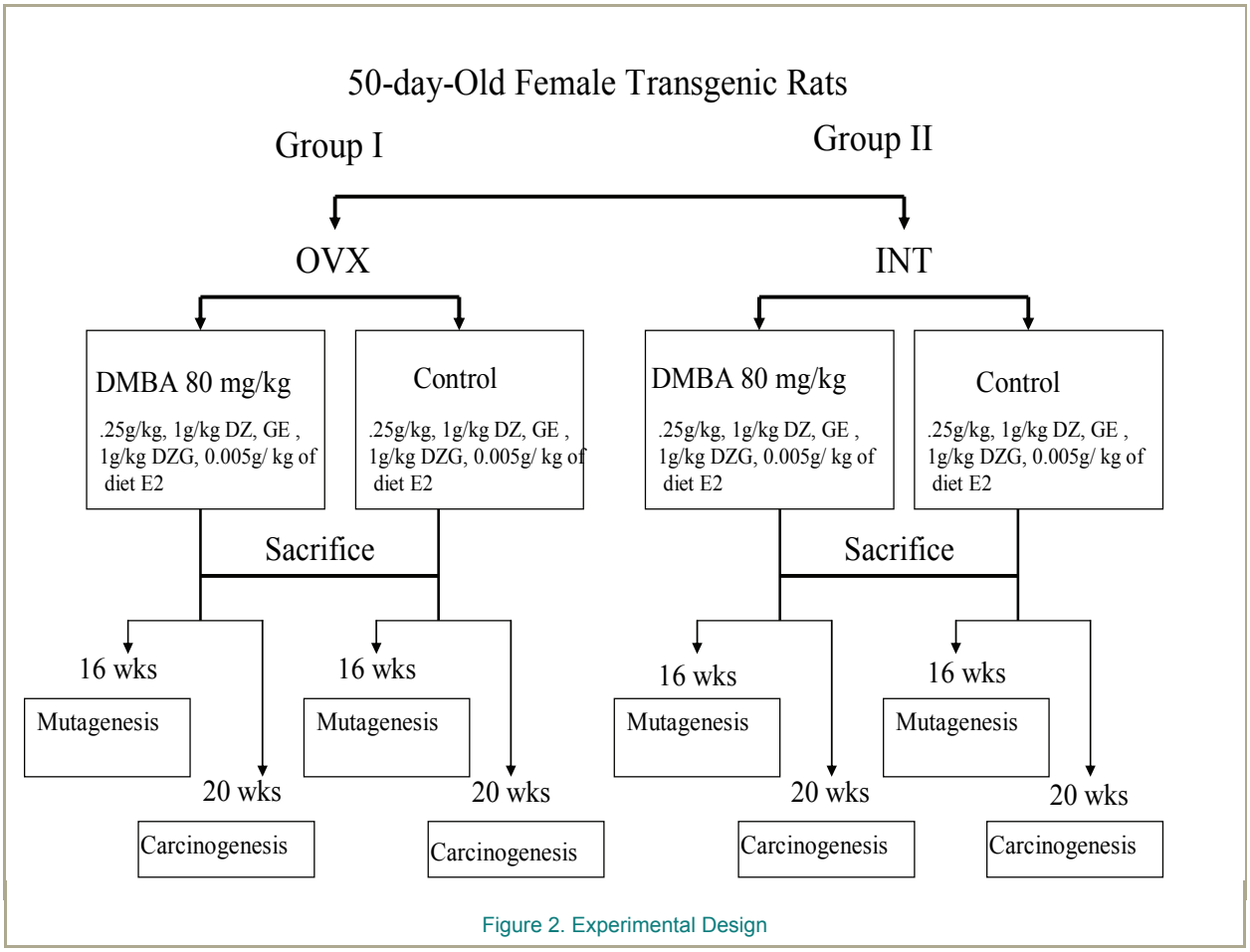

Figure 2. Experimental Design

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crine tissue of intact rats (Lyn-Cook LE., unpublished information). At postnatal day (PND) 50, the rats were gavaged with a single dose of 80 mg/kg DMBA suspended in sesame oil (Figure 2). This dose of DMBA has been shown to produce tumors in female Fischer 344 rats that are infrequently used for DMBA carcinogenesis compared to female Sprague-Dawley rats. The PND50 treatment was based on carcinogenesis studies indicating that rats at this age have a high density of terminal end buds: ductal structures that are more sensitive to chemically induced mammary tumors [33]. The animals were maintained on isoflavone/estradiol-supplement or the isoflavone/estradiol-free diets until the experiments were terminated.

Animals were divided into two groups two weeks after DMBA treatment (Figure 2). Group I rats were bilaterally ovariectomized (OVX) under ketamine/xylazine (100 mg/kg and 15 mg/kg, respectively) anesthesia. Group II rats were kept intact (INT). The rationale for treating the animals two weeks prior to ovariectomy is based on the fact that sensitivity of the rat-to-mammary tumor induction by DMBA is in part dependent on the hormonal state of the animal [34, 35]. Animals continued to have free access to food and water. Food consumption and body weight were recorded weekly. Animals were killed at 16 or 20 weeks following DMBA treatment by CO_2 anesthesia, and tissues or organs were aseptically harvested. The spleens were used immediately for *Hprt* mutagenesis assay. Portions of other tissues were either frozen in liquid nitrogen and stored at - 80°C for the *lac*I or *cII* mutagenesis assay or preserved in 10% neutral-buffered formalin for histopathological analysis.

For the mammary and uterus histopathology, tissues were examined grossly, removed, and preserved in 10% neutral buffered formalin. Lesion descriptions were recorded on the IANR form (Individual Animal Necropsy Record). Tissues were trimmed, processed, and embedded in Tissue Prep II, sectioned at 4-6 microns, and stained with hematoxylin and eosin. Slides were microscopically examined and, when applicable, non-neoplastic lesions were graded for severity.

Table 1. Mean Values of Serum Isoflavones (µM)

Treatment Groups	Daidzein	Genistein	Equol
Control diet	0.0	0.0	0.0
DZ 0.25 g/kg	0.13	0.0	1.26
GE 0.25 g/kg	0.0	0.01	0.0
DZ 1.0 g/kg	0.1	0.0	5.24
GE 1.0 g/kg	0.01	0.6	0.0
DZG 1.0 g/kg	0.013	0.03	1.51
E2 0.005 g/kg	0.0	0.0	0.0
DMBA 80 mg/kg	0.0	0.0	0.0
DMBA+DZ 0.25 g/kg	0.1	0.0	1.05
DMBA+GE 0.25 g/kg	0.0	0.0	0.0
DMBA+DZ 1.0 g/kg	0.27	0.0	6.3
DMBA+GE 1.0 g/kg	0.0	0.12	0.0
DMBA+DZG 1.0 g/kg	0.6	0.6	2.3
DMBA+E2	0.01	0.0	0.0

Mortality

In the OVX group, two animals died early in the study (DMBA plus 0.25 and 1.0 g/kg DZ); the cause of death was due to either surgery or gavage error, and the animals were excluded from the study. In ovary-intact animals, one rat in the group treated with DMBA and fed E2 died early in the study, but the cause of death was undetermined. Six other animals from the INT group treated with DMBA were found to be moribund between the 11th and 16th week after DMBA treatment and therefore euthanized. All of these DMBA-treated INT rats bore mammary gland adenocarcinomas and were included in the analysis of tumor-bearing animals.

Assessment of isoflavone levels in serum

Isoflavones undergo extensive metabolism in the intestinal tract prior to absorption (35). Following absorption, the metabolites and/or the parent compounds are transported to the liver where they are removed from the portal blood. However, a percentage of the isoflavones in the portal blood can escape uptake by the liver and enter the peripheral circulation. The effectiveness of hepatic first-pass clearance influences the amount that reaches peripheral tissues [36]. Therefore the concentration of DZ, GE, and equol, a metabolite of DZ, were measured by HPLC/MS. Table 1 shows the mean serum concentrations of DZ, GE, and equol, 16 weeks after the commencement of isoflavone feeding. The levels detected in the serum were physiologically relevant and biologically active, because they were within the range of isoflavone concentrations that significantly modify clinical markers of cardiovascular disease and osteoporosis [37]. The

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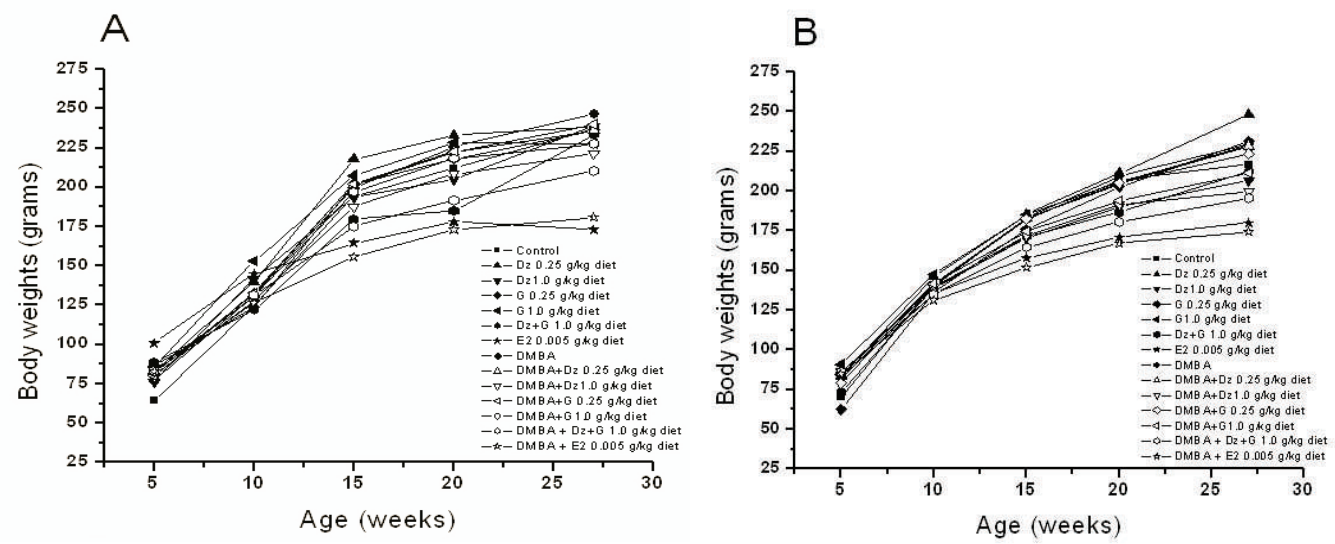

Figure 3. Body weights of OVX and INT BB rats fed DZ, GE, or E2 with or without DMBA treatment.

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biological activity of these isoflavone levels was also demonstrated by an increase in cell proliferation and a reduction in the severity of atrophy (discussed under histopathological changes) observed in OVX rats fed DZ or GE.

Food intake, body, and organ weights

Food intake and body weight were measured weekly during the course of the study. DZ or GE supplementation alone had no effect on body weight gain (Figure 3). However, the body weight gains of E2-fed rats in both OVX (Figure 3A) and INT (Figure 3B) groups with or without DMBA treatment were markedly diminished. The E2-mediated reduction in body weight

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Table 2A. Mean organ weights (grams) derived from OVX rats. All organ weights were essentially similar with the exception of E2-treated rats where the uterine weight was increased.

Group	Organs									
	Brain	Liver	Heart	Spleen	Thymus	Adrenal	Kidney	Pituitary	Thyroid	Uterus
Control	1.9	4.6	0.7	0.5	0.2	0.06	1.5	0.01	0.03	0.2
DZ 0.25 g/kg	1.9	5.0	0.7	0.5	0.2	0.07	1.5	0.01	0.02	0.2
DZ 1.0 g/kg	1.9	5.2	0.7	0.5	0.2	0.05	1.4	0.01	0.03	0.2
GE 0.25 g/kg	2.0	5.2	0.6	0.5	0.2	0.07	1.5	0.01	0.02	0.1
GE 1.0 g/kg	2.0	5.1	0.7	0.5	0.3	0.07	1.3	0.01	0.02	0.2
DZ/GE 1.0 g/kg	2.1	5.4	0.7	0.5	0.2	0.06	1.4	0.01	0.03	0.2
E2	1.9	5.6	0.7	0.5	0.2	0.06	1.4	0.02	0.02	0.4
DMBA	2.0	5.0	0.8	0.5	0.3	0.05	1.4	0.01	0.02	0.1
DMBA+DZ 0.25 g/kg	1.9	4.9	0.7	0.5	0.3	0.05	1.4	0.01	0.02	0.2
DMBA+GE 0.25 g/kg	1.9	5.1	0.7	0.5	0.3	0.05	1.4	0.01	0.03	0.1
DMBA+DZ 1.0 g/kg	1.9	4.6	0.7	0.5	0.2	0.05	1.3	0.01	0.02	0.2
DMBA+GE 1.0 g/kg	2.0	5.0	0.7	0.5	0.3	0.05	1.4	0.01	0.02	0.1
DMBA+DZ/GE 1.0 g/kg	1.9	4.7	0.7	0.5	0.2	0.05	1.3	0.02	0.02	0.2
DMBA+E2	1.9	5.5	0.7	0.5	0.2	0.05	1.4	0.02	0.02	0.5

Table 2B. Mean organ weights (grams) derived from INT rats. Organ weights were essentially similar for all treatment groups.

Group	Organs									
	Brain	Liver	Heart	Spleen	Thymus	Adrenal	Kidney	Pituitary	Thyroid	Uterus
Control	1.9	5.4	0.7	0.5	0.2	0.06	1.4	0.02	0.03	0.5
DZ 0.25 g/kg	1.9	5.6	0.7	0.5	0.2	0.05	1.4	0.02	0.02	0.5
GE 0.25 g/kg	1.9	5.3	0.7	0.5	0.2	0.07	1.4	0.02	0.03	0.5
DZ 1.0 g/kg	1.9	5.0	0.7	0.5	0.2	0.06	1.3	0.01	0.03	0.5
GE 1.0 g/kg	2.0	5.2	0.7	0.5	0.2	0.06	1.5	0.02	0.03	0.6
DZ/GE 1.0 g/kg	1.9	5.4	0.6	0.5	0.2	0.05	1.4	0.01	0.03	0.5
E2	1.9	5.2	0.7	0.4	0.3	0.05	1.3	0.02	0.02	0.5
DMBA	2.0	5.6	0.5	0.5	0.2	0.07	1.4	0.02	0.02	0.6
DMBA+DZ 0.25 g/kg	2.0	5.9	0.7	0.5	0.2	0.07	1.5	0.02	0.02	0.6
DMBA+GE 0.25 g/kg	1.9	5.8	0.7	0.5	0.2	0.07	1.5	0.02	0.02	0.5
DMBA+DZ 1.0 g/kg	2.0	5.6	0.7	0.6	0.2	0.06	1.4	0.02	0.02	0.5
DMBA+GE 1.0 g/kg	2.0	5.6	0.7	0.5	0.2	0.06	1.4	0.02	0.02	0.6
DMBA+DZ/GE 1.0 g/kg	2.0	5.2	0.7	0.4	0.2	0.07	1.4	0.02	0.02	0.5
DMBA+E2	1.7	5.4	0.6	0.5	0.2	0.06	1.2	0.02	0.02	0.5

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gain was not statistically significant. However, it is intriguing to note that this response by E2 has been associated with the suppression of the expression of neuropeptide-Y in the hypothalamus that regulates appetite in rats [38, 39]. We did not investigate this brain substance in the present study; however, we observed that the amount of food consumed per rat was essentially similar in all the treatment groups, and it is possible that the suppression of the neuropeptide may not be the sole determinant of E2-mediated decrease in body weight gain.

In order to determine organ weights, all necropsy livers, kidneys, hearts, brains, uteri, thymuses, and adrenal glands were removed, examined, and weighed wet as soon as possible after dissection, while the thyroid/parathyroid and pituitary glands were weighed after fixation. In the OVX rats, the only gross observation other than decreased uterine weight was the occurrence of mammary gland (lymph node) and clitoral gland adenomas in rats fed the E2 diet. Table 2 shows the mean organ weights. While most of the treatment groups, including daidzein and genistein, demonstrated organ weights comparable to the control diet group, E2 treatment markedly increased uterine weight of OVX rats (Table 2A). The E2 related effects in the OVX group clearly suggest exogenous estrogen-induced dysplasia. In the INT animals, the organ weights, including uterine, were essentially similar in all the treatment groups (Table 2B).

Effect of GE on DMBA mutagenesis in the heart

Heart disease among women increases at menopause. Relatively little is known about the role of gene mutations in heart disease, probably due to the fact that the heart is a post-mitotic tissue, and mutations are generally thought to be associated with mitotic processes of DNA replication. Recent findings, however, indicate that mutations may be involved in cardiovascular disease. A gene mutation has been detected in the LDL receptor in a patient with familial hypercholesterolemia, a condition associated with coronary artery disease [40]. Researchers at the Cleveland Cardiovascular Clinic have reported that a deletion mutation in MEF2A gene is linked to coronary artery disease [41]. A mutation in the cholesteryl ester transfer protein gene has been shown to increase coronary heart disease in Japanese-American men [42]. Moreover, chemically induced mutation has been demonstrated in the heart tissue of laboratory mice [43]. These findings suggest that genetic mutations may be directly or indirectly

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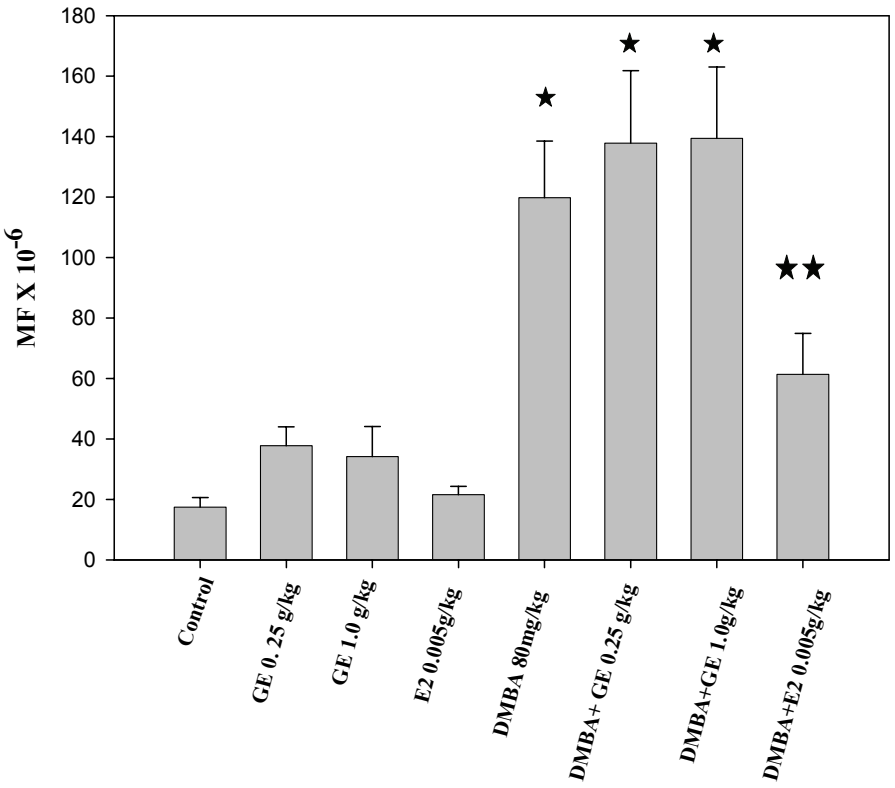

Figure 4. Mean *lac*I MFs measured in the heart of OVX BB rats fed control diet containing 0, 25, 1.0 g/kg GE, 0.005 g/kg E2 with or without DMBA exposure. Each point represents the mean ± SD of five rats. Abbreviations: GE1, genistein, E2, 17β-estradiol, and DMBA 7,12-dimethylbenz[*a*]anthracene.
★ Significantly different from control ($p<0.05$)
★★ Significantly different from control and DMBA-treated rats

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involved in the development of heart disease. Bearing in mind the increase of heart disease in women after menopause due to estrogen decline, we decided to determine the mutagenic activity of the potent carcinogen DMBA in the heart and whether the response could be modified by dietary GE or E2 in OVX rats.

The procedure for the BB *lac*I assay as applied to the heart and other tissues is briefly described here. DNA extraction, lambda packaging, and plating for *lac*I mutant plaques were carried out in a "blocked" manner in order to minimize bias from day-to-day variations in experimental procedures. The *lac*I shuttle-containing vector was recovered by mixing the genomic DNA extracted from different tissues such as liver, mammary gland, uterus, heart with Transpack™ *in vitro* lambda phage packaging extract as described previously [28]. The resulting phages were preadsorbed to E. coli SCS-8 cells for 20 minutes at 37°C, mixed with prewarmed NZY top agar containing 1.5 mg/ml of X-gal, and poured into 250-mm assay trays containing Big Blue media. The plates were incubated overnight at 37°C and scored for mutant blue plaques. Color control mutants were included in all plating, and the results were accepted only if mutant CMI could be detected. Packaging and plating were repeated for the DNA samples until at least 2 x 10^5 plaques were scored for each data point.

The mutant blue plaques were picked into individual tubes containing 0.5 ml of SM buffer and 50 µl of chloroform. To confirm the mutant phenotypes, and for future use in DNA sequence analysis, all recovered putative mutant phages from the 250-mm assay plates were diluted 1:100 and replated on 100-mm plates with 3.5 ml of top agarose containing 1.5 mg/ml of X-gal. The sectored plaques were also verified for their phenotype as specified in previous experiments [44]; the confirmed sectored plaques were separately scored. The *lac*I mutant frequency was calculated by dividing the number of verified mutant plaques by the total number of plaques analyzed.

DMBA treatment caused an eight-fold increase in MF in the heart ($p < 0.001$) compared to the response seen in the control diet (Figure 4). GE or E2 intake alone did not produce significant *lac*I MF in the heart, although 0.25 g/kg and 1.0 g/kg doses of GE induced higher *lac*I MFs relative to control diet. Our results and those of Cruz-Munoz *et al*. [43], indicating that carcinogens induce mutations in a generally nonreplicative tissue, suggest that post-mitotic tissues like the heart tend to accumulate mutations probably due to errors associated with mismatch repair. Although gene mutations in the heart may not cause cancer, they can change the genetic code for amino acid sequence in proteins, thus introducing biochemical errors that lead to disease [40-42]. Feeding GE diet to rats treated with DMBA did not significantly affect DMBA mutagenicity; however, dietary E2 resulted in a significant reduction in DMBA mutagenic response ($p < 0.05$), suggesting that E2 is protective. If this response could be extrapolated to the human setting, one would assume that the apparent protective effect of E2 in the heart stands strikingly at variance with the report from the Women's Health Initiative Study that suggested increased risk for cardiovascular disease among women taking HRT [1].

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Table 3. Summary of types of *lac*I independent mutations in the heart of OVX BB rats fed control diet containing GE (0.25 g/kg) or E2 (0.005 g/kg) with or without DMBA treatment.

	Control	GE 0.25 g/kg	DMBA	DMBA+E2 00.5 g/kg
Types of Mutations	No. of mutations (% total mutations)	No. of mutations (% total mutations)	No. of mutations (% total mutations)	No. of mutations (% total mutations)
Transitions				
G:C → A:T (CpG sites)	9 (50) 6 (65)	19 (54) 9 (47)	7 (13) 2 (28)	13 (24) 8 (61)
A:T → GC	1 (5.5)	1 (3)	4 (8)	11 (21)
Transversions				
G:C → T:A	1 (5.5)	2 (6)	10 (19)	7 (13)
G:C → C:G	2 (11)	1 (3)	0 (0)	0 (0)
A:T → C:G	1 (5.5)	1 (3)	3 (6)	4 (7)
A:T → T:A	1 (5.5)	1 (3)	22 (42)	12 (23)
-1 Frame Shifts	1 (5.5)	3 (9)	3 (6)	0 (0)
+1 Frame Shifts	2 (11)	5 (14)	0 (0)	2 (4)
Deletion	0 (0)	1 (3)	2 (4)	2 (4)
Insertion	0 (0)	1 (3)	1 (2)	2 (4)
Total	18 (100)	35 (100)	52 (100)	53 (100)

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The unexpected reduction in the *lac*I MF seen in rats fed the E2 diet and treated with the potent mutagen prompted us to analyze the mutants to determine the molecular nature of the mutations. DNA sequence analysis revealed that the majority of DMBA-induced mutations, which are consistent with DMBA-dA and DMBA-dG adducts reported in mammary gland [44], were A:T→T:A (40%) and G:C → T:A (25%) transversions. Interestingly, E2 plus DMBA treatment altered the levels of signature mutations associated with DMBA mutagenesis [27]. We observed A:T → T:A and G:C → T:A transversions reduced from 42% to 23% and 19% to 13%, respectively; whereas A:T → G:C transitions were increased from 8% to 21% in the E2 plus DMBA rats compared to rats treated with only DMBA (Table 3). Dietary GE did not have an effect on the types of mutations induced in the heart by DMBA. Although the isoflavones did not have any significant effect on DMBA-induced mutagenicity in the heart of OVX rats, the ability of E2 to reduce the DMBA mutagenic response in this organ appears intriguing and suggests the potential benefit of E2 in conditions of ovariectomy or menopause when estrogen levels are low. This experiment was conducted in the OVX rats only, thus further studies in the heart of rats with intact ovaries could explain why the isoflavones did not produce significant changes in DMBA mutagenicity and also elucidate the underlying mechanism associated with decreased DMBA mutagenic response in OVX rats fed the E2 diet.

Effect of GE on DMBA mutagenesis in the liver

The liver is the major organ of drug/chemical metabolism, and it is widely accepted that the metabolic activation of DMBA and other polycyclic aromatic hydrocarbons (PAHs) is an essential step in the initiation of cancer by PAHs. However, hepatic metabolism in the rat is known to be influenced by sex hormones and other factors [45, 46]. Estrogen also has been suggested as a promoter of liver carcinogenesis in rats [47-49]. It is also suggested that phytoestrogens could act as hormone agonists and elicit hormone-dependent effects [50, 51]. It is therefore conceivable that isoflavones may act as antagonists in premenopausal women in whom circulating levels or endogenous estrogen are high, and they may act as agonists in postmenopausal women with lower estrogen levels [52]. Given the multistep processes involved in carcinogenesis wherein the accumulation of multiple genetic alterations or mutations in critical genes play a large role, understanding the effect of steroidal estrogens and plant-derived estrogen such as genistein on chemical mutagenesis in the liver could be important.

We evaluated DMBA mutagenic activity in this tissue to determine if the response would be influenced by dietary isoflavones in OVX and INT rats. For liver, we evaluated the MF in transgenic rats fed diets containing GE or E2. No significant difference in hepatic MF was found between the control and the GE diet groups in either the OVX or the INT rats, although in the OVX rats the MF for GE alone appeared to be lower than that of control diet (Figure 5). DMBA-treated rats showed a significant increase in MF ($p < 0.01$) relative to that of controls in both OVX and INT rats. GE did

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not have an effect on DMBA-induced mutagenicity. Surprisingly, DMBA mutagenic response was significantly higher in OVX rats compared to the INT group. The increase in DMBA-induced MF in the liver of OVX rats relative to the INT group, irrespective of GE supplementation (Figure 5), is little understood. It should be noted, however, that the mutagenic response to DMBA seen in the present study is consistent with the chemically induced hepatic DNA adduct levels previously reported in OVX rats [53].

The observation that DMBA-induced mutagenic response was relatively higher in OVX rats, led us to analyze the mutants to determine if differences exist between the types of mutations induced by DMBA in the OVX and in the INT groups. Molecular analysis of the mutants recovered from DMBA alone and DMBA+GE (OVX and INT) showed no significant difference in MFs between any two groups for paired comparison among the four DMBA treatment groups (Table 4). However, the spectrum of mutations from any group of the four DMBA-treated groups (INT DMBA alone, INT DMBA+GE, OVX DMBA alone, OVX DMBA+GE) was significantly different from that of the controls. The common types of mutations induced by DMBA were G:C → T:A transversions, A:T → G:C transitions, and A:T → T:A transversions, and these types of mutations are consistent with DNA adduct formation associated with DMBA treatment. G:C → A:T transition predominated in the control (Table 4). Overall, the results demonstrate that DMBA is significantly mutagenic in the liver of both OVX and INT rats, and the response was higher in the OVX rats compared to the INT group. The DMBA-induced mutation spectra were significantly different from those of control animals, and dietary genistein did not modify these responses.

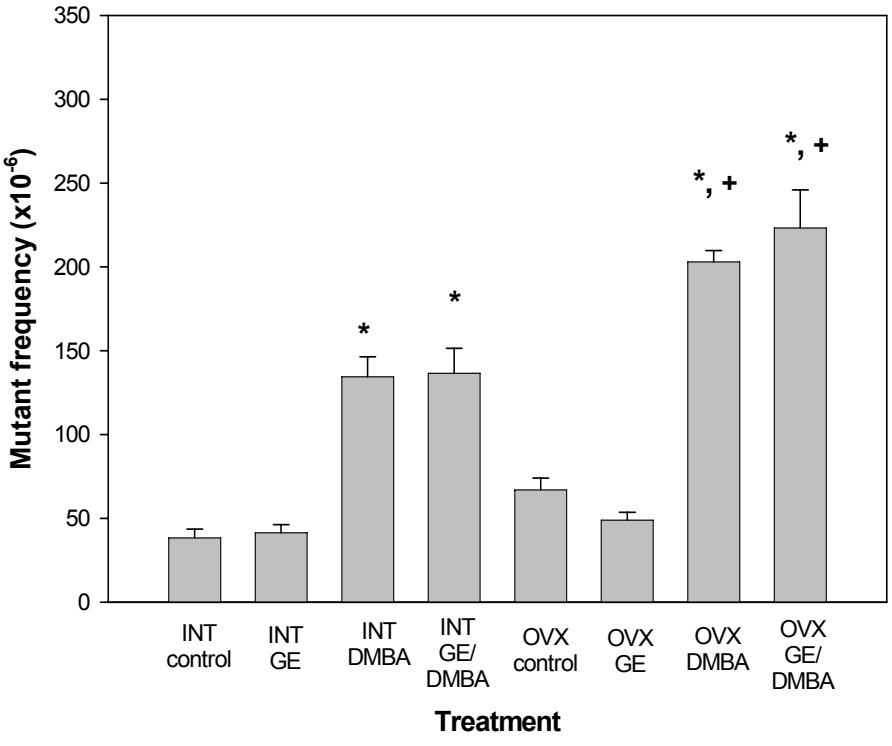

Figure 5. Mean MFs in the liver *cII* gene of INT and OVX BB rats treated with DMBA or vehicle control with or without dietary GE.
* Indicates that the treatment group is significantly different from its concurrent control group ($p < 0.001$), while + indicates that the OVX treatment group is significantly different than the comparable INT treatment group ($p < 0.001$).

Effect of DZ and GE on DMBA mutagenesis and carcinogenesis in the mammary gland

Breast cancer is the most common cancer and possibly the second-leading cause of cancer mortality in women with approximately one-in-nine affected in their lifetime [54]. Populations consuming soy beans have reduced rates of breast and other cancers possibly due, in part, to the presence of isoflavones, DZ and GE. However, GE has been shown to enhance mammary tumor growth in rats [21, 22]. Therefore, we investigated the effects of DZ and GE or E2 as positive control on DMBA-induced mutagenicity and carcinogenicity in the mammary tissues.

The results indicate that DMBA treatment significantly increased *lac*I MFs compared to those seen in the animals fed the control diet without DMBA, and that feeding BB rats with low and high doses of DZ, GE, or 0.005 g/kg E2 separately did not alter either spontaneous or chemically induced *lac*I MFs in rat mammary gland (Table 5). However, feeding the animals with a diet containing the mixture (DZG, 1g/kg GE + 1 g/kg DZ) resulted in a significant reduction in the DMBA-induced *lac*I MF in the mammary glands of the OVX rats ($p < 0.05$, Table 5). The *lac*I MFs in the DMBA-treated groups, with or without isoflavone supplements, were significantly higher in the INT rats compared to the OVX group ($p < 0.05$) indicating a significant enhancement by endogenous estrogens on the MF induced by DMBA; ovariectomy did not significantly alter the MFs of any of the diet groups not receiving DMBA.

Mild atrophy was detected in 10 of the 14 treatment groups in the OVX animals (Figure 6A); however

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this condition was non-neoplastic and appeared to be attributable to ovariectomy and not to dietary treatment. Animals exposed to a high-dose of DZ or DZG and treated with DMBA had a mildly reduced severity in mammary gland atrophy. Since atrophy was absent in animals fed the E2 diet, the reduction seen with the phytoestrogens clearly suggests an enhanced estrogenic action of DZ and GE when administered as a mixture. Also, the incidence of mammary gland ductal hyperplasia (defined here as a relative increase per unit area of hypodermis of branching intralobular and/or interlobular ducts) was highest in OVX rats fed the 1.0 g/kg DZ or the DZG diet without DMBA treatment (Figure 6A). However, ductal hyperplasia was absent in OVX animals fed the control diet, the low dose DZ and GE diets, and the high-dose GE diet; DMBA treatment reduced the hyperplasia seen in the high-dose DZ and the DZG diets. Ductal hyperplasia of the mammary can be considered as a precursor to the development of ductal carcinoma *in situ* [55, 56]; it is not clear why the incidence was high in animals treated with isoflavone alone. Hyperplasia is generally initiated by hormonal stimuli or other factors and even though it reflects non-neoplastic cellular proliferation with neoplastic transformation potential, it can also serve as a physiologic and adaptive response useful to organisms. Thus, ductal hyperplasia seen in OVX rats fed control diet containing isoflavones alone may relate to a physiologic or adaptive response to the estrogenic action of DZ and GE and not a pathological condition.

Despite the significant DMBA mutagenic response seen in the OVX rats (Table 6), histopathological examination of the mammary tissues in this group revealed that DMBA exposure was not associated with significant mammary tumor induction (Figure 6A). The lack of DMBA tumorigenicity in the mammary gland of OVX rats was not surprising because previous studies have indicated that the development and growth of mammary tumors by DMBA is greatly influenced by the presence or absence of estrogen [29, 30, 57, 58]. It also has been demonstrated that administration of exogenous estrogen is accompanied with mammary tumor development in OVX rats exposed to DMBA [57]. Feeding DMBA-treated rats with E2 in the present study resulted in adenoma (20%) in the mammary of OVX rats, whereas in the DMBA-treated rats fed DZ or GE no tumors were seen (Figure 6A). This response was associated with a 3-fold increase in PCNA: apoptosis ratio, suggesting increased cell proliferation (Table 7A). The virtual absence of neoplasia in the DMBA-treated OVX rats also fed DZ and GE suggests that isoflavones are strongly estrogen receptor competitive weak agonists (< 0.1% of estradiol), and they may be less toxic in a menopausal condition when estrogen levels are low [52].

Consistent with the responses observed in the isoflavone-treated animals in the OVX group, ductal hyperplasia also was prominent in the INT rats given isoflavones with or without DMBA exposure (Figure 6B). However, unlike OVX rats, a majority of INT rats treated with DMBA developed mammary tumors by 20 weeks following carcinogen treatment (Figure 6B). These mammary pathologic lesions, classified as either adenoma or adenocarcinoma, were combined, and their percentages are presented in Figure 6B. Among the DMBA-treated animals, 40% developed adenocarcinoma while the rest displayed ductal hyperplasia (Figure 6B). In the rats fed DZ or GE, the DMBA-induced mammary tumors were increased, but the responses were not statistically significant. Feeding

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Table 4. Summary of independent mutations induced by DMBA in the liver *cII* gene of OVX and INT rats fed GE.

Type of Mutation	Number (%) of independent mutations				
	Intact		Ovariectomized		Control [a]
	DMBA	DMBA+GE	DMBA	DMBA+GE	
Transitions					
G:C → A:T	15 (21)	8 (14)	2 (6)	4 (9)	27 (42)
% at CpG	67%	63%	100%	50%	74%
A:T → G:C	12 (16)	18 (32)	7 (21)	11 (25)	8 (12)
Transversions					
G:C → T:A	17 (23)	13 (23)	11 (32)	13 (30)	10 (15)
G:C → C:G	5 (7)	7 (12)	6 (18)	4 (9)	7 (11)
A:T → T:A	16 (22)	5 (9)	6 (18)	10 (23)	0 (0)
A:T → C:G	2 (3)	1 (2)	0	0	5 (8)
Frame Shifts	5 (7)	5 (9)	2 (6)	2 (5)	8 (12)
Others	1 (1)	0 (0)	0	0	0 (0)
Total mutations	73 (100)	57 (100)	34 (100)	44 (100)	65 (100)

[a] Data for control from Harbach *et al*., 1999.

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DMBA-treated rats with the mixture decreased mammary tumor incidence, perhaps due to synergy inherent in mixtures. The carcinogenic potency of DMBA in the INT rats could be due to the presence of endogenous ovarian hormones, including estrogen, which may have augmented the effect of DMBA. Like the OVX rats, the INT rats were also given exogenous E2. Even though 17β-estradiol is considered a weak mutagen [59], its metabolism by cytochrome P450 isoforms has been shown to generate catechol estrogens that can directly or indirectly interact with DNA and may initiate the carcinogenesis process [60]. It also has been shown that the metabolites of estrogens can directly or indirectly, through redox cycling processes, generate reactive radical species that cause oxidative DNA damage [61-63].

Although the observed increase in DMBA carcinogenicity by the isoflavones was not significant, it may reflect the intrinsic estrogenic activity of phytoestrogens. The fact that isoflavones can increase DMBA-induced carcinogenicity suggests that, like estrogens, these compounds can act as co-carcinogens or tumor promoters in tissues with preexisting DNA damage or exposed to carcinogens. An increase in DMBA-induced mammary adenocarcinoma by 1.0 g/kg GE in wild-type, but not in estrogen receptor-α-knockout mice, has been reported [21]. Chronic intake of 0.75 g/kg GE administered six weeks following carcinogen treatment and ovariectomy (when tumors had already developed) increased the growth of these tumors in Sprague-Dawley rats [22]. In addition, dietary GE at a level of 0.25 g/kg fed to female Fischer 344 rats also treated with azoxymethane-enhanced colon carcinogenesis [20]. In contrast in the present study, DZ or GE feeding starting two weeks before DMBA treatment did not cause significant changes in mammary gland carcinogenesis in the INT rats (Figure 5). Our results suggest that DZ and GE are comparatively weak estrogens since the mixture containing both high-dose of DZ and GE slightly decreased DMBA carcinogenicity in the mammary gland compared to E2. Also, differences such as experimental design, duration of treatment, use of transgenic animals, and the weak estrogenic activity of the phytoestrogens may have contributed to a lack of significant tumorigenic effect of the isoflavones in the present study.

Table 5. Mammary *lac*I MFs measured 16 weeks post DMBA treatment in OVX and INT rats fed control diet containing DZ, GE, and mixture (DZG) or E2.

Treatment and Dose	Mutant frequency (x 10^{-6})			
	OVX[a]		INT[a]	
	Vehicle	DMBA	Vehicle	DMBA
Control/oil	22.5 ± 3[b]	201.7 ± 34*	29.5 ± 2[b]	263.5 ± 18*
DZ 0.25 g/kg diet	33.7 ± 7	140 ± 6*,≠	33 ± 6	215 ± 42*
GE 0.25 g/kg diet	30 ± 10	227.6 ± 66*	34.2 ± 6	295.7 ± 42*
DZ 1.0 g/kg diet	24 ± 19	149.4 ± 43*,≠	27 ± 7	264.5 ± 17*
GE 1.0 g/kg diet	27.5 ± 9	197.5 ± 39*	41.5 ± 4	307.5 ± 32*
DZG 1.0 g/kg diet	21.2 ± 6	127.4 ± 31**	27.5 ± 6	247 ± 39*
E2 0.005 g/kg diet	40 ± 10	186.6 ± 98*	39.5 ± 11	288.4 ± 33*,≠

[a] Significant difference between OVX and INT groups as determined by *t*-tests ($p < 0.05$).

[b] Values are means ± SD of five rats for all treatment groups except those marked '≠', which had only four rats per group.

* Significant differences were found between the rats treated with DMBA and those treated with vehicle only ($p < 0.01$).

** Significant changes were found among the groups treated with DMBA and among the groups treated with vehicle ($p < 0.05$).

Effect of DZ and GE on DMBA mutagenesis and carcinogenesis in the uterus

An association between soy consumption and the risk for endometrial cancer has been discussed [64-66]; however, comparatively little is known about the possible action of naturally occurring phytoestrogens in chemical carcinogenesis in the uterus. Since the uterus appears especially sensitive to neoplastic transformation by estrogens [67], experiments were performed to determine the effects of DZ, GE, or E2 on DMBA-induced mutagenicity and carcinogenicity in the uterus of OVX and INT rats. The uterine tissues from rats sacrificed at 16 or 20 weeks following DMBA treatment were processed to assess *lac*I mutant frequencies and histopathological parameters, respectively. The results are presented in Table 6 and Figure 7. The data indicate that in the uteri of both OVX and INT rats, DMBA treatment significantly increased *lac*I MFs compared to the animals that were not exposed to DMBA, while DZ, GE, or E2 diets did not alter either the DMBA-induced or spontaneous MFs in the uterus (Table 6). However, the MF of the DMBA-treated INT rats was greater than that seen in the corresponding OVX animals.

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Histopathological analysis of uterine samples derived from OVX rats revealed no neoplastic transformation, malignant or nonmalignant, in the uterus of rats exposed to DMBA alone or those fed isoflavone and treated with the carcinogen (Figure 7). DMBA is a multiorgan carcinogen, and it is possible that its failure to induce tumors in the uterus may be due, in part, to toxicological factors such as the inefficiency of local or distant metabolism of DMBA, removal of DNA adducts by excision repair, cell proliferation rate, or doubling time of initiated cells in the uterus. Besides the latter hypothesis, the other factors appear to be unlikely because DMBA exposure resulted in a significant MF in uterus, liver, and also in a surrogate tissue, lymphocytes (data not shown) of OVX rats, with cell proliferation increased in DMBA-treated rats (Table 7B). Further, the lack of DMBA carcinogenic effect in the uterus may not be due to estrogen deficiency as a result of ovariectomy: in rats fed E2 and exposed to DMBA, the only pathologies observed were a developmental malformation in the uterus in one rat and a clitoral gland adenoma in another. Moreover, in similar experiments with INT rats, DMBA treatment alone induced only a clitoral gland carcinoma in one rat and no uterine tumors (data not shown).

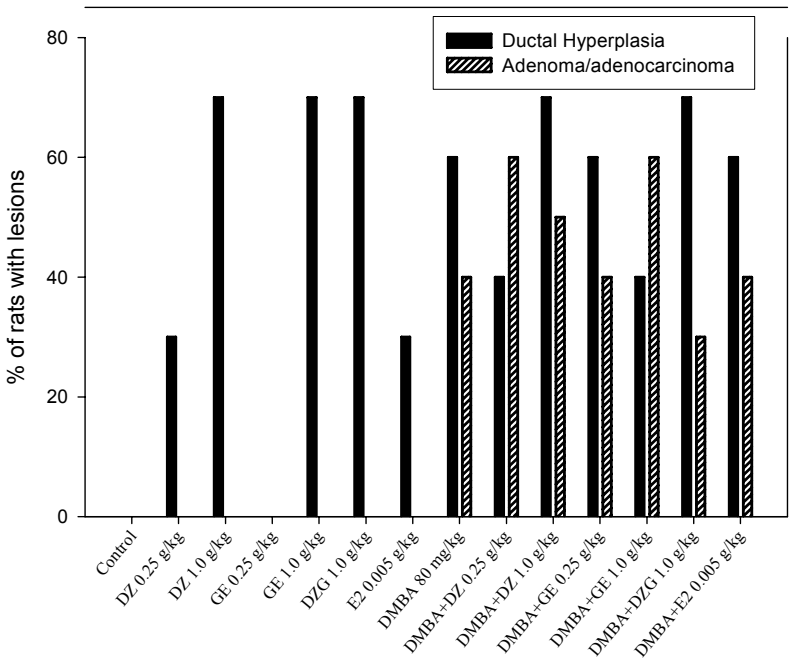

Figure 6B. Neoplastic and non-neoplastic lesions in the mammary glands of INT BB rats fed control diet containing DZ, GE, and mixture (DZG) or E2. Animals were sacrificed 20 weeks following DMBA treatment.

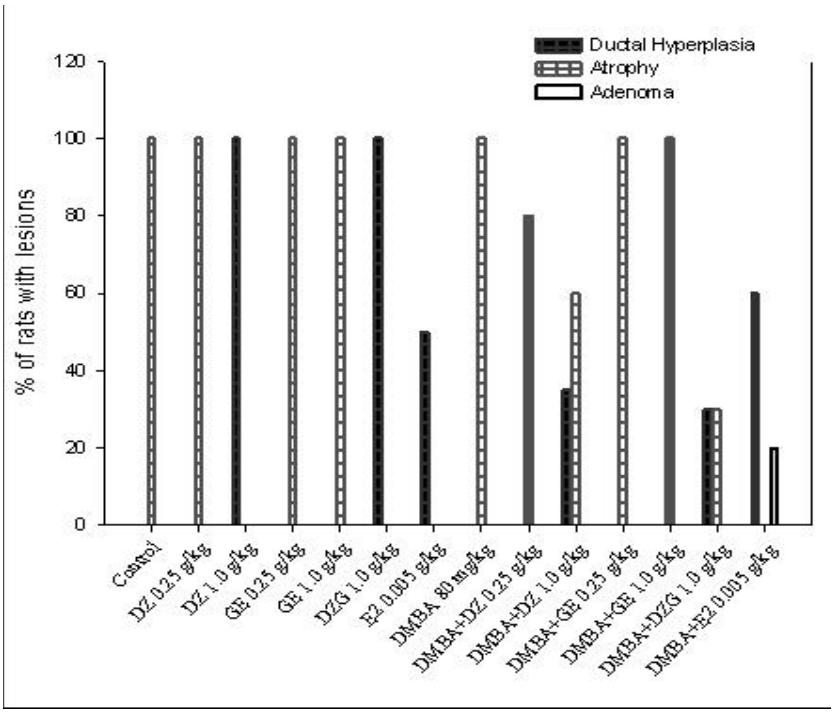

Figure 6A. Neoplastic and non-neoplastic lesions in the mammary glands of OVX BB rats fed control diet containing DZ, GE, and mixture (DZG) or E2. Animals were sacrificed 20 weeks following DMBA treatment.

As shown in Figure 7, uterine atrophy was prominent in the OVX group with or without the supplements or DMBA treatment as expected in ovariectomy. Although the percentage of OVX rats with atrophy as depicted in Figure 7 appears essentially similar in all the treatment groups, except E2, the degree of severity, graded as 3-4 (high) and 2 or below (low), was found to be high in rats receiving the control diet and those fed the isoflavones or treated with DMBA alone (data not shown). Interestingly, the degree of severity was only reduced in DMBA-treated rats fed the isoflavones, while it was completely eliminated in E2-fed rats exposed to DMBA, suggesting lower estrogenic activity of the isoflavones compared to E2.

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This finding is consistent with the absence of uterine dysplasia in rats exposed to the phytoestrogens or DMBA alone in both OVX (Figure 6 and INT not shown) groups. Dietary E2, however, caused a high incidence of dysplasia in OVX rats (55% in E2-fed alone and 100% in E2-fed plus DMBA treatment).

The high incidence of dysplasia found in OVX rats fed E2 and treated with DMBA essentially implies that E2 does not only induce dysplasia, by itself, but also is capable of promoting chemically initiated cells into dysplasia. Dysplasia is an abnormal, atypical cellular proliferation, and while it is not a tumor and does not cause health problems, if left untreated it sometimes can progress to an early form of cancer. These findings have important health implications due to the fact that one of the major adverse effects of estrogen replacement therapy among menopausal women is endometrial cancer [68]. In contrast, in the INT group dysplasia was not detected, but asynchronistic growth pattern was observed in the uterus possibly due to exposure to the isoflavones and E2; uterine hemangiosarcoma also was detected in one INT rat fed 1.0 g/kg DZ and treated with DMBA. In addition, isolated incidences of mild-to-marked dilatations were seen in some animals exposed to DMBA with or without the isoflavones. No incidence of dilatation, mild or marked, was seen in E2-fed rats in this group. The incidence of these pathologic lesions was sporadic and too low to be interpreted as treatment related.

Dietary DZ and GE combined with DMBA treatment also produced dysplasia in the uterus of OVX rats (Figure 7), but the incidence was relatively low (ranging from 10-18%). The low incidence of dysplasia in this group illustrates the relatively weak estrogenic potency of phytoestrogens and indicates that they may be safer than estradiol. However, under the conditions of these experiments, the period of isoflavone administration was not long enough to actually determine whether they were carcinogenic by themselves. Nonetheless, their ability to induce dysplasia, even on a small scale when a carcinogen is present, clearly indicates that they can potentially influence the growth of initiated cells in rat uterus. The underlying mechanism of action of isoflavones is not clearly understood; however, they can function both as estrogen agonists and antagonists [69, 70] depending on many factors, including hormonal milieu or receptor occupancy, treatment regimen, and tissues under investigation. From a genotoxic perspective, this result may imply that when estrogen levels are low, as they tend to be in ovariectomy or menopause, isoflavones can substitute for the organism's own estrogen and act as co-carcinogens (already discussed above).

Table 7A. Effect of DZ, GE or E2 on cell proliferation and apoptosis in the mammary glands of OVX and INT BB rats.

Treatment Group	OVX			INT		
	S phase	G1	Apoptosis	S phase	G1	Apoptosis
Control diet	4.5 ± 0.7	7.5 ± 0.7	3.5 ± 0.7	7.5 ± 1.4	13.5 ± 2.1	10 ± 2.8
DZ 0.25 g/kg	5.6 ± 0.9	5.0 ± 0.5	4.8 ± 0.5	7.5 ± 2.1	12.0 ± 1.4	8.5 ± 0.7
DZ 1.0 g/kg	4.0 ± 1.4	6.5 ± 0.7	5.5 ± 0.7	6.0 ± 1.4	11.0 ± 0.0	8.0 ± 4.2
GE 0.25 g/kg	9.5 ± 1.2	7.2 ± 1.0	5.8 ± 0.6	6.0 ± 1.4	10.0 ± 1.4	14.5 ± 6.3
GE 1.0 g/kg	5.5 ± 0.7	4.5 ± 0.7	2.5 ± 0.7	16.0 ± 2.8	24.5 ± 3.5	9.5 ± 6.3
DZ/GE 1.0 g/kg	4.3 ± 0.6	10.3 ± 1.5	5.4 ± 0.4	9.5 ± 2.1	15.0 ± 2.8	8.5 ± 4.2
E2 0.005 g/kg	7.6 ± 0.9	9.7 ± 0.5	4.9 ± 0.5	12.0 ± 1.4	15.5 ± 2.1	5.5 ± 2.1
DMBA	12.4 ± 4.8	20.6 ± 8.0	6.6 ± 4.1	8.8 ± 3.1	14.1 ± 3.7	10.4 ± 2.4
DMBA + DZ 0.25 g/kg	19.4 ± 6.3	28.7 ± 8.6	8.0 ± 3.3	12.3 ± 3.4	19.2 ± 5.2	12.6 ± 3.9
DMBA + DZ 1.0 g/kg	24.1 ± 3.6*	33.4 ± 5.1*	7.9 ± 2.7	17.4 ± 2.6*	26.3 ± 5.3*	11.6 ± 3.2
DMBA + GE 0.25 g/kg	23.7 ± 8.2*	33.0 ± 9.1*	8.5 ± 4.0	13.7 ± 4.0	20.7 ± 4.9	11.1 ± 2.8
DMBA + GE 1.0 g/kg	18.6 ± 4.7	28.3 ± 8.4	9.4 ± 2.4	15.0 ± 5.9	24.8 ± 8.3	10.1 ± 4.1
DMBA + DZGE 1.0 g/kg	12.5 ± 3.9	22.3 ± 8.6	7.5 ± 2.3	9.4 ± 3.1	16.0 ± 6.0	12.8 ± 3.2
DMBA + E2 0.005 g/kg	18.2 ± 2.3	28.5 ± 3.0	6.5 ± 2.6	20.6 ± 1.4*	30.4 ± 5.8*	11.6 ± 4.0

Abbreviations: DZ, Daidzein; GE, Genistein; E2, 17 β-Estradiol; DMBA, 7, 12-Dimethylbez[α]anthracene.
Values are mean ± standard deviation
* Significantly different from DMBA alone at $P < 0.05$.

Since DZ, GE, and E2 did not increase DMBA-induced MFs, and DMBA alone did not induce uterine dysplasia in OVX rats, it is possible that the mechanism of action for the induction of dysplasia by E2 and/or the phytoestrogens results from their ability to increase cell proliferation. The most widely established role of estrogen in carcinogenesis involves increased cell proliferation [71-73]; therefore, we assessed cell proliferation by PCNA immunochemistry. As expected, the percentage of PCNA-positive cells in S-phase and G1-phase of the cell cycle in the uterus of OVX rats fed E2 diet and treated with DMBA was significantly higher than the other groups (Table 7B, $p < 0.05$). There also was a corresponding increase in uterine weight in E2-fed animals treated with DMBA (Table 2A); this finding was in contrast to the atrophy seen in the OVX rats fed the

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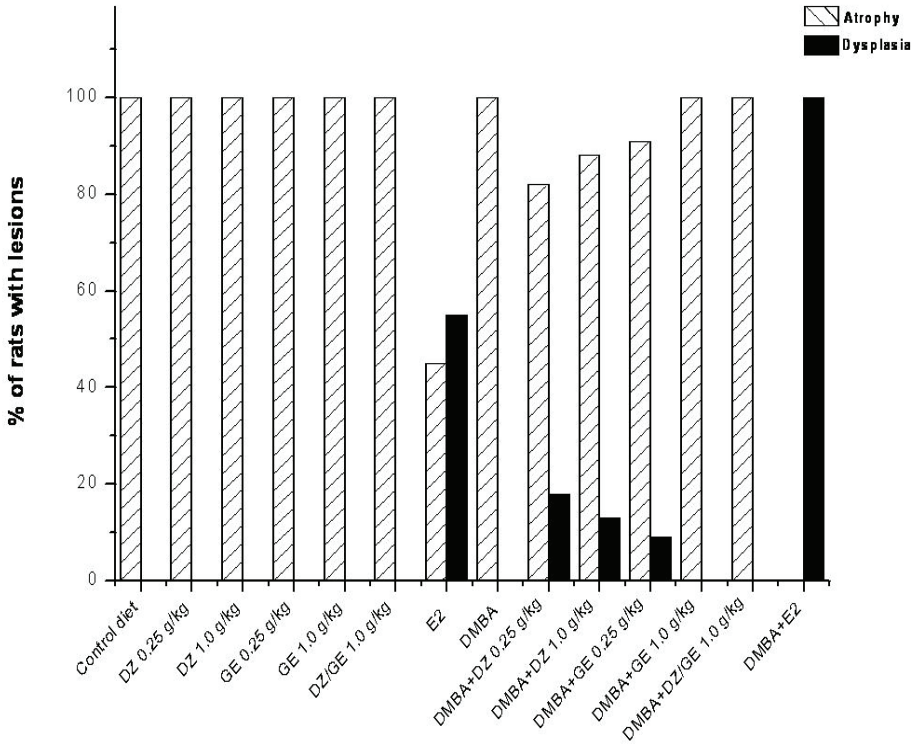

Figure 7. Histopathology of uterine tissues derived from ovariectomized rats sacrificed at 20 weeks post-DMBA treatment. Atrophy was marked in most of the treatment groups including the control, but the incidence was less marked in rats fed E2 diet alone. Atrophy was virtually absent when E2 feeding was combined with DMBA treatment; in this group, 100% dysplasia was detected, while 10-18% incidence of dysplasia was observed in rats fed the isoflavones and treated with DMBA.

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isoflavone and the control diets. Cell proliferation also was increased in the isoflavone-fed animals treated with DMBA in the OVX group (Table 7B). In the INT rats, no significant changes in G1 and S phases were observed in any of the treatment groups (Table 7B). These results suggest that cell proliferation is partly responsible for the induction of dysplasia in the uterus of OVX rats by dietary E2 and by the isoflavones, and that DMBA-induced cytolethality also contributes to the observed pathologic lesion.

Considering the fact that prolonged exposure to synthetic or endogenous steroidal hormones or their metabolites is causally linked to several human cancers, including endometrial cancer [71, 74, 75], it was unexpected that dysplasia was virtually absent in the uterus of INT rats treated with DMBA and fed E2 or the phytoestrogens. One possible explanation for this phenomenon is hormone balance. It has been shown that postmenopausal hormone replacement therapy using unopposed estrogen significantly increases endometrial cancer risk. This risk, however, is markedly reduced when estrogen is administered in conjunction with progestin [8]. Also, E2 is metabolized along two competing pathways to form 2-hydroxylated and 16α-hydroxylated metabolites. Because of their different biological activities, the ratio of these metabolites, 2-hydroxy-estrogen:16ahydroxy-estrone has been used as a biomarker for breast cancer risk [76, 77]. Since the ovary produces many other hormones in addition to E2, it is also possible that some of these hormones or their metabolites may function against estrogenic action and inhibit cell proliferation in the uterus of INT rats [68].

Effect of DZ and GE on histopathological lesions in other organs/tissues

In addition to the mammary gland and the uterus, other tissues were examined for the presence of pathologic lesions in rats killed 20 weeks after DMBA treatment. In the OVX rats treated with DMBA alone, no neoplastic lesion was seen except a mild duct dilatation in the clitoral gland. However, a neural crest tumor was observed in the ear of one rat fed 0.25 g/kg DZ diet and treated with DMBA; another rat in the DMBA-treated group fed E2 had clitoral gland adenoma. In the INT group, ovarian cysts were seen in all the treatment groups, including those fed the control diet. Also, GE feeding caused cellular infiltration in lymph node in one rat and abscess in the oral mucosa in another. DMBA treatment resulted only in clitoral gland tumor in one rat and nonneoplastic lesions, such as fibrosis in the liver, and marked hematopoietic cell proliferation in the spleen. In rats treated with DMBA and fed 1.0 g/kg DZ diet, lung carcinoma in one rat and skin carcinoma in another was detected. However, these lesions, observed in 27-week-old rats, were low and sporadic to suggest treatment-related effects and could represent background alterations in female rats of this age.

Summary and conclusions

Isoflavones are naturally occurring estrogens that are believed to exhibit predominantly beneficial effects by preventing hormone-dependent cancers and relieving menopausal symptoms. As naturally occurring and specific modulators of estrogen receptors, they appear to play a significant role in modifying the molecular processes involved in the pathogenesis of hormone-dependent cancers. The phy-

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toestrogens, DZ and GE, are increasingly used by women. Previous studies raised concerns that DZ and GE may cause adverse effects at physiologically relevant concentrations. This study thus evaluated the effects of DZ, GE, or E2 supplementation on chemical mutagenesis and carcinogenesis in the mammary and uterus of OVX and INT BB transgenic rats.

DZ and GE were found to be biologically active as determined by increased PCNA positive cells and concomitant decrease in atrophy in the OVX rats, as well as levels detected in serum. Feeding rats diets containing DZ or GE was not associated with significant changes in animal or organ weights, although there was a concurrent reduction in animal weights and an increase in uterine weight in the animals fed E2. Both

Table 6. Mutant frequencies in the uterus *lac*I gene measured 16 weeks post DMBA treatment in ovariectomized (OVX) and intact (INT) rats fed with control, daidzein (DZ), genistein (GE), daidzein + genistein (DZG), and 17 β-estradiol (E2) diets.

Treatment	Mutant frequency (x 10^{-6})			
	OVX[a]		INT[a]	
	Vehicle	DMBA	Vehicle	DMBA
Control/oil	13.3 ± 2.9 [b]	83 ± 12.5*	15.3 ± 3.6 [b]	100.0 ± 37*
DZ 0.25 g/kg diet	16 ± 6.5 [b]	64 ± 47 *,≠	16.2 ± 5.9	70.0 ± 19.2*
GE 0.25 g/kg diet	17 ± 4.5	94 ± 25*	20.7 ± 11.2	123.7 ± 61*
DZ 1.0 g/kg diet	11.2 ± 4.8	71 ± 11.5*,≠	11.8 ± 5.2	89.7 ± 12.9*
GE 1.0 g/kg diet	18.7 ± 7.5	89.5 ± 16.7*	25.5 ± 6.2	135.5 ± 62*
DZG 1.0 g/kg diet	8.4 ± 2.9	60.5 ± 49*	16.9 ± 5.6	81 ± 42*
E2 0.005 g/kg diet	23.3 ± 2.9	95.7 ± 37.8*	32 ± 9.2	140.0 ± 44*,≠

[a] No significant difference between OVX and INT groups as determined by *t*-tests ($p > 0.14$).

[b] Values are means ± SD of five rats for all treatment groups except those marked '≠', which had only four rats per group.

* Significant differences were found between the rats treated with DMBA and those treated with vehicle only ($p < 0.05$). No significant changes were found among the groups treated with DMBA or among the groups treated with vehicle.

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Table 7B. Effect of DZ, GE, and E2 on cell proliferation and apoptosis in the uterus of OVX and INT BB rats.

Treatment Group	OVX			INT		
	S phase	G1	Apoptosis	S phase	G1	Apoptosis
Control diet	6.0 ± 1.4	12.5 ± 0.7	22.0 ± 0.0	57.5 ± 16.3	67.0 ± 19.8	30.5 ± 4.9
DZ 0.25 g/kg	18.9 ± 4.5**	20.4 ± 2.6	15.8 ± 3.2	37.5 ± 18.9	42.0 ± 26.8	37.5 ± 9.2
DZ 1.0 g/kg	27.0 ± 5.7**	50.0 ± 8.5**	9.5 ± 0.7**	45.5 ± 17.7	54.5 ± 19.1	28.5 ± 10.6
GE 0.25 g/kg	21.3 ± 3.9	29.5 ± 9.8**	11.6 ± 1.8	75.0 ± 19.8	73.5 ± 16.3	35.0 ± 1.4
GE 1.0 g/kg	14.0 ± 1.4	24.0 ± 1.4	10.5 ± 0.7	29.5 ± 9.2	39.0 ± 2.8	31.0 ± 2.8
DZ/GE 1.0 g/kg	20.4 ± 2.8**	31.8 ± 8.7**	13.4 ± 2.0	74.0 ± 7.0	76.5 ± 9.2	22.5 ± 6.4
E2 0.005 g/kg	29.5 ± 7.8**	33.8 ± 12.8	9.8 ± 1.6**	55.5 ± 7.8	71.5 ± 7.8	21.0 ± 11.3
DMBA	19.8 ± 10.1	30.3 ± 11.2	29.3 ± 5.9	58.1 ± 18.2	65.0 ± 21.7	28.4 ± 8.2
DMBA + DZ 0.25 g/kg	24.5 ± 7.9	39.5 ± 10.7	25.3 ± 6.6	50.3 ± 22.5	56.7 ± 23.3	27.9 ± 6.3
DMBA + DZ 1.0 g/kg	39.8 ± 10.6*	52.1 ± 14.7*	28.1 ± 5.1	50.0 ± 18.4	56.7 ± 19.9	29.2 ± 6.9
DMBA + GE 0.250 g/kg	31.1 ± 6.9	42.8 ± 9.7	28.0 ± 7.3	51.8 ± 26.6	51.6 ± 18.7	28.9 ± 7.9
DMBA + GE 1.0 g/kg	31.4 ± 7.7	47.1 ± 10.4	28.4 ± 5.6	54.2 ± 16.5	62.2 ± 15.6	32.7 ± 5.7
DMBA + DZ/GE 1.0 g/kg	46.5 ± 8.9*	62.7 ± 12.1*	22.9 ± 9.5	39.9 ± 9.1	44.8 ± 11.3	32.5 ± 6.6
DMBA + E2 0.005 g/kg	59.0 ± 8.0*	84.4 ± 11.4	24.5 ± 10.5	49.3 ± 23.6	60.9 ± 29.8	32.7 ± 4.4

Abbreviations: DZ, Daidzein; GE, Genistein; E2, 17 β-Estradiol; DMBA, 7, 12-Dimethylbez[α]anthracene.
Values are mean ± standard deviation
* Significantly different from DMBA alone at $P < 0.05$.
** Significantly different from control diet at $P < 0.05$.

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OVX and INT rats fed control diets containing the isoflavones (except DZG in the OVX) did not significantly alter the mutagenicity of DMBA in the hormone-responsive tissues (heart, liver, mammary, and uterus) examined. E2 feeding effectively reduced DMBA-induced mutagenicity in the heart, and molecular analysis of the mutants showed a shift in the mutational spectra of DMBA from the common type (A:T → T:A) to G:C → A:T and A:T→ G:C.

The DMBA treatment induced tumors only in the mammary gland of INT animals, and neither DZ nor GE given separately resulted in any significant changes in DMBA-induced tumorigenicity. The DZG diet, however, reduced DMBA tumorigenic response in the mammary gland. DMBA treatment alone failed to induce tumors in the uterus; while feeding E2 to rats treated with DMBA resulted in 100% incidence of uterine dysplasia in the OVX rats. In comparison with E2, dietary DZ and GE were associated with low incidence of uterine dysplasia in OVX rats, suggesting weak estrogenic effects of these phytoestrogens. Taken together, DZ and GE given separately resulted in nonsignificant increase in the DMBA-mediated carcinogenicity in the mammary gland, while the mixture reduced DMBA response. This finding suggests the potential benefit of eating soy products containing the isoflavones mixture as opposed to ingestion of single isolated supplements.

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Glossary:

Big Blue® transgenic rats — These rats are obtained from Stratagene; they are F344 rats with multiple copies of Lambda vector with *lac*I transgene inserted in the genome of every cell. The transgene can be retrieved from the DNA of any tissue after treating the rats with drugs or chemicals and screened for mutation accumulation. The screening of mutant plaques is done on a Petri dish, and the selection of mutants is accomplished by blue color using X-gal, hence the name Big Blue®.

Chemical mutagenesis/tumorigenesis — Many natural and synthetic chemicals can directly damage DNA by forming DNA adducts, which eventually alter the DNA sequence and induce irreversible genetic damage or mutations that could set the stage for tumor development. Tumorigenesis or carcinogenesis is the molecular process by which cancer develops. Most chemical mutagens are carcinogens (agents that cause cancer), thus it is important to evaluate chemicals or drugs for their ability to induce mutation as an index of carcinogenic risk to humans.

7,12-dimethylbenz[*a*]anthracene (DMBA) — DMBA is a synthetic polycyclic aromatic hydrocarbon (PHA) that

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has been widely evaluated for its carcinogenicity and is often used as a model compound for breast, skin, and other cancers in rodents. DMBA is metabolically activated to form reactive metabolites that bind to DNA-causing mutations (changes in the base pair sequence of genetic material) and cancer initiation. Unlike other PHAs that are common environmental pollutants, DMBA is not found in the environment; however, recent studies indicate that cigarette smoke contains small amounts of the carcinogen.

17ß-Estradiol (E2) — E2 is a sex hormone that is derived from cholesterol and predominates in females compared to males. E2 is one of the three estrogen hormones in the body; the other two are estrone (E1) and estriol (E3). E2 binds to estrogen receptors, alpha and beta, and plays a critical role in reproduction and sexual functioning, but it also has effects on other organs, including heart, liver, and bone structure. In serum, E2 is largely bound to sex hormone binding globulin and albumin, thus only a fraction is free and biologically active.

Hormone replacement therapy (HRT) — HRT is a type of medical treatment for women to prevent discomfort and other health problems associated with diminished levels of estrogen and progesterone hormones due to surgical, perimenopausal, or postmenopausal conditions.

***Lac*I mutagenesis assay** — The Big Blue® transgenic mice and rats contain multiple copies of the *lac*I genes inserted into the lambda vector. The transgene *lac*I is the target gene, and the mutation in this gene is evaluated by retrieving the transgenes from the treated animals following exposure to test chemicals and screening for blue plaques on a lawn of *E.coli* bacteria. The plaques that are blue carry a mutation in the *lac*I gene, which in turn allows the *lac*Z gene to transcribe and produce B-gal enzyme. The B-gal enzyme hydrolyzes the X-gal in the media turning the plaques blue. Transgene mutant frequency is measured by dividing the number of blue plaques by the total number of white or non-blue plaques.

Phytoestrogens — Phytoestrogens, such as isoflavones, are chemicals found in plants and have a chemical structure similar to steroidal estrogens. Thus when ingested, they bind to estrogen receptors alpha and beta and act like the body's own estrogens; however, they have a weak estrogenic effect. In the body isoflavones can also exert their effects through estrogen-independent mechanism. Research indicates that isoflavones may play a role in reducing risk for certain diseases or preventing menopausal symptoms. The most investigated phytoestrogens that are believed to have beneficial effects in menopause are the isoflavones, genistein and daidzein, found in soybeans and other legumes with higher concentrations observed in roasted soybeans. Food sources of soy isoflavones include tofu, tempeh, veggie burgers, textured soy protein, and soy milk.

The Authors

Anane Aidoo, Ph.D.

Anane Aidoo, Ph.D. is a research biologist at the FDA's National Center for Toxicological Research (NCTR), Division of Genetic and Reproductive Toxicology. In 1986 he earned his B.S. in biology from Georgia State University and his Ph.D. in cell biology from Clark Atlanta University. After an ORISE/NCTR postdoctoral fellowship in genetic toxicology, with emphasis in the development of a rat T-lymphocyte model system for measuring *in vivo* somatic mutations in the hypoxanthine-guanine phosphoribosyl transferase (*hprt*) gene, he became a staff fellow and was promoted to the position of research biologist in 1992. The *Hprt* gene in T-lymphocytes is one of the few genes suitable as a marker for mutation induction in animal systems and humans. It has been used extensively as a reporter gene for human *in vivo* mutagenesis and biomarker for genetic effects of genotoxic exposures in human population. Thus, it is imperative to develop such an assay in rodents to facilitate genotoxicity evaluation of chemicals or drugs relevant to the FDA. Dr. Aidoo's current research interests include mitochondrial DNA mutations and the role of dietary agents in mutation induction and the modification of cellular function. He has published in peer-reviewed scientific journals; he is a member of the Environmental Mutagen Society and a recipient of the FDA Equal Opportunity Achievement and Commendable Service Awards.

Dr. Tao Chen received his Ph.D. degree in toxicology from the University of Arkansas for Medical Sciences and was a postdoctoral scientist at Duke University. He joined the Division of Genetic and Reproductive Toxicology, National Center for Toxicological Research, U.S. Food and Drug Administration, as a staff fellow in 2000 and became a research toxicologist in 2002. Dr. Chen has been a diplomat of the American Board of Toxicology since 1999. He is also an adjunct professor in Shanghai Jiao Ton University, Shanghai, China, and in Shantou University Medical Col-

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lege, Shantou, China. Dr. Chen serves as an Ad hoc reviewer for many journals. He is a member of the Society of Toxicology and Environmental Mutagen Society. Dr. Chen has published many articles in peer-reviewed journals and books. His research addressed fundamental issues concerning *in vivo* mutation detection and analysis, including development of methods for molecular characterization of chemical-induced and spontaneous mutations in the rodent endogenous genes and transgenic genes for safety assessment of FDA-relevant drugs and chemicals. His research interests also include fundamental mechanisms of mutagens and the molecular basis for mutations, effects of age and hormones on *in vivo* mutation inductions by carcinogens, and elucidating the molecular mechanisms of the mutagenesis and carcinogenesis using gene expression profiles and bioinformatics, and defining the relationships among different biomarkers like expression of marker genes, DNA adducts, mutations, and tumors.

Michelle E. Bishop

Michelle E. Bishop is a support research biologist in the Division of Genetic and Reproductive Toxicology at the National Center for Toxicological Research (NCTR), Jefferson, Ark. Ms. Bishop began working at the FDA/NCTR in 1991 as a student intern in the Division of Chemistry. After receiving her B.S. in chemistry from the University of Arkansas at Pine Bluff in 1993, she became a full-time employee at NCTR. She is recognized as having established both *in vitro* and *in vivo* micronucleus assay techniques for evaluating drugs and chemicals relevant to the FDA. Ms. Bishop is applying the techniques in micronucleus assay to assist principal investigators at NCTR. She is currently involved in the National Toxicology Program and The National Institute of Child Health and Development projects being conducted at the Center.

The Authors

Sharon D. Shelton

Sharon D. Shelton began her career in 1993 as a biologist in the Division of Genetic and Reproductive Toxicology at the FDA's National Center for Toxicological Research (NCTR), Jefferson, Ark. She graduated with a B.S. in Biology from the University of Arkansas at Pine Bluff. Most of her research is focused on the study of spontaneous and induced mutations in transgenic animals, indicative of her expertise in conducting mutagenesis studies, which utilize Stratagene's Big Blue® transgenic rodent mutation assays. By using Big Blue rats, chemical- or drug-induced mutations can be measured in virtually any tissue, thus enabling the identification of tissue-specific sensitivity to chemicals or drugs. She is currently involved in the National Institute of Child Health and Development study aimed at evaluating the genotoxicity of methylphenidate male Big Blue® mice.

Lascelles Lyn-Cook is a biologist in the Division of Genetic and Reproductive Toxicology at the FDA's National Center for Toxicological Research, Jefferson, Ark. He graduated from Howard University with a B.S. in zoology in 1976 and attended graduate school until 1979 (Zoology). He was employed at the University of North Carolina (UNC) at Chapel Hill, Department of Physiology, where his research focused on the role of microtubules in inter- and intra-cellular transport. Also, in the

Lascelles Lyn-Cook

Department of Pediatric Gastroenterology at UNC, he was involved in research aimed at evaluating the role of epidermal growth factor in the developing small bowel. Mr. Lyn-Cook began working at FDA/NCTR in 1988 in the Division of Genetic and Reproductive Toxicology, focusing on mutation induction at the *Hprt* gene in lymphocytes. His current research work involves the evaluation of the effects of genistein and daidzein on the genotoxic and carcinogenic activity of the model mammary carcinogen 7, 12-dimethylben[*a*]anthracene (DMBA).

Dr. Mugimane G. Manjanatha received a Ph.D. in microbiology and genetics from Iowa State University, Ames, Iowa, in 1990. He joined the Division of Genetic and Reproductive Toxicology (DGRT) at the National Center for Toxicology Research (NCTR) to pursue an ORISE postdoctoral training. At NCTR, as a postdoc, he was responsible for designing and carrying out experiments for evaluating several chemical agents for their mutagenic potential using the *in vitro* CHO/H*prt* and the *in vivo/in vitro* rat lymphocyte *Hprt* mutagenicity assays. In addition, he developed methods for molecular characterization of chemically induced mutations in the tumor suppressor gene *p53* and protooncogenes H- and K-*ras* in rodent tumors induced by FDA-relevant chemi-

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cals. Following his postdoctoral training, Dr. Manjanatha has continued as a tenured staff member at the DGRT/NCTR.

Dr. Manjanatha is a recognized leader in the field of Genetic Toxicology, especially in the area of hazard identification and risk assessment using transgenic systems. He has been leading a team of researchers involved in evaluating transgenic systems for their sensitivity and specificities as mutational targets for safety assessment of FDA-relevant drugs and chemicals. He has obtained many IAG grants from the National Toxicology Program for hazard identification and risk assessment of drugs or chemicals, such as Leucomalachite green, Malachite green, Acrylamide, etc. Currently, he is working on the genotoxicity evaluation of methylphenidate (Ritalin) in mice (IAG grant from NICHD). He is currently working on developing a method to augment the transgenic *lac*I assay so that additional types of mutations can be detected for a better human risk assessment. Dr. Manjanatha serves on many FDA, local, and national committees dealing with transgenic systems used in Genetic Toxicology. He is an adjunct Assistant Professor in the Department of Pharmacology & Toxicology at the University of Arkansas Medical Sciences, Little Rock, Ark. He has published numerous scientific articles, serves as a reviewer for several journals on Toxicology, and has received several major awards, the most recent being the Commendable Service Award from the FDA.

The Authors

Mugimane G. Manjanatha, Ph.D.

RRP's Research Spotlight

Dr. Varsha G. Desai earned her M.S. and Ph.D. in Biochemistry from the University of Bombay, India, in 1990. In 1993, she came to the U.S. to continue her scientific career as a postdoctoral fellow at the Oak Ridge Institute for Science and Education in the Division of Genetic and Reproductive Toxicology at the National Center for Toxicological Research (NCTR), Jefferson, Ark. During her postdoctoral career, she focused on the role of mitochondrial oxidative stress in aging, age-associated degenerative diseases, and various drug- and chemical-induced toxicities in animal models and humans. She has developed a number of biochemical assays aimed at delineating the mechanisms of mitochondrial dysfunction and the influence of various dietary interventions in modulating oxidative stress. In 2001, in her new position as a staff fellow, she played a significant role in the development of high-throughput DNA microarray technology in the Center for Functional Genomics at NCTR. Dr. Desai was promoted to the position of Research Biologist in the Division of Systems Toxicology in 2004 and, utilizing her knowledge in mitochondria function and expertise in DNA microarray technology, she developed a mouse *MitoChip*, an oligonucleotide microarray containing mitochondrial and nuclear genes associated with mitochondrial function. The mouse *MitoChip* is capable of simultaneously measuring the expression level of 542 mitochondrial genes and has the potential to understand the mechanisms of mitochondrial dysfunction associated with a number of age-related debilitating diseases, including neurological disorders, cardiovascular disease, obesity, and type 2 diabetes, as well as various drug- and chemical-induced toxicities.

Varsha Desai, Ph.D.

Dr. Desai explores the utility of the *MitoChip* in various research initiatives at NCTR, and this approach has broadened the scope of her research interests. Recently she investigated the effect of short-term exposure to the anti-HIV drug, AZT, on expression level of mitochondrial genes in skeletal muscle of neonatal mice. The results of the study may have implications in clinical settings where children born to HIV-infected mothers are treated with AZT during the first few weeks of life. In another study, funded by the FDA Office of Women's Health (OWH), Dr. Desai is utilizing the *MitoChip* system to elucidate the role of mitochondria in the gender-based differences in the adverse effects of AZT in a mouse model. The mouse *MitoChip* has broad applications. It is also being used to determine, at the transcriptional level, the mechanism of 3-Nitropropionic acid (3-NPA)-induced mitochondrial dysfunction in specific regions of the brain. In addition, Dr. Desai is also collaborating with Dr. Greg Khitrov at Mount Sinai School of Medicine and Dr. Irwin Kurland at SUNY Stony Brook to assess the expression level of mitochondrial genes in fatty liver dystrophy lipin-deficient mouse and the Pten-deficient mouse models. Moreover, she has an ongoing collaboration with Dr. Joseph

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Garcia at the University of Texas Southwestern Medical Center at Dallas to evaluate gene expression patterns of mitochondrial genes in various tissues derived from HIF-1a and HIF-2a deficient mice.

Baiting Ning, Ph.D.

Dr. Baiting Ning is a research chemist in the Division of Personalized Medicine and Nutrition at the National Center for Toxicological Research (NCTR), Jefferson, Ark. In April 2007, Dr. Ning's project titled: *Mechanisms of gender difference in aspirin effects: metabolizing enzymes and therapeutic targets* was funded by the Office of Women's Health (OWH). Dr. Ning and co-authors, Drs. K. Barry Delclos, Beverly Lyn-Cook, Lei Guo, and James Chen's project hypothesizes "that the gender difference in therapeutic effects and adverse drug reactions associated with aspirin treatment is mainly determined by sex hormone modulation of aspirin-targeting enzymes. Furthermore, we hypothesize, that sex hormones modulate aspirin's actions."

Dr. Ning received his doctoral degree in biochemistry and molecular biology at the University of Arkansas for Medical Sciences, Little Rock, Ark. in 2000. His major research interest is functional genomics and genetics, by which he uses biochemical technologies to investigate inter-individual disparities in cancer susceptibilities and drug metabolisms.

Dr. Neera Gopee and co-authors, Drs. Beverly Lyn-Cook, William Tolleson, Tucker Patterson, Edward Treadwell, and James Chen were recently awarded funding from the OWH to conduct a study titled: *Sex differences in Systemic Lupus Erythematosus (SLE): Effects of a single nucleotide polymorphism (SNP) in the prolactin (PRL) gene on individual response to prasterone therapy*. There is currently no cure for SLE, a chronic and debilitating autoimmune disease, due to the multi-factorial etiology involved in its pathogenesis. They postulate that a more complete understanding of gene-drug relationships will improve efficacy of SLE treatment strategies by directing patients to therapeutic modalities tailored to their genetically determined characteristics. However, the absence of physiologic explanations for the sex and ethnic bias of SLE towards African-American female phenotype complicates the design of novel therapies.

They hypothesize that the sex differences observed in SLE and therapeutic response to prasterone is due in part to the presence of the PRL SNP. The study will evaluate the molecular basis for the sex differences and correlation between the responses of SLE patients to prasterone and their PRL genotype. If successful, results obtained from this study will demonstrate that PRL SNP may serve as a genetic biomarker to predict susceptibility of the female phenotype to SLE and response to prasterone. Improved efficacy of prasterone therapy, by tailoring its use to the patient's PRL genotype, will significantly decrease morbidity and improve the quality-of-life for women with the potential for expanded regulatory FDA use towards personalized medicine.

Dr. Gopee obtained her D.V.M. at the University of the West Indies, Trinidad, in 1998 and her Ph.D. in toxicology at

Neera Gopee, D.V.M., Ph.D.

the University of Georgia in 2002. She subsequently joined NCTR as an ORISE postdoctoral fellow and currently is a visiting scientist at the NCTR in the Division of Biochemical Toxicology, Jefferson, Ark. Her research interests also include investigating the immunology and photo- and dermal-toxicity of nanoscale titanium dioxide and quantum dots, tattoo pigments, and furocoumarins in lemon and lime oil.

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