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Chemoprevention by Grape Seed Extract and Genistein in Carcinogen-induced Mammary Cancer in Rats Is Diet Dependent^{1,2}

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ABSTRACT Many popular dietary supplements are enriched in polyphenols such as the soy isoflavones, tea catechins, and resveratrol (from grape skins), each of which has been shown to have chemopreventive activity in cellular models of cancer. The proanthocyanidins, which are oligomers of the catechins, are enriched in grape seeds and form the basis of the dietary supplement grape seed extract (GSE). Evidence suggests that the proanthocyanidins may be metabolized to the monomeric catechins. This study was carried out to determine whether GSE added to rodent diets protected against carcinogen-induced mammary tumorigenesis in rats and whether this was affected by the composition of the whole diet. Female rats were begun on 5%, 1.25%, or 0% (control) GSE-supplemented diets at age 35 d. At age 50 d they were administered 7,12-dimethylbenz[a]anthracene (DMBA) in sesame oil at 80 mg/kg body weight. They were weighed and monitored weekly for tumor development until 120 d after DMBA administration. Administration of GSE in AIN-76A diet did not show any protective activity of GSE against DMBA-induced breast cancer. However, administration of GSE in a laboratory dry food diet (Teklad 4% rodent diet) resulted in a 50% reduction in tumor multiplicity. In similar experiments, genistein administered in AIN-76A diet also failed to show chemopreventive activity against the carcinogen *N*-methyl-*N*-nitrosourea; however, when administered at the same dose in the Teklad 4% rodent diet, genistein exhibited significant chemopreventive activity (44–61%). These results demonstrate that GSE is chemopreventive in an animal model of breast cancer; moreover, the diet dependency of the chemopreventive activity for both GSE and genistein suggests that whether or not a compound is chemopreventive may depend on the diet in which the agent is administered. *J. Nutr.* 134: 3445S–3452S, 2004.

KEY WORDS: • cancer • DMBA • MNU • grapes • proanthocyanidins

Despite tremendous advances in medicine, cancer remains a major health concern in the United States and the rest of the

world (1). In many cases, cancer is a long drawn-out disease that emotionally drains both patient and family. Although enormous energies have been invested in treating existing cancer by chemotherapy (with some success in certain cancers), prevention of cancer is the preferred option. Because the incidence of cancer varies dramatically from country to country (2), and epidemiological studies show that ethnicity is at most a small part of the overall risk equation (3), much emphasis has been placed on understanding the environmental factors that influence cancer risk. Of these, diet is considered to have a major role. Epidemiological studies have suggested that certain dietary components are associated with lower cancer risk (4,5). These include vitamins as well as other phytochemicals, particularly polyphenols. Because phyto-

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chemicals are safe at levels found in the diet, they are of interest to those investigating the role of chemoprevention in lowering cancer risk.

Animal models of cancer have been used to investigate the chemopreventive potential of phytochemicals and related synthetic molecules, such as 13-*cis*-retinoic acid and *N*-(4-hydroxyphenyl)retinamide (6,7). However, the role of the diet in which a potential chemopreventive agent is administered has not been systematically addressed in studies using animal models of cancer. Indeed, in most animal studies the administered chemicals are mixed into diets with little consideration of whether the composition of the diet was a determining factor in the experimental outcome. Paradoxically, the American public places great emphasis on the quality and composition of the diet, particularly with regard to the prevention of chronic disease. This diet consciousness is reflected in the advocacy by both the National Institutes of Health and the National Cancer Institute for diets containing at least 5 daily servings of fruits and vegetables, both rich sources of phytochemicals (8). In the mid 1980s concerns about variation in the laboratory diet negatively affecting the efficacy of potential chemopreventive agents in cancer research led to the use of AIN-76A diet, a purified diet based on casein as the sole source of protein, in place of laboratory dry food diets. The chemoprevention experiments that followed using AIN-76A indicated a notably higher incidence and numbers of tumors in *N*-methyl-*N*-nitrosourea (MNU)⁴-induced mammary cancer in rats (9). This led to the appreciation that the soy component of the rodent diet may have a chemopreventive effect (10). However, during studies to determine synergies between chemoprevention agents administered in the AIN-76A diet, certain well-described chemoprevention agents did not exhibit their predicted effects (C. J. Grubbs, University of Alabama at Birmingham, 1987, unpublished data). Inspection of previous data revealed that the experiments detecting the chemopreventive activities of these agents had been carried out using a laboratory dry food diet. These data provided a rationale for directly examining the role that the diet plays in chemoprevention experiments.

The diet contains many types of phytochemicals, including vitamins and other compounds that cannot be synthesized by humans or animals. Currently, the health benefits of vitamins are widely appreciated, but the benefits of other phytochemicals such as the polyphenols have yet to be clearly understood or accepted, in part because of the complexity of their actions. The polyphenols include a wide range of closely related compounds synthesized by plants; these include the flavonoids found in tea leaves (catechins), isoflavonoids in soybeans (genistein and daidzein), and stilbenes in red grapes (resveratrol). Each of these has been shown to have anticancer properties in cell culture models of cancer (11–13).

The original goal of this study was to evaluate the chemopreventive potential of grape seed extract (GSE), a preparation extracted from grape seeds that is highly enriched in the proanthocyanidins (Fig. 1). These complex polyphenols have *in vitro* antioxidant activity (14); inhibit aromatase enzyme activity (15); inhibit the growth of cancer cells in cell culture (16,17); and prevent or attenuate disease in various animal models of disease, including atherosclerosis (18), cataract formation (19), and skin cancer (20). Because GSE inhibits the growth of human breast cancer cells in culture (16,17), we

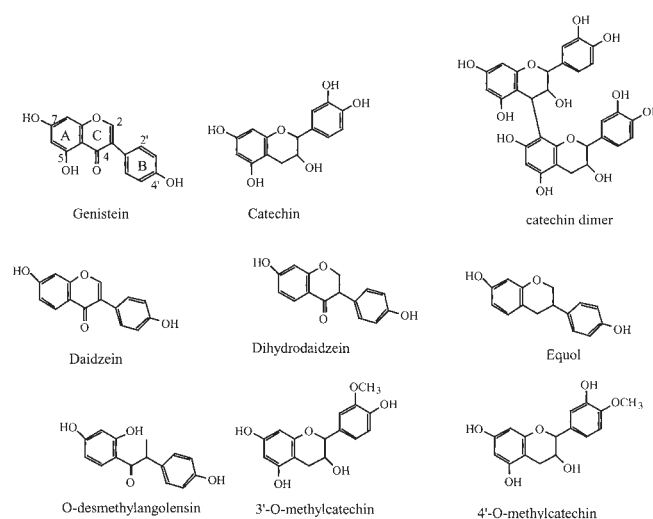


FIGURE 1 Chemical structures of polyphenols and their metabolites. Dihydrodaidzein, O-desmethylangolensin, and equol are the metabolites of the soy isoflavones daidzein and genistein, respectively. 3'- and 4'-O-Methylcatechins are urinary metabolites of GSE catechin dimers and higher oligomers.

hypothesized that GSE would have chemopreventive activity in an animal model of breast cancer. In an initial experiment, however, GSE, at up to 5% by weight administered in AIN-76A diet, did not prevent the incidence or number of mammary tumors induced by 7,12-dimethylbenz[*a*]anthracene (DMBA), an established carcinogen for the initiation of mammary tumors (21). Similar results were observed in an experiment with genistein, where up to 1 g/kg AIN-76A diet had no effect on MNU-induced mammary cancer. This led to the decision to reexamine the effect of these 2 polyphenol preparations when administered in a laboratory diet (4% rodent diet, Teklad Industries).

MATERIALS AND METHODS

Materials

DMBA and *Helix pomatia* β -glucuronidase/sulfatase were obtained from Sigma-Aldrich Chemical. MNU was purchased from Ash Stevens. Daidzein was purchased from LC-Labs. Equol, dihydrodaidzein, and O-desmethylangolensin were provided by Dr. Kristiina Wahaala, University of Helsinki, Finland.

Grape seed extract preparation. A powdered GSE preparation was provided by Kikkoman and stored at 4°C in light-tight containers until used. The composition of this preparation was previously described (14) to consist of >95% flavanols, of which 86% were proanthocyanidins.

Genistein. A concentrate enriched (40% by weight) in genistein, the β -glucoside of genistein, derived from soy molasses was prepared and generously donated by Protein Technologies International (now Solae). Genistein was further purified as follows (22). The concentrate was decolorized by boiling with activated charcoal. After volume reduction by evaporation, it was subjected to fractional crystallization from hot 60% aqueous ethanol. The purity of the final product was determined to be >98% genistein from its absorbance at 262 nm. It was then hydrolyzed by refluxing in methanol:1 mol/L HCl. Genistein was extracted into diethyl ether and after the solvent was removed, it was recrystallized from 60% aqueous ethanol. The final product was analyzed by reverse-phase HPLC and was found to contain >98% genistein.

Animals. Sprague-Dawley rats aged 21 d were purchased from Charles River and fed an AIN-76A diet (Teklad Industries). For the second round of experiments, they were fed a 4% rodent diet (Teklad

⁴ Abbreviations used: DMBA, 7,12-dimethylbenz[*a*]anthracene; GSE, grape seed extract; LC-ESI-MS, LC-electrospray ionization mass spectrometry; MNU, *N*-methyl-*N*-nitrosourea; MRM, multiple-reaction ion monitoring; TFA, trifluoroacetic acid.

Industries). They were housed in animal quarters at 22°C with a 12-h light/dark cycle. Animals were given free access to water and the powdered diets. These animal studies were approved by the UAB Institutional Animal Care and Use Committee.

GSE dose selection study. At age 35 d the rats were segregated into 5 groups receiving diet supplemented with 0%, 0.1%, 1%, 5%, or 10% GSE. The animals were maintained on these diets for 6 wk, after which they were killed by carbon dioxide asphyxiation and necropsied. Body weight gain was monitored weekly and organs were weighed at necropsy, snap frozen in liquid nitrogen, and archived at -80°C. No differences in body weights or organ weights were detected after 6 wk; this was confirmed during the subsequent chemoprevention study reported here. The maximum tolerated dose for GSE was determined to be 10% GSE in the diet. Although in several animal studies the GSE was administered at $\leq 1\%$, we decided to test as high a dose as the animals could tolerate, to maximize the chances of obtaining chemopreventive activity. Because there appeared to be a very slight reduction (although not statistically significant) in brain weight normalized to 100 g body weight at the 10% GSE dose, doses up to 5% but not 10% were chosen for the chemoprevention study.

A similar study was performed where genistein was added to the AIN-76A diet from age 25 d. No toxic effects were observed in a 6-wk study with doses up to 2.0 g/kg diet. This was the highest dose used in subsequent studies.

Chemoprevention studies. Female rats (20 per group) were given AIN-76A diet supplemented with either 0%, 1.25%, or 5% GSE, beginning at age 35 d. At age 50 d, DMBA (80 mg/kg body weight) in sesame oil was administered by gavage to all animals. Two additional groups of 5 animals each received AIN-76A alone or AIN-76A plus 5% GSE but did not receive DMBA. All animals were maintained in their dietary groups for 120 d after DMBA administration, during which time body weights were monitored and tumors were palpated and counted weekly. Two days before sacrifice, 5 animals selected randomly from each group were placed in metabolic cages, and urine was collected over 48 h into 50-mL plastic tubes sitting in crushed dry ice. Urine samples were collected at 24 and 48 h and stored at -80°C until analyzed. At 120 d post-DMBA, the animals were killed by carbon dioxide asphyxiation and final body and organ weights as well as mammary tumor numbers and sizes were recorded. For the second round of analysis, the same doses of GSE were administered in powdered 4% Teklad rodent diet; all other variables were the same as for the initial study where GSE was given in the AIN-76A diet.

In the genistein chemoprevention studies, for the first experiment female Sprague-Dawley rats were started on isoflavone-free AIN-76A diet at age 22 d. Diet supplementation with genistein (0, 0.8, and 1.6 g/kg diet) was started 3 d later in groups of 30 animals. At age 50 d, the direct-acting carcinogen MNU dissolved in acidified saline (pH 5) at 50 mg/kg body weight was administered intravenously into the jugular vein (23). In the second experiment, the animals were fed a 4% Teklad diet throughout the study. Supplementation with genistein (0, 0.2, and 2.0 g/kg diet) began on day 25. All animals were monitored weekly for overall health and their body weights; tumors were palpated and counted weekly. At the end of the experiment (108 d post-MNU), the animals were killed by carbon dioxide asphyxiation and final body and organ weights as well as mammary tumor incidence, number, and sizes were recorded. Tumors were histologically classified as adenocarcinomas or benign. Five random animals from each group were anesthetized with ether and blood (1–2 mL) was removed by cardiac puncture. After being allowed to clot for 1 h, the blood samples were centrifuged at $3000 \times g$ for 10 min and the serum was carefully removed and stored at -80°C until further analysis.

Analysis of isoflavones

Diet samples (0.5 g) were extracted with 10 volumes of ice-cold 80% aqueous methanol for 2 h. The ice-cold temperature prevented degradation of the isoflavone malonyl- β -glucosides into their corresponding β -glucosides, which occurs at room temperature or during extraction with boiling solvents (24). Dichlorofluorescein was added to the extraction solvent as an internal standard. The diet:solvent

mixtures were centrifuged at $3000 \times g$ for 10 min and samples (20 μ L) of the supernatant were analyzed by HPLC on a Hewlett Packard 1100 instrument (Agilent Technologies). Analyses were carried out on a 25 cm \times 4.6 mm i.d. Rainin C₈ reverse-phase column equilibrated with 10% aqueous acetonitrile in 0.1% (v/v) trifluoroacetic acid (TFA). Bound compounds were eluted by a linear 10–50% gradient of acetonitrile in 0.1% TFA over 30 min. The column was regenerated by washing with 90% aqueous acetonitrile–0.1% TFA and then reequilibrated with 10% aqueous acetonitrile–0.1% TFA. The mobile phase flow rate was 1.5 mL/min. Eluted compounds were detected by absorbance using a diode array detector. Quantitation was determined from their absorbances at 262 nm. All areas were corrected for that of the internal standard and were compared with those in a mixture of known isoflavone standards. Data were recorded in aglucone units as nmol/g of diet.

Serum and urine samples (2 \times 0.5 mL) obtained from genistein and GSE-treated animals were analyzed by LC-electrospray ionization mass spectrometry (LC-ESI-MS) (25,26). In brief, phenolphthalein glucuronide, 4-methylumbelliferyl sulfate, and apigenin were added as internal controls to each serum sample before hydrolysis with 400 U of β -glucuronidase and aryl sulfatase in 150 mmol/L ammonium acetate buffer, pH 5, for 16 h at 37°C. After acidification with glacial acetic acid, the samples were extracted with hexane to remove neutral lipids and then with diethyl ether to recover the isoflavonoid aglucones. For the samples from the GSE-treated animals, the metabolites were initially extracted with ethyl acetate instead of diethyl ether, then evaporated to dryness, and finally reconstituted in 80% aqueous methanol. Aliquots were analyzed by LC-ESI-MS using multiple-reaction ion monitoring (MRM) for specific detection of daidzein, equol, O-desmethylangolensin, dihydrodaidzein, genistein, catechin, and methylcatechins. Analyses were performed using a Shimadzu SIL-HT gradient HPLC and a Sciex API III triple quadrupole mass spectrometer. Chromatography was carried out on a 10 cm \times 2.1 mm i.d. C₈ reverse-phase column under isocratic conditions using a 35% aqueous acetonitrile in 10 mmol/L ammonium acetate at a flow rate of 0.4 mL/min. The eluate was split so that 100 μ L/min was diverted to the Ionspray™ interface operating at -2700 V. MRM experiments for catechin and its metabolites were performed in the positive ion mode. Selected molecular ions were passed to a collision cell containing Ar/N₂ (90:10) and fragment ions detected in the third quadrupole. Isoflavonoids were detected using MRM with the following precursor ion/product ion transitions: daidzein (m/z 253/223), dihydrodaidzein (m/z 255/149), O-desmethylangolensin (m/z 257/108), equol (m/z 241/119), genistein (m/z 269/133), apigenin (m/z 269/149), phenolphthalein (m/z 317/93), and 4-methylumbelliferone (m/z 175/125). Similarly, MRM analysis was conducted by monitoring transitions from precursor ion to product ion—291/139 (catechin), 291/123 (catechin), 305/139 (methylcatechin), and 305/137 (methylcatechin). Areas under the peaks were determined using the MacQuan program provided by Sciex. They were normalized to the area of the internal standard apigenin and also to areas for known standards prepared fresh for each analysis. Mean concentrations and standard deviations were calculated for duplicate aliquots of each sample. The limits of detection for each isoflavonoid were 4–15 nmol/L starting with 500 μ L serum.

Statistical analysis

The significance of the effects of GSE and genistein in this study was evaluated using either Student's *t* test (2-way comparisons with *P* < 0.05 considered a significant difference) or Tukey's adjustment for multiple comparisons (27).

RESULTS

Dietary supplementation of GSE at doses up to 5% was well tolerated by adult female rats (Fig. 2A); there was no significant difference in body weight gain over the course of the chemoprevention study or in the final body weights at the end of the study among all dietary groups. Moreover, there were no significant differences in the weights per 100 g body weight for

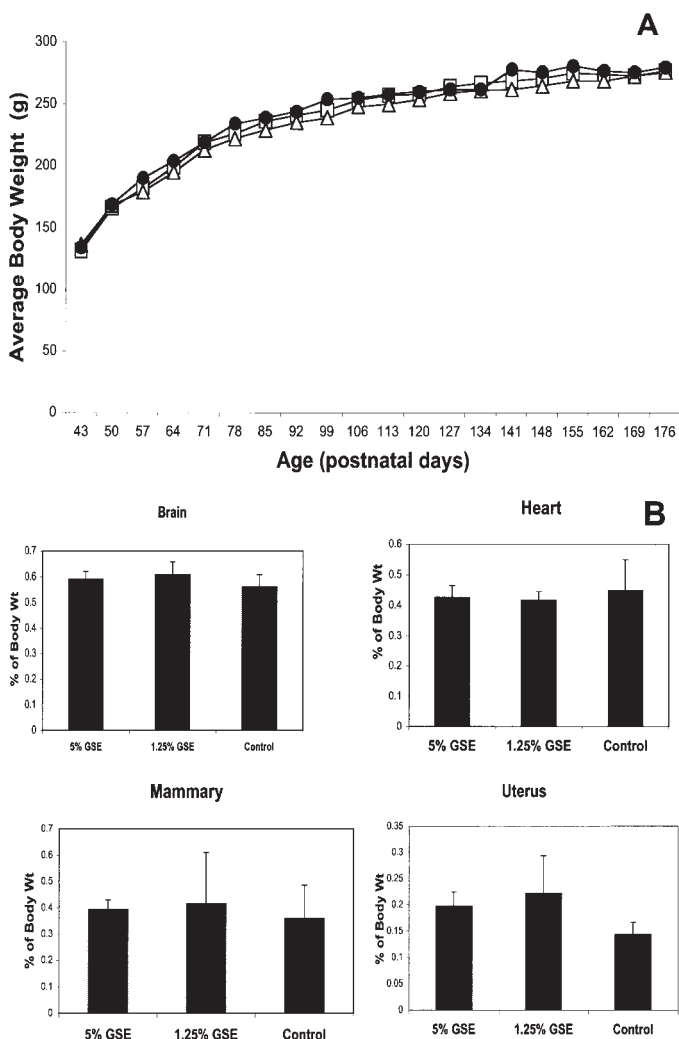


FIGURE 2 Effect of GSE on weight gain and organ weights in female rats. No significant difference in body weight gain was detected among the 3 groups (A); filled circles, DMBA and no GSE supplementation; open triangles, DMBA and 1.25% GSE; and open squares, DMBA and 5% GSE (B). At the end of the chemoprevention study, the animals were euthanized and organs were removed and weighed. Weights were normalized to 100 g body weight. Organ weights for animals on GSE and control diets did not differ.

brain, liver, heart, or uterus among the different dietary groups (Fig. 2B).

LC-ESI-MS analysis of the GSE preparation indicated that catechin and its oligomers (up to heptamers) were present in the starting material (data not shown). In the urine, however, only catechin and methylated derivatives of catechin (3'- and 4'-O-methylcatechins) were detected (Fig. 3). These were identified by product ion analysis and by comparison with published data (28). Higher oligomers of catechin were not detected under the conditions used in this analysis.

The chemopreventive efficacy of GSE against DMBA-induced mammary tumorigenesis was tested using 1.25% and 5% GSE supplementation. The GSE was initially administered in AIN-76A, a defined rodent diet where the 20% protein component is casein. This diet has been used extensively in animal experiments because of its defined composition. The 5% GSE had no effect on the multiplicity of DMBA-induced mammary tumors (Fig. 4). When GSE was administered in the 4% rodent diet, however, DMBA-induced mammary tumorigene-

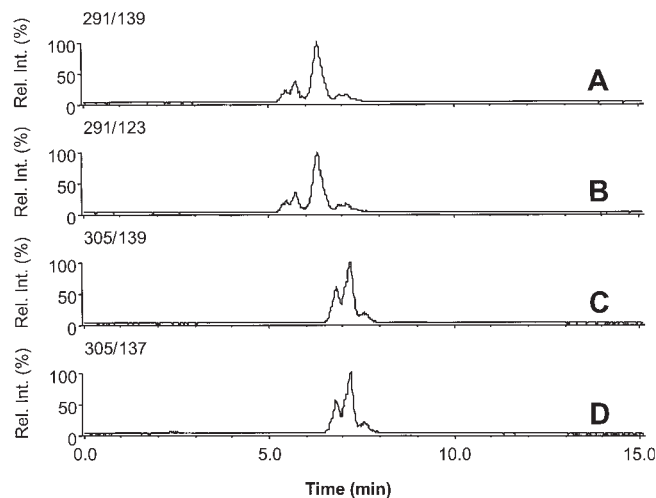


FIGURE 3 LC-MS-MRM ion chromatograms of urinary catechins from a rat given 5% GSE in AIN-76A diet. A and B are the chromatograms for precursor/product ion transitions for catechins; C and D are the chromatograms for the methylated catechin metabolites.

sis was significantly attenuated; the tumor multiplicity was 45% lower in the group that received 5% GSE relative to the group that did not receive GSE (Fig. 5). The 1.25% GSE supplementation did not have a detectable chemopreventive effect.

Similar experiments were carried out with genistein. Previous experiments showed that prepubertal exposure to genistein afforded significant chemoprotection against DMBA-induced mammary tumorigenesis in rats (29). An earlier study carried out in our laboratory showed that administering genistein in the AIN-76A diet to adult rats did not protect against MNU-induced mammary tumors (Fig. 6). A similar result was also reported in studies using the DMBA model of breast cancer (30). However, when the 4% Teklad

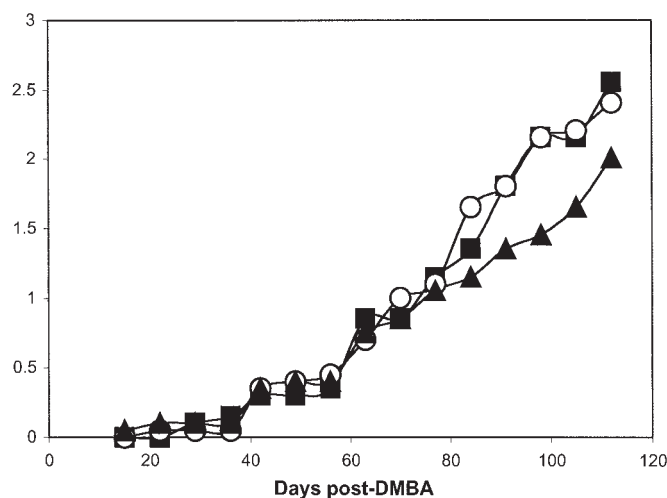


FIGURE 4 GSE supplementation in AIN-76A diet was not protective against mammary tumors induced by DMBA in Sprague-Dawley rats. Rats were begun on 0, 1.25%, or 5% GSE in AIN-76A diet on day 35 and given DMBA on day 50. They were palpated for tumors weekly for 4 mo post-DMBA. Closed triangles, no GSE; open circles, 1.25% GSE; and closed squares, 5% GSE. There was no statistical difference between the 3 curves.

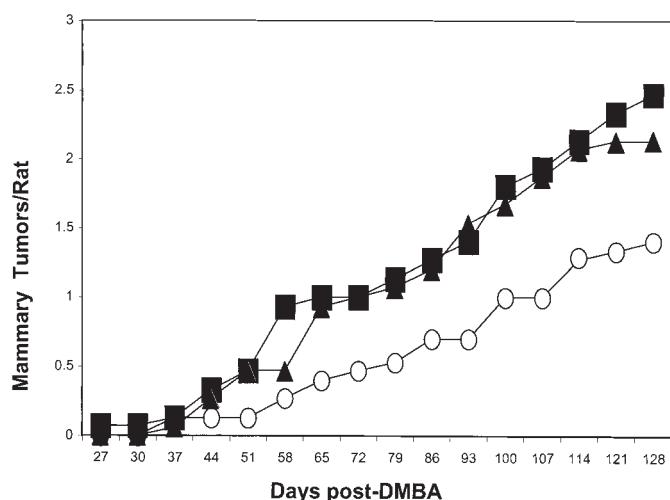


FIGURE 5 Chemopreventive activity of GSE administered in Teklad 4% diet against mammary tumors induced by DMBA in female rats. The doses of GSE were as in Figure 4. Animals that received 5% GSE had a 44% reduction in the number of mammary tumors (circles), relative to those that received no GSE (triangles). There was no significant protection against the tumors in the animals that received 1.25% GSE (squares).

diet was used instead of the AIN-76A diet, supplementation with 0.2 and 2.0 g/kg diet doses of genistein inhibited MNU-induced mammary adenocarcinomas by 44% and 61%, respectively (Fig. 7), in a 108-d study.

Because intact soy protein is part of the 4% Teklad diet and therefore can contribute isoflavones, it was important to ascertain the isoflavone composition of the diets used in this study. Reverse-phase HPLC analysis of the 4% Teklad diet

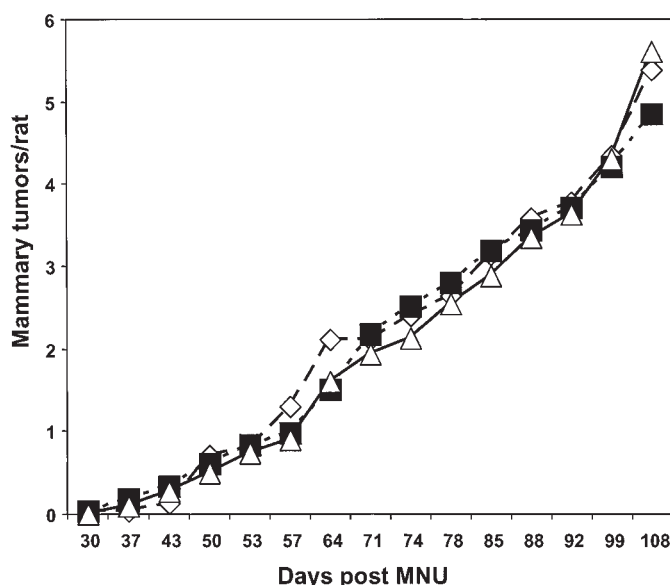


FIGURE 6 Effect of genistein in AIN-76A diet on the multiplicity of mammary tumors induced by MNU in female Sprague-Dawley rats. Rats were begun on 0, 0.8 g genistein or 1.6 g genistein per kg AIN-76A diet on day 25 and given MNU on day 50. They were palpated for tumors weekly for 108 d post-MNU. Open triangle, no genistein; open diamonds, 0.8 g/kg genistein; and closed squares, 1.6 g/kg diet genistein. There was no statistical difference between the 3 curves.

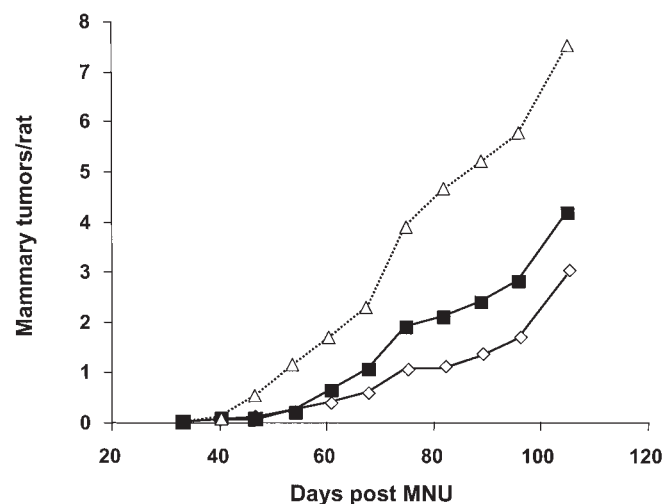


FIGURE 7 Chemopreventive action of genistein given in Teklad 4% diet against MNU-induced mammary tumors in female Sprague-Dawley rats. When genistein was administered in 4% Teklad diet at either 0.2 or 2.0 g/kg diet, the animals that received genistein exhibited lower tumor multiplicity; the animals that received 0.2 g/kg diet genistein (squares) had 44% fewer tumors, and those that received 2.0 g/kg diet genistein (diamonds) had 61% fewer tumors per animal than those that received no genistein (triangles).

revealed that it contained 429 $\mu\text{g/g}$ diet of isoflavones (expressed in aglycone units), the majority being 6''-O-malonylglucoside, 6''-O-acetylglucoside, and β -glucoside conjugates of daidzein and genistein) (Table 1). Unconjugated genistein was low (16 $\mu\text{g/g}$ diet). Supplementation with genistein did not significantly alter the concentrations of any of the other isoflavones in the diet; however, the mean total genistein content rose from 200 $\mu\text{g/g}$ in the unsupplemented diet to 420 $\mu\text{g/g}$ in the 0.2 g/kg supplemented diet and to 2315 $\mu\text{g/g}$ in the 2.0 g/kg supplemented diet.

The isoflavone composition of blood taken at the time of

TABLE 1

Isoflavone content in 4% Teklad diets (control and genistein-supplemented)¹*

Isoflavone	Control	2000 mg/kg	
		200 mg/kg	2000 mg/kg
$\mu\text{g/g}$			
Dz-Glc ²	89 \pm 2	94 \pm 1	92 \pm 6
Dz-GlcMal	62 \pm 3	65 \pm 2	65 \pm 4
Dz-GlcAc	50 \pm 1	51 \pm 1	50 \pm 3
Dz	nd	nd	nd
Gly-Glc	12 \pm 1	15 \pm 1	14 \pm 1
Gly-GlcMal	9 \pm 1	10 \pm 1	10 \pm 1
Gly-GlcAc	7 \pm 1	8 \pm 1	7 \pm 1
Gly	nd	nd	nd
Gen-Glc	92 \pm 1	95 \pm 1	95 \pm 7
Gen-GlcMal	68 \pm 1	70 \pm 1	69 \pm 5
Gen-GlcAc	24 \pm 1	25 \pm 1	25 \pm 2
Gen	16 \pm 1	230 \pm 18	2126 \pm 113

1 *Expressed as the aglucone equivalent. Values are means \pm SD.

2 Dz-Glc, daidzin; Dz-GlcMal, 6''-O-malonyldaidzin; Dz-GlcAc, 6''-O-acetyldaidzin; Dz, daidzein; Gly-Glc, glycitin; Gly-GlcMal, 6''-O-malonylglycitin; Gly-GlcAc, 6''-O-acetylglycitin; Gly, glycitein; Gen-Glc, genistin; Gen-GlcMal, 6''-O-malonylgenistin; Gen-GlcAc, 6''-O-acetylgenistin; Gen, genistein; nd = not determined.

TABLE 2

Serum isoflavone concentrations at the time of necropsy in rats on 4% Teklad diet supplemented with genistein^{1,2}

Isoflavone	Control	200 mg/kg	2000 mg/kg
	<i>nmol/L</i>		
Equol	2730 ± 283	2910 ± 171	3459 ± 740
Daidzein	307 ± 60	514 ± 165	401 ± 80
Dihydrodaidzein	21 ± 9	43 ± 5	76 ± 13**
O-Desmethylangolensin	64 ± 4	96 ± 7*	200 ± 77
Daidzein ± metabolites	3122 ± 301	3562 ± 275	4136 ± 834
Genistein	372 ± 88	1182 ± 445	5352 ± 1126**
Genistein/daidzein	0.12 ± 0.02	0.31 ± 0.09	1.38 ± 0.20**

¹ Values are means ± SEM, *n* = 5.

² **P* < 0.05; ***P* < 0.02.

euthanasia was determined by LC-ESI-MS (Table 2). In animals on the unsupplemented 4% rodent diet, serum total daidzein and genistein concentrations were 307 ± 60 nmol/L and 372 ± 88 nmol/L (mean ± SEM, *n* = 5), respectively. The concentration of the daidzein metabolite equol was strikingly higher (2730 ± 283 nmol/L, mean ± SEM, *n* = 5); other daidzein metabolites were present in lower concentrations. The addition of genistein to the 4% Teklad diet led to significant increases in the serum genistein concentration but did not affect the concentrations of daidzein and its metabolites (Fig. 8). Concentrations of the isoflavones and their metabolites in their unconjugated forms ranged from 2 to 4% (data not shown).

DISCUSSION

There are 2 central findings in the present study: chemopreventive activity is demonstrated for GSE in an established carcinogen-induced animal model of breast cancer, and the chemopreventive effects of both genistein and GSE in animal models of breast cancer are dependent on the background diet. Previous experiments showed that extracts of GSE inhibited growth of MDA-MB468 breast cancer cells in culture by a mechanism involving cell cycle arrest and ultimately differentiation and nonapoptotic cell death (16). However, results from the present study are the first to demonstrate a chemopreventive activity of GSE against breast cancer in vivo. The results obtained with GSE confirm that the proanthocyanidin fraction extracted from grape seeds has significant health benefits, as initially suggested by Masquelier et al. (31,32). It should be noted that Concord grape juice added to rat drinking water significantly attenuated tumor multiplicity in the DMBA rat model of breast cancer (33), suggesting that the monomeric anthocyanins enriched in grape juice have similar bioactivities as the proanthocyanidin-rich GSE. Because of the potentially high biomedical value of these bioactivities of the grape phenolic compounds, it is anticipated that future research will address the questions of mechanisms and identification of the bioactive entities from grape juice and seeds. An interesting experiment will be, for example, to determine whether administering both grape juice and GSE has nonadditive, additive, or synergistic chemopreventive actions in an animal model of breast or other cancer.

Topical application of a DMSO-soluble extract of proanthocyanidin-enriched GSE significantly protected against phorbol ester-induced tumor promotion in a mouse model of skin cancer (19), suggesting that anticancer cell growth activity resided in this oligomeric polyphenol fraction from grape

seeds. In addition, the GSE oligomers were suggested to be the active agents inhibiting growth of MDA-MB468 cells (16). However, the uptake of the oligomeric proanthocyanidins was not demonstrated in either study. Moreover, catechin and epicatechin were 10% of the GSE extract used in these experiments, raising the possibility that the inhibition of growth of the cells may have been due to the monomeric polyphenols. ESI-MS analysis in the present study revealed that whereas GSE contained oligomers (up to heptamers) of polyphenols, as well as monomeric catechins, only monomeric catechin-based polyphenols and their metabolites were detected in the urine (under the same extraction conditions) (Fig. 3). Follow-up experiments need to be carried out using proanthocyanidin preparations from grape seed depleted of monomeric catechins to confirm that the catechin in the urine from animals that had ingested GSE was generated by metabolism of the higher oligomers. Our initial ESI-MS results suggest that the proanthocyanidins in GSE are metabolized to catechins and catechin derivatives, and that these metabolites may be the chemopreventive agents. These results are consistent with 2 previous studies in rats in which ingested procyanidin dimers were not detected in the urine (34,35). Our findings are also consistent with those of Rice-Evans and co-workers (36), who found monomers but not higher oligomers of epicatechin in the brains of rats after GSE ingestion. However, uptake and metabolism of procyanidins may be different in humans because procyanidin B1 dimers were detected in serum after GSE intake (37); similarly, procyanidin B2 dimer was detected in plasma after consumption of flavanol-rich cocoa (38).

Recently, Eng et al. (15) found that pretreatment of athymic ovariectomized mice with a polyphenol preparation enriched in procyanidin-dimers purified from red wine inhibited proliferation of MCF-7 cells overexpressing aromatase. The suggestion that the procyanidin dimers inhibited the cells via inhibition of aromatase was supported by the dimers directly inhibiting the enzyme in vitro (15). The failure to detect procyanidin dimers in the animals in the present study, however, suggests that monomeric catechin metabolites of the proanthocyanidins may also inhibit aromatase or that other targets in addition to aromatase may be important. This is consistent with the observation that other monomeric flavonoids inhibit aromatase in vitro (39).

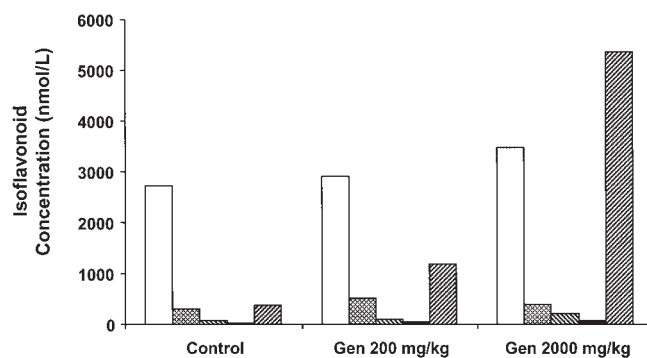


FIGURE 8 Serum levels of isoflavones in rats after ingestion of 4% Teklad diet supplemented with 0.2 and 2.0 g/kg diet genistein. Serum from rats that had ingested genistein-supplemented 4% Teklad diet was extracted and analyzed for isoflavones. The isoflavones and their metabolites were, from left to right, equol, daidzein, O-desmethylyangolensin, and dihydrodaidzein and genistein. Serum from rats that ingested genistein-supplemented diets (200 and 2000 mg/kg diet) contained 2–15 times as much genistein as serum from the rats on the unsupplemented 4% rodent diet (Control).

Earlier experiments showed that prepubertal exposure to genistein afforded significant protection against DMBA-induced mammary tumorigenesis (29). However, whereas administering genistein in AIN-76A to adult rats that had been bred and grown in a genistein-free environment did not protect against carcinogen-induced mammary tumors, exposure to genistein during neonatal and prepubertal development combined with exposure in adult life afforded greater protection against DMBA than did the neonatal and prepubertal exposure alone (30). The results presented here suggest that the genistein administered only to the adult rats might have had efficacy against the DMBA-induced breast cancer had it been administered under different dietary conditions. Most investigators obtain the animals for experimentation from commercial breeders and have no control of the diet during the critical early days of development; this could be an important source of variation in their experiments because prepubertal exposure of rats to genistein leads to increased mammary differentiation (29,40).

The mechanism by which dietary composition influences chemopreventive efficacy of GSE or genistein remains to be determined. The AIN-76A and 4% Teklad diets, although providing roughly the same amount of protein and same overall nutritional content, differ substantially in the complexity and source of their protein (milk casein for AIN-76A vs. a mixture of soy, wheat, and barley proteins for the Teklad lab diet) as well as in a number of other components. Therefore, it is not unreasonable that different bioactivities might be detected for the same chemical administered in the 2 diets.

The 4% Teklad diet contained ~0.4 mg/g diet isoflavones (Table 1). The animals on this diet, unsupplemented, were thus exposed to a basal level of isoflavones, as shown by a mean genistein serum concentration of 372 nmol/L. Switching from this diet to the isoflavone-free AIN-76A diet may have been the basis for the increased number of MNU-induced mammary tumors observed in previous animal studies (9). In the present study, the supplementation of the 4% Teklad diet

with genistein increased the serum genistein concentration to 1182 nmol/L for the 0.2 g/kg dietary dose and to 5327 nmol/L for the 2.0 g/kg dose. These increases were directly proportional to the dose of genistein (Fig. 9).

In addition to isoflavones, the 4% Teklad diet contains multiple other factors contributed by soy, wheat, and barley protein, not contained in AIN-76A (where milk casein is the only protein), that could have either enhanced the bioavailability of genistein and GSE components, allowing their chemopreventive action in adult rats, or otherwise contributed synergistically to their bioactivities. The purpose of this report is not to discuss the potential roles of multiple other phytochemicals in complex animal diets but to document our initial results, which suggest that dietary composition may be important in the bioactivity of chemicals administered in the diet.

In summary, we demonstrate that GSE is chemopreventive against DMBA-induced breast cancer in adult rats, that genistein exhibits similar activity in the MNU rat model of breast cancer, and that the effects of both grape seed and genistein depend on the diet in which they are administered. The diet dependency of the chemopreventive actions of grape seed and genistein suggests that previous studies where no chemopreventive activity was noted for a compound of interest may warrant retesting in a different diet to ensure that the sought bioactivity was not missed. The modulatory role of the diet in these rat studies suggests that clinical chemopreventive or chemotherapeutic interventions against breast or other cancers might need to consider the overall diet of the patient while such interventions are administered.

LITERATURE CITED

1. Arias, E., Anderson, R. N., Kung, H. C., Murphy, S. L. & Kochanek, K. D. (2003) Deaths: final data for 2001. *Natl. Vital Stat. Rep.* 52: 1-115.
2. Shibuya, K., Mathers, C. D., Boschi-Pinto, C., Lopez, A. D. & Murray, C. J. (2002) Global and regional estimates of cancer mortality and incidence by site: II. Results for the global burden of disease 2000. *BMC Cancer* 2: 37.
3. Maskarinec, G. (2000) Breast cancer-interaction between ethnicity and environment. *In Vivo* 14: 115-123.
4. Doll, R. (1980) The epidemiology of cancer. *Cancer* 45: 2475-2485.
5. Gerber, M. (2001) The comprehensive approach to diet: a critical review. *Nutrition* 131: 3051S-3055S.
6. Tallman, M. S. & Wiernik, P. H. (1992) Retinoids in cancer treatment. *J. Clin. Pharmacol.* 32: 868-888.
7. Moon, R. C., Thompson, H. J., Becci, P. J., Grubbs, C. J., Gander, R. J., Newton, D. L., Smith, J. M., Phillips, S. L., Henderson, W. R., Mullen, L. T., Brown, C. C. & Sporn, M. B. (1979) *N*-(4-Hydroxyphenyl)retinamide, a new retinoid for prevention of breast cancer in the rat. *Cancer Res.* 39: 1339-1346.
8. Eat 5 to 9 A Day for Better Health [Online]. National Cancer Institute. <http://www.5aday.gov/> [accessed Aug. 17, 2004].
9. Grubbs, C. J., Juliana, M. M., Hill, D. L. & Whitaker, L. M. (1987) Effect of laboratory diets on methylnitrosourea (MNU)-induced mammary cancers and on the efficacy of a chemopreventive agent (retinyl acetate). *Proc. Am. Assoc. Cancer Res.* 28: 160.
10. Barnes, S., Grubbs, C., Setchell, K.D.R. & Carlson, J. (1990) Soybeans inhibit mammary tumors in models of breast cancer. *Prog. Clin. Biol. Res.* 347: 239-253.
11. Park, O. J. & Surh, Y. J. (2004) Chemopreventive potential of epigallocatechin gallate and genistein: evidence from epidemiological and laboratory studies. *Toxicol. Lett.* 150: 43-56.
12. Barnes, S. & Peterson, T. G. (1995) Biochemical targets of the isoflavone genistein in tumor cell lines. *Proc. Soc. Exp. Biol. Med.* 208: 103-108.
13. Mgbonyebi, O. P., Russo, J. & Russo, I. H. (1998) Antiproliferative effect of synthetic resveratrol on human breast epithelial cells. *Int. J. Oncol.* 12: 865-869.
14. Yamaguchi, F., Yoshimura, Y., Nakazawa, H. & Ariga, T. (1999) Free radical scavenging activity of grape seed extract and antioxidants by electron spin resonance spectrometry in an H₂O₂/NaOH/DMSO system. *J. Agric. Food Chem.* 47: 2544-2548.
15. Eng, E. T., Ye, J., Williams, D., Phung, S., Moore, R. E., Young, M. K., Gruntmanis, U., Braunstein, G. & Chen, S. (2003) Suppression of estrogen biosynthesis by procyanidin dimers in red wine and grape seeds. *Cancer Res.* 63: 8516-8522.
16. Agarwal, C., Sharma, Y., Zhao, J. & Agarwal, R. (2000) A polyphenolic fraction from grape seeds causes irreversible growth inhibition of breast carcinoma

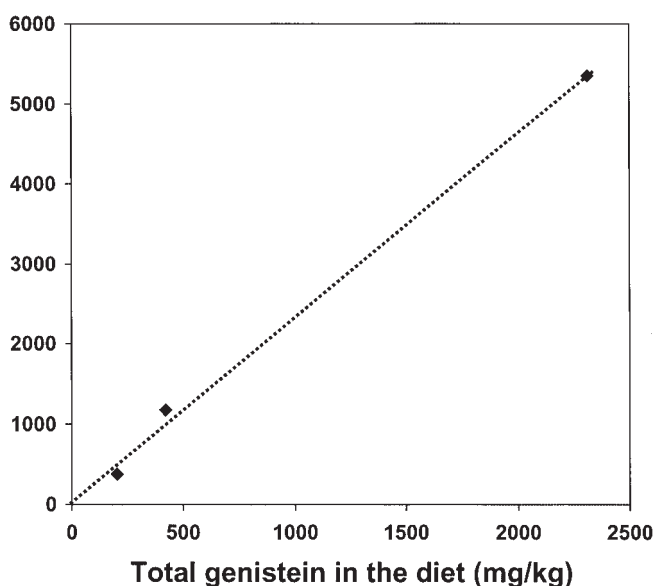


FIGURE 9 Linear relationship between serum concentrations of genistein and levels in the starting diet. The concentrations of genistein in the serum (Table 2) were plotted against total genistein doses in the diet (Table 1). At dietary doses up to 2500 mg/kg genistein, the serum concentration of genistein increased linearly in proportion to the levels in the diet.

noma MDA-MB468 cells by inhibiting mitogen-activated protein kinases activation and inducing G1 arrest and differentiation. *Clin. Cancer Res.* 6: 2921–2930.

17. Sharma, G., Tyagi, A. K., Singh, R. P., Chan, D. C. & Agarwal, R. (2004) Synergistic anti-cancer effects of grape seed extract and conventional cytotoxic agent doxorubicin against human breast carcinoma cells. *Breast Cancer Res. Treat.* 85: 1–12.

18. Zhao, J., Wang, J., Chen, Y. & Agarwal, R. (1999) Anti-tumor-promoting activity of a polyphenolic fraction isolated from grape seeds in the mouse skin two-stage initiation-promotion protocol and identification of procyanidin B5-3'-gallate as the most effective antioxidant constituent. *Carcinogenesis* 20: 1737–1745.

19. Yamakoshi, J., Saito, M., Kataoka, S. & Tokutake, S. (2002) Procyanidin-rich extract from grape seeds prevents cataract formation in hereditary cataractous (ICR/f) rats. *J. Agric. Food Chemistry* 50: 4983–4988.

20. Bomser, J. A., Singletary, K. W., Wallig, M. A. & Smith, M.A.L. (1999) Inhibition of TPA-induced tumor promotion in CD-1 mouse epidermis by a polyphenolic fraction from grape seeds. *Clin. Cancer Res.* 135: 151–157.

21. Huggins, C., Moon, R. C. & Morii, S. (1962) Extinction of experimental mammary cancer. I. Estradiol-17 beta and progesterone. *Proc. Natl. Acad. Sci. U.S.A.* 48: 379–386.

22. Peterson, T. G. & Barnes, S. (1991) Genistein inhibition of the growth of human breast cancer cells: independence from estrogen receptors and the multi-drug resistance gene. *Biochem. Biophys. Res. Commun.* 179: 661–667.

23. Moon, R. C., Grubbs, C. J., Sporn, M. B. & Goodman, D. G. (1977) Retinyl acetate inhibits mammary carcinogenesis induced by *N*-methyl-*N*-nitrosourea. *Nature* 267: 620–621.

24. Coward, L., Smith, M., Kirk, M. & Barnes, S. (1998) Chemical modification of isoflavones in soy foods during cooking and processing. *Am. J. Clin. Nutr.* 68: 1486S–1491S.

25. Coward, L., Kirk, M., Albin, N. & Barnes, S. (1996) Analysis of plasma isoflavones by reversed-phase HPLC-multiple reaction ion monitoring-mass spectrometry. *Clin. Chim. Acta* 247: 121–142.

26. Urban, D., Irwin, W., Kirk, M., Markiewicz, M. A., Myers, R., Smith, M., Weiss, H., Grizzle, W. E. & Barnes, S. (2001) The effect of isolated soy protein on plasma biomarkers in elderly men with elevated serum prostate specific antigen. *J. Urol.* 165: 294–300.

27. Tukey, J. (1991) The philosophy of multiple comparisons. *Stat. Sci.* 6: 100–116.

28. Cren-Olive, C., Deprez, S., Lebrun, S., Coddeville, B. & Rolando, C.

(2000) Characterization of methylation site of monomethylflavan-3-ols by liquid chromatography/electrospray ionization tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* 14: 2312–2319.

29. Fritz, W. A., Coward, L., Wang, J. & Lamartiniere, C. A. (1998) Dietary genistein: perinatal mammary cancer prevention, bioavailability and toxicity testing in the rat. *Carcinogenesis* 19: 2151–2158.

30. Lamartiniere, C. A., Cotroneo, M. S., Fritz, W. A., Wang, J., Mentor-Marcel, R. & Elgavish, A. (2002) Genistein chemoprevention: timing and mechanisms of action in murine mammary and prostate. *J. Nutr.* 132: 552S–558S.

31. Masquelier, J., Michaud, J., Laparra, J. & Dumon, M. C. (1979) Flavonoids et pycnogenols. *Intl. J. Vitamin Nutritional Research* 49: 307–311.

32. Murray, M. & Pizzorno, J. (1999) Procyanidolic oligomers. In: *The Textbook of Natural Medicine*, 2nd ed. (Murray, M. & Pizzorno, J., eds.), pp. 899–904. Churchill Livingstone, London, UK.

33. Singletary, K. W., Stansbury, M. J., Giusti, M., Van Breemen, R. B., Wallig, M. & Rimando, A. (2003) Inhibition of rat mammary tumorigenesis by Concord grape juice constituents. *J. Agric. Food Chem.* 51: 7280–7286.

34. Nakamura, Y. & Tonogai, Y. (2003) Metabolism of grape seed polyphenol in the rat. *J. Agric. Food Chem.* 51: 7215–7225.

35. Donovan, J. L., Manach, C., Rios, L., Morand, C., Scalbert, A. & Remesy, C. (2002) Procyanidins are not bioavailable in rats fed a single meal containing a grape seed extract or the procyanidin dimer B3. *Br. J. Nutr.* 87: 299–306.

36. Abd El Mohsen, M. M., Kuhnle, G., Rechner, A. R., Schroeter, H., Rose, S., Jenner, P. & Rice-Evans, C. A. (2002) Uptake and metabolism of epicatechin and its access to the brain after oral ingestion. *Free Rad. Biol. Med.* 33: 1693–702.

37. Sano, A., Yamakoshi, J., Tokutake, S., Tobe, K., Kubota, Y. & Kikuchi, M. (2003) Procyanidin B1 is detected in human serum after intake of proanthocyanidin-rich grape seed extract. *Biosci. Biotechnol. Biochem.* 67: 1140–1143.

38. Holt, R. R., Lazarus, S. A., Sullards, M. C., Zhu, Q. Y., Schramm, D. D., Hammerstone, J. F., Fraga, C. G., Schmitz, H. H. & Keen, C. L. (2002) Procyanidin dimer B2 [epicatechin-(4beta-8)-epicatechin] in human plasma after the consumption of a flavanol-rich cocoa. *Am. J. Clin. Nutr.* 76: 798–804.

39. Pelissero, C., Lenczowski, M. J., Chinzi, D., Davail-Cuisset, B., Sumpter, J. P. & Fostier, A. (1996) Effects of flavonoids on aromatase activity, an *in vitro* study. *J. Steroid Biochem. Mol. Biol.* 57: 215–223.

40. Cotroneo, M. S., Wang, J., Fritz, W. A., Eltoun, I. E. & Lamartiniere, C. A. (2002) Genistein action in the prepubertal mammary gland in a chemoprevention model. *Carcinogenesis* 23: 1467–1474.