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Soy Processing Affects Metabolism and Disposition of Dietary Isoflavones in Ovariectomized Balb/c Mice

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Soy foods and nutritional supplements are widely consumed for potential health benefits. It was previously shown that isoflavone-supplemented diets, which contained equal genistein equivalents, differentially stimulated mammary tumor growth in athymic mice based on the degree of processing. This paper reports plasma pharmacokinetic analysis and metabolite identification using the parental mouse strain fed the same diets, which contained genistin, mixed isoflavones, Novasoy, soy molasses, or soy flour plus mixed isoflavones. Whereas the degree of soy processing did affect several parameters reflecting isoflavone bioavailability and gut microflora metabolism of daidzein to equol, stimulation of tumor growth correlated significantly with only the plasma concentration of aglycon genistein produced by the diets. This conclusion is consistent with the known estrogen agonist activity of genistein aglycon on mammary tumor growth. Conversely, plasma equol concentration was inversely correlated with the degree of soy processing. Although antagonism of genistein-stimulated tumor growth by equol could explain this result, the very low concentration of aglycon equol in plasma (12-fold lower relative to genistein) is inconsistent with any effect. These findings underscore the importance of food processing, which can remove non-nutritive components from soy, on the pharmacokinetics and pharmacodynamics of isoflavones. Such changes in diet composition affect circulating, and presumably target tissue, concentrations of genistein aglycon, which initiates estrogen receptor-mediated processes required for the stimulation of tumor growth in a mouse model for postmenopausal breast cancer.

KEYWORDS: Soy; isoflavone; genistein; equol; pharmacokinetics; mass spectrometry

INTRODUCTION

Soybeans and soy products are rich sources of isoflavones that are widely consumed for potential health benefits (*1*). The two main isoflavones, genistein and daidzein, are present in soy primarily as β-D-glycosides, genistin and daidzin (*2*). After ingestion, glycosidic bonds are hydrolyzed by glucosidases of intestinal bacteria and in the intestinal wall to produce aglycons (*3*−*6*). Aglycons are further metabolized to glucuronide conjugates in the intestine and liver (*7*). Intestinal microflora present in rodents, monkeys, and 30−40% of humans convert daidzein to equol, and associations between beneficial outcomes from soy consumption and equol formation in women have been suggested (*8*). However, in general, epidemiological associations between isoflavone ingestion based on food frequency forms and beneficial health effects have often had mixed results, suggesting that associations may be strengthened by including other factors, particularly by including analytical measurements of isoflavones and metabolites (*9*).

We recently described the effect of processing soy-based products on the stimulation of implanted estrogen-dependent human breast cancer cells (MCF-7) in an ovariectomized athymic Balb/c mouse model for postmenopausal breast cancer (*10*). This preclinical model has previously been used to demonstrate the role of dietary genistein in the stimulation of tumor growth at blood levels that are relevant to human exposures from soy foods and dietary supplements (*3*, *10*−*12*). In Allred et al. (*10*) different diets containing a constant amount of genistein equivalents (750 mg/kg of diet) were formulated by supplementation with the following isoflavone products prepared by successive removal of non-isoflavone components through processing. The products investigated included soy flour supplemented with mixed isoflavones to reach the target concentration of 750 ppm (SF); soy molasses, an alcohol extract

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of SF (MOL); Novasoy, a product containing 40% isoflavones made from MOL by the removal of carbohydrates; a mixture of isoflavones produced by crystallization of Novasoy (MI); and purified genistin produced by further crystallization (GIN). Despite identical total genistein content in each diet, the diets containing more processed products produced increased stimulation of estrogen-dependent breast tumor growth in athymic mice (i.e., more processed isoflavone components stimulated tumor growth more). These findings suggested that consumption of whole soy-derived foods, as opposed to purified isoflavone dietary supplements, could have lower risks for stimulating estrogen-dependent breast tumors in postmenopausal women. Although the previous study correlated a number of indices for tumor cell proliferation and estrogen-dependent gene expression with tumor growth, the mechanism for different responses among diets is still unclear.

The present paper extends these findings by determining the changes in metabolism and disposition of isoflavones that result from the consumption of diets that contain different soy components. These results suggest that the degree of processing can affect phase II metabolism of isoflavones, bioavailability, and gut microflora metabolism of daidzein to equol and that such factors are likely to be important determinants of biological effects caused by both isoflavones and non-isoflavone soy components.

MATERIALS AND METHODS

Reagents. Archer Daniels Midland Co. (Decatur, IL) provided all of the processed soy isoflavone preparations used to formulate the diets, which were prepared as described previously (*10*). All of the products used in this study were prepared by Archers Daniel Midland Co. from soy flour, which is made when soybeans are successively cracked, dehulled, extracted with hexane to remove fats, heated to remove the residual solvent, toasted, and ground (*13*). Soy molasses is the soluble material produced by washing soy flour with 60% aqueous ethanol followed by condensation to remove residual solvent (*13*). Novasoy is a commercially available product containing ~40% isoflavones that is isolated from soy molasses by the removal of carbohydrates through a proprietary process. Novasoy was further processed by recrystallization from ethanolic solution to a mixed isoflavone product that is not currently available commercially. Similarly, the purified genistin, which is also not currently available commercially, was produced by further recrystallization of the mixed isoflavones from ethanolic solution. Isoflavones were quantified in each product by HPLC-UV at the Archer Daniels Midland Co. Sigma Chemical Co. (St. Louis, MO) was the source for all enzymes and buffers used, Cambridge Isotope Laboratories (Andover, MA) supplied the deuterated genistein and daidzein, and d_4-equol was generously provided by Dr. Kristiina Wahala, University of Helsinki. The enzymes used were almond β-glucosidase (G-0395, 12.4 units/mg), *Helix pomatia* glucuronidase/sulfatase (S3009, 23 units/mg), *Aerobacter aerogenes* sulfatase (S1629, 5 units/mg), and *Escherichia coli* glucuronidase (G8396, 5000 units/mg).

Diet Formulation. American Institute of Nutrition 93 growth (AIN 93G) semipurified diet, with corn oil substituted for soy oil, was selected as a basal diet for control animals because it has been established as meeting all of the nutritional requirements of mice (*14*). Although the soy flour and molasses contained macronutrients, each was added to an AIN 93G diet without affecting total fat, total carbohydrates, total protein, and total caloric content. All other products were also added to the AIN 93G diet, but they provided no nutritive value. All diets meet the nutritional requirements of the mouse. The soy products were added to each diet to provide 750 mg of genistein equiv/kg of diet (**Table 1**), a dose that is sufficient to stimulate growth of implanted MCF-7 tumors in vivo (*11*). When the maximum amount of SF was added, this diet contained only 433 mg/kg genistein equiv; therefore, SF was supplemented with the MI such that the final concentration of genistein was 750 mg/kg in the SF + MI diet. Daidzein content in the diets was not specifically controlled but was nearly identical for SF + MI, MOL, and NS diets, ~30% lower in the MI diet, and nearly absent from the GIN diet (85% lower, **Table 1**).

Table 1. Isoflavone Analysis of Various Treatment Diets[a]

	diet[b]				
	SF + MI	MOL	NS	MI	GIN
Isoflavones (Milligrams per Kilogram of Diet)					
genistein	9.7	9.3	15.7	8.3	1.5
genistin	750.3	1015.1	1054.3	1148.0	1178.7
malonylgenistein	299.3	62.4	7.0	3.0	4.0
acetylgenistin	196.2	120.3	118.8	26.5	8.4
daidzein	10.8	9.1	20.2	5.6	0.0
daidzin	502.3	804.4	798.8	619.9	134.4
malonyldaidzein	319.5	68.8	20.9	0.0	0.0
acetyldaidzin	170.6	115.4	120.9	30.8	0.0
glycitein	3.3	4.9	16.7	2.6	0.0
glycitin	112.1	171.2	244.3	88.7	6.0
malonylglycitein	82.0	23.0	16.1	0.0	0.0
acetylglycitin	21.8	11.1	11.5	2.7	0.0
Total Aglycon Equivalents (Milligrams per Kilogram of Diet)					
daidzein	574	599	586	402	82
genistein	750	750	751	748	751

[a] Isoflavone profiles were determined by the manufacturer, Archer Daniels Midland Co. [b] GIN, genistin; MI, mixed isoflavones; MOL, soy molasses; NS, Novasoy; SF, soy flour.

Animal Study Design. Ovariectomized female Balb/c mice were purchased from Harlan Sprague−Dawley (Indianapolis, IN) and delivered at 35 days of age. Animals were housed individually and kept on a reverse light cycle (12 h dark/12 h light). All experiments were approved by the Institutional Animal Care and Use Committee of the University of Illinois. After a 7-day adjustment period, mice were randomly assigned to one of six treatment groups. The treatment groups were (a) control (NC), (b) soy flour + mixed isoflavones (SF + MI), (c) molasses (MOL), (d) Novasoy (NS), (e) mixed isoflavones (MI), and (f) genistin (GIN). Control animals were fed the basal AIN 93G diet. The remaining animals were put on one of the six dietary treatments that were individually formulated and balanced for energy and GIN concentration (~750 mg/kg aglycon equiv). Mice were acclimated to a meal feeding regimen over a 10-day period prior to the day in which plasma samples were collected. During this time, mice were given bowls of individual treatment diets for two 1-h periods each day. The first was at 8:00 a.m., the beginning of the dark cycle in the animal facility; the second was at 8:00 p.m., the end of the dark cycle.

Each treatment group consisted of 42 mice (i.e., 6 mice in each of 7 time points). On the day of sample collection, diets were placed in cages, and after 30 min, the timer was restarted to define subsequent blood collection times. Mice were sedated, and blood samples were collected by cardiac puncture at 0.5, 1, 2, 4, 8, 12, and 24 h after completion of the 30-min feeding period. Food intake for individual mice was determined by measuring the amount of diet in each bowl, and spillage from the bowls was collected to ensure accurate consumption values. Ten mice were fed the control diet (AIN 93G) to determine if there were differences in food intake between the treatments and control diet. No significant differences in food consumption were observed by comparison with the control diet group using the two-tailed *t* test (data not shown). All mice used for the 24-h plasma samples were fed basal AIN 93G diet at 8:00 p.m. as their second meal. Body weights were measured for all animals prior to plasma collection.

Quantification of Plasma Isoflavones. Plasma concentrations of total isoflavones were determined after complete enzymatic hydrolysis using a *H. pomatia* preparation containing glucuronidase, sulfatase, and β-glucosidase activities using a previously validated LC-ES/MS/MS method (*15*). The *H. pomatia* glucuronidase/sulfatase preparation contained significant β-glucosidase activity using genistin as a substrate and was found to hydrolyze the β-glucoside even more rapdily than genistein glucuronides. Measurements of isoflavone aglycon concentration in plasma were done without enzymatic treatment. Isoflavone glucuronides and sulfates were quantified after selective hydrolysis to the respective aglycon using 2 units of either purified sulfatase or

glucuronidase preparations as previously described (*16*). Isoflavone glucoside conjugates were similarly quantified following treatment with β-glucosidase (10 units). The β-glucosidase preparation contained small amounts of glucuronidase activity (~2% of the *H. pomatia* enzyme) using phenolphthalein glucuronide as a substrate, so glucoside conjugate determinations may be slightly overestimated. The limit of quantification (LOQ) from analysis of 10 μL of plasma was 0.005 μM for genistein, daidzein, and equol. The precision of isoflavone measurements (relative standard deviation) was in the range of 3−13% across the concentration ranges previously reported, and the corresponding accuracies were 88−99% (*15*). The glucuronidase activity was verified using phenolphthalein glucuronide as an alternate substrate (*16*). Quality control procedures included concurrent analysis of isoflavone-fortified rat serum, blank rat serum, and a mixture of labeled and unlabeled standards interspersed throughout each sample set.

Liquid Chromatography (LC). LC separations were performed using a 2795 liquid handling system (Waters, Milford, MA). Chromatography was performed using an Ultracarb ODS column (either 2 × 30 or 2 × 150 mm, 3-μm particle size, Phenomenex, Torrance, CA) with isocratic elution for the aglycons (65% of aqueous 0.1% formic acid/35% of acetonitrile). The flow rate for all analyses was 0.3 mL/min, and the entire effluent was introduced into the electrospray (ES) probe.

Mass Spectrometry. A Quattro Ultima triple-quadrupole mass spectrometer (Waters, Manchester, U.K.) equipped with an ES interface was used with an ion source temperature of 150 °C. The method, which was previously validated (*14*), used a collision energy of 25 eV for the $(M + H)^+$ transitions and monitored MRM transitions for d0/d4 geinstein (*m*/*z* 271 → 215 and 275 → 219, respectively), d0/d3 daidzein (*m*/*z* 255 → 199 and 258 → 202, respectively), and d0/d4 equol (*m*/*z* 243 → 133 and 247 → 135, respectively) using a dwell time of 0.3 s, sampling cone-skimmer potential of 25−60 V, Ar gas cell pressure of 1.1×10^{-3} mbar, and collision energies between 20 and 27 eV. For the identification of isoflavone conjugates, positive and negative ion ES/MS product ion and precursor ion spectra were acquired over the mass range of *m*/*z* 85−300 or 250−630, respectively, using a collision energy of 40 eV.

Pharmacokinetic Analysis. Plots of average total isoflavone concentrations in plasma ($n = 6$), as a function of time, were analyzed using a model-independent spreadsheet approach (*17*). The first-order elimination rate constants (k_{elim}) were determined for genistein and daidzein from the slope of the terminal phase of the ln−linear serum concentration−time curve. Maximal isoflavone concentrations (C_{max}) were determined by inspection. Internal exposures to genistein, daidzein, and equol (AUC_{0-24}, area under the time−concentration curves from 0 to 24 h) were estimated from all data points using the trapezoidal rule (*17*).

Statistical Analysis. For all calculated means in the graphs and tables, individual measurements that were more or less than 2 times the calculated standard deviation of the mean were considered to be outliers. These data were removed from the final analysis. One-way ANOVA, using the Bonferroni *t* test for multiple comparisons, was used to determine significant differences ($p < 0.05$) in plasma isoflavone concentrations resulting from different diets.

RESULTS

Pharmacokinetics of Total Plasma Isoflavones. The pharmacokinetics of total plasma isoflavones were determined for genistein and daidzein, the principal components of the soy product-fortified diets, as well as equol, the microbial metabolite of daidzein. **Figures 1−3** show the time−concentration curves. The pharmacokinetic parameters derived are shown in **Table 2**. C_{max} values for genistein and daidzein were typically observed at the 1-h point (range = 0.5−2 h), reflecting rapid absorption from the intestine; however, for equol as long as 8 h was required to attain peak values after the consumption of some diets, reflecting the additional time required for metabolism by the gut microflora. Determination of elimination half-times for genistein and daidzein was typically done using 1−12-h points

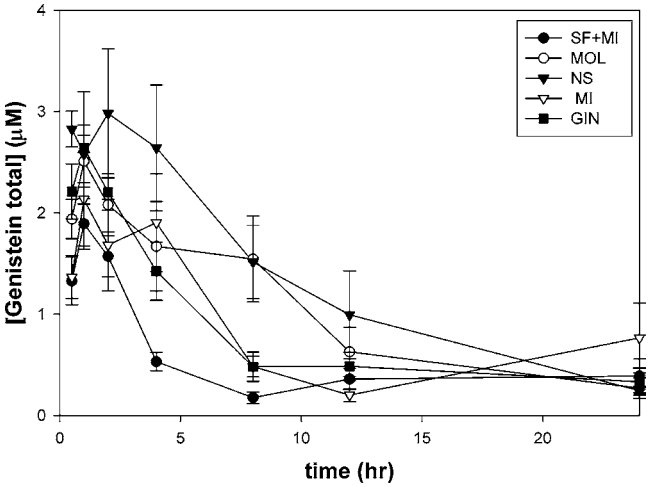

Figure 1. Time−concentration profile for genistein in plasma from mice fed different soy-containing diets. Average plasma total genistein concentrations and error bars representing the SEM are shown ($n = 6$) for the various time points following ingestion of diets containing identical genistein-aglycon equivalents.

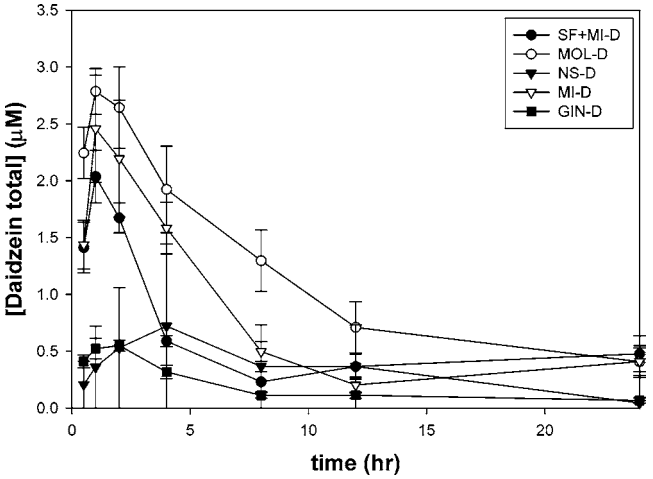

Figure 2. Time−concentration profile for daidzein in plasma from mice fed different soy-containing diets. Average plasma total daidzein concentrations and error bars representing the SEM are shown ($n = 6$) for the various time points following ingestion of diets containing similar daidzein-aglycon equivalents (except GIN).

because of variability in the 24-h time points, possibly due to enterohepatic recycling (*7*). The complex equol concentration−time profiles, which resulted from superposition of formation, elimination, and possibly recirculation processes, made the determination of elimination kinetics impossible. The elimination half-times for genistein in each diet group varied within a factor of ~5. It appeared that mice on the NS diet showed the slowest elimination of genistein and those on the soy molasses diet showed the fastest elimination. Similar variation in elimination half-times was observed for daidzein except that the SF + MI diet produced the fastest elimination. The internal exposures were calculated as AUC_{0-24}, and not $AUC_{0-\infty}$, to avoid extrapolation errors using 24-h time points that showed considerable variability.

Bioavailability. Despite the constant amount of genistein equivalents in each diet, the relative bioavailability (i.e., ratio of AUC_{0-24}) varied considerably. Bioavailability of genistein from the SF + MI diet was lowest, and all other diets produced values 1.5−2.5-fold higher. Although the diets were not designed to investigate effects of daidzin content, some com-

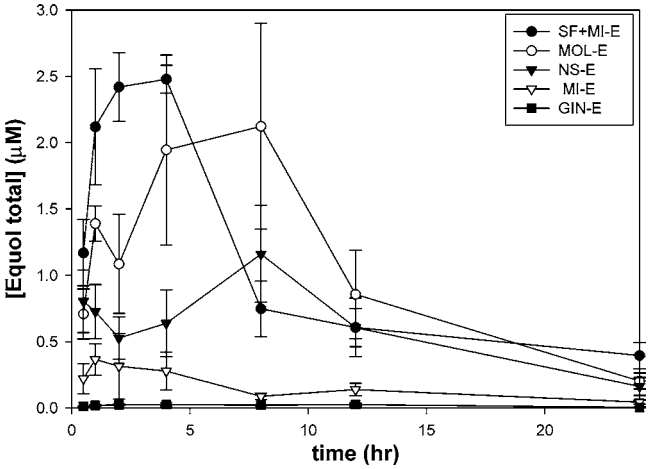

Figure 3. Time–concentration profile for equol in plasma from mice fed different soy-containing diets. Average plasma total equol concentrations and error bars representing the SEM are shown ($n = 6$) for the various time points following ingestion of different soy diets containing similar daidzein-aglycon equivalents (except GIN).

Table 2. Pharmacokinetic Parameters for Plasma Isoflavones from the Administration of Isoflavone-Supplemented AIN Diets

diet[a]	genistein AUC[b] (μmol·h·L^{-1})	daidzein AUC[b] (μmol·h·L^{-1})	equol AUC[b] (μmol·h·L^{-1})	genistein $t_{1/2elim}$[b] (h)	daidzein $t_{1/2elim}$[b] (h)
SF + MI	11.9	13.2	23.5	3.9	2.2
MOL	23.8	26.2	25.4	2.0	8.0
NS	31.2	36.8	14.1	11	6.3
MI	18.6	16.7	3.4	5.8	2.9
GIN	18.5	4.1	0.4	4.1	8.1

[a] GIN, genistin; MI, mixed isoflavones; MOL, soy molasses; NS, Novasoy; SF, soy flour. [b] Elimination half-life and area under the time concentration curve (AUC_{0-24}) were determined as described under Materials and Methods.

parisons are possible. The SF + MI, MOL, and NS diets contained essentially identical daidzein equivalents (**Table 1**), and the mixed isoflavones diet had 30% less; however, the GIN diet had much lower levels of daidzin than the other diets (86% less), so that comparisons to the other diets were not made. In general, daidzein AUC was comparable to that of genistein for each diet (**Table 2**). Finally, the observed metabolism of daidzein to equol (equol AUC_{0-24}) was also markedly affected by the diet. The production of equol, with or without normalization for daidzin content in each diet, was highest in mice consuming the SF + MI and MOL diets but decreased in the more processed diets.

Isoflavone Conjugates and Aglycons in Plasma. The distribution of plasma isoflavones as glucuronide/sulfate conjugates and aglycons was determined at the 1-h time point. This time point was chosen because it is the time when most total genistein and daidzein C_{max} values were achieved for the diets and also provided a reasonable compromise for comparison with maximal equol concentrations. **Figure 4** shows the isoflavone concentrations determined before glucuronidase/sulfatase-catalyzed hydrolysis (aglycon) and after (total). The concentration of genistein aglycon, but not total genistein, was found to increase regularly as the degree of isoflavone processing was increased. Significant differences ($p < 0.05$) in plasma genistein aglycon concentration were observed between SF + MI and either GIN or MI diet groups. Significant differences in aglycon, but not total, daidzein were observed between SF + MI and the NS and MI diet groups. The plasma concentrations of total

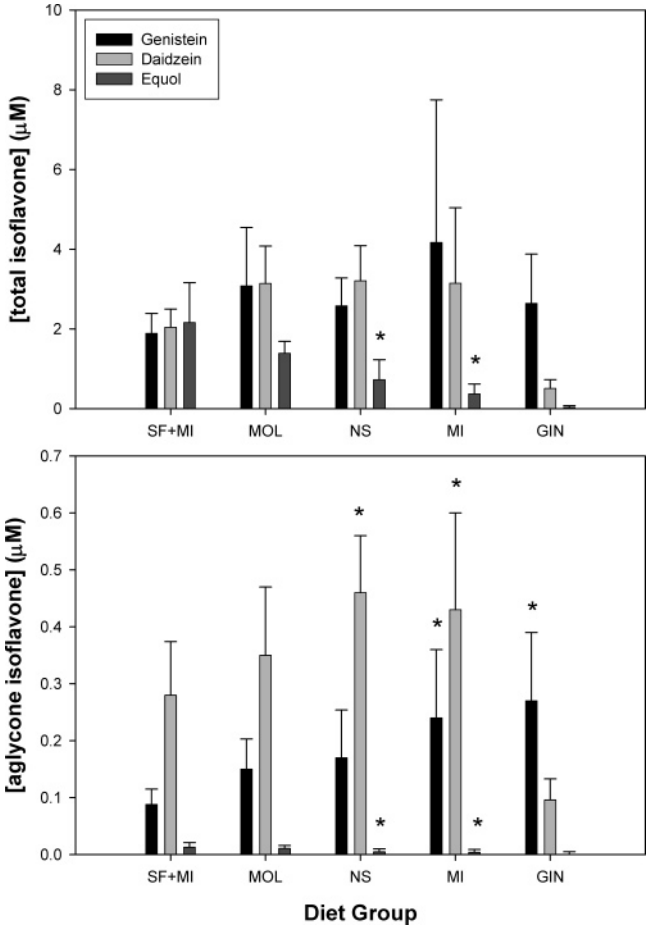

Figure 4. Near-peak concentrations of total and aglycon isoflavones/equol in plasma. Plasma was collected from mice 1 h after ingestion of the respective diets and analyzed for total isoflavones/equol (glucuronidase/sulfatase hydrolysis) or aglycons (no enzymatic hydrolysis). Mean values ± SD are shown ($n = 6$). Significant differences ($p < 0.05$) with the SF + MI group are noted (*). Note: no comparisons were made for daidzein and equol plasma concentrations from mice fed the GIN diet because of the very low daidzin content.

and aglycon equol observed at 1-h time points decreased regularly as the degree of isoflavone processing increased in the diets; significant differences in either total or aglycon equol plasma concentrations were observed between the SF + MI group and the NS and MI diet groups. The average fraction of aglycon genistein, daidzein, and equol observed in 1-h plasma samples from individual diet groups ranged from 5 to 12%, from 11 to 18%, and from 0.5 to 0.9%, respectively.

The composition of isoflavones among various conjugated forms (i.e., those quantified following glucuronidase/sulfatase hydrolysis and following subtraction of the aglycon content determined in the absence of enzyme treatment) was estimated using selective hydrolysis with either purified sulfatase or glucuronidase preparations as previously described (*16*). **Table 3** shows the results for genistein, daidzein, and equol contained in a pool of 30-min samples from mice fed a soy flour diet. This pooled plasma sample, obtained from mice fed a diet in a different study, was selected because it contained similar concentrations of total isoflavones and equol (1.10, 1.11, and 1.26 μmol/L, respectively). The fractions of total isoflavones present as the respective aglycon, sulfate, or glucuronide did not differ greatly for genistein and daidzein; glucuronides were the predominant metabolites in both cases. In contrast, equol sulfate was present at levels greater than the corresponding

Table 3. Distribution of Isoflavones and Equol into Glucuronide, Sulfate, and Aglycon Forms in a Single Pooled Plasma Sample Obtained from Mice Fed a Soy Flour Diet Collected at 0.5 h[a]

form	genistein (%)	daidzein (%)	equol (%)
aglycon	9	23	1
sulfate	38	33	78
glucuronides	53	44	21

[a] Isoflavones and equol were quantified following treatment by either no enzyme (aglycon), glucuronidase/sulfatase (total), sulfatase (aglycone + sulfate), or glucuronidase (aglycon + glucuronides). Data presented are means of replicate analyses ($n = 3$–6).

glucuronide, and the aglycon was present at much lower levels (<0.9%) than seen for genistein and daidzein (5−12 and 11−18%, respectively, see above).

Conjugates of genistein, daidzein, and equol in mouse plasma samples that had not been subjected to enzymatic hydrolysis were further analyzed using precursor ion MS scans. **Figure 5** shows reconstructed ion chromatograms from precursor ion scans that identify the glucuronide (parent *m*/*z* 447 and 431, respectively) and sulfate (parent *m*/*z* 351 and 335, respectively)-conjugates of genistein (left panel) and daidzein (right panel) as previously reported (*14*) along with several other new plasma conjugates. These components were subsequently verified from product ion scans (not shown) as the glucoside and malonyl glucoside conjugates of genistein (parent *m*/*z* 433 and 519, respectively) and daidzein (parent *m*/*z* 417 and 503, respectively). The product ion tandem mass spectra were identical to those previously reported from a commercial soy dietary supplement (*16*) and authentic samples of genistin and daidzin (not shown). In addition, treatment of the plasma samples with β-glucosidase eliminated the glucoside peaks (not shown), and the glucoside peaks coeluted on an LC column with authentic genistin and daidzin standards (not shown). In contrast, only the sulfate conjugate of equol was observed using both positive and negative product or precursor ion scans (not shown).

The amount of glucoside conjugates present in mouse serum was investigated by using β-glucosidase hydrolysis of 1-h time points in subsets from all diet groups and for all time points for the mixed isoflavones diet group. The glucoside + aglycon concentration−time profiles for genistein and daidzein (data not shown) rose and declined much as did the total isoflavone profiles shown in **Figures 1** and **2**. **Figure 6** shows the quantification of all forms of isoflavones for the different diet groups obtained using either no enzyme (aglycones), glucuronidase/sulfatase (glucuronides + sulfates + aglycons), or β-glucosidase (glucosides + aglycons) at the 1-h time point. The average percentages of genistein and daidzein present as glucoside conjugates at the 1-h time point were 11 ± 0.80 and 17 ± 2.6%, respectively, values comparable to those of the respective aglycons.

DISCUSSION

This study using the parental strain of mice (i.e., not athymics) is a follow-up investigation to our previous study (*10*) in which diets formulated to contain identical amounts of total genistein (as aglycon equalivents), but which varied in the degree of processing of the soy-based components, were fed to ovariectomized athymic mice as an animal model for postmenopausal breast cancer. This unique experimental design allowed us to examine factors that affect metabolism and disposition of isoflavones not possible in previous studies that compared bioavailability of purified isoflavone aglycons as opposed to their glucoside forms (*18*−*20*). Administration of diets containing equal genistein equivalents (750 mg/kg, **Table 1**) stimulated tumor growth, and the level of stimulation increased with the degree of soy processing (*10*). One possible explanation offered for these tumor growth data was that differences in diet could affect the bioavailability of genistein. However, in the present study no such relationship was observed for total plasma genistein AUCs (**Table 2**). Although the relative bioavailability (i.e., AUC ratio) for total plasma genistein varied across the diet groups by 2.6-fold, there was no correlation between total genistein AUC (μM × h) and tumor growth (mm^2 × week^{-1}; ref *10*) for the different diets ($r^2 = 0.06$, $p < 0.70$, data not shown). This finding indicated bioavailability alone could not explain the effects of soy processing on tumor growth.

The dietary source of isoflavones also affected the kinetics of isoflavone disposition. The elimination half-times for genistein and daidzein varied across the diet groups by 5.5- and 3.7-fold, respectively, but not in concert with the changes in bioavailability or tumor growth stimulation (*10*). This finding indicated changes in kinetics alone could not explain the effects of soy processing on tumor growth.

The distribution of genistein, daidzein, and equol between conjugated and aglycon forms was determined at a single time point (1 h) that best reflected the time for peak concentrations of genistein and daidzein and was within 50% of the peak for equol. **Figure 4** shows that peak concentrations of genistein aglycon, but not total genistein, increased consistently in the plasma of Balb/c mice as the degree of soy processing in the diet increased. The average peak plasma concentrations of genistein aglycon correlated linearly with average tumor growth previously reported in athymic mice fed the same diets ($r^2 = 0.96$ and $p < 0.001$, data not shown; ref *10*). As seen with AUCs, no significant correlation was observed between average tumor growth and total plasma genistein concentrations ($r^2 = 0.55$ and $p < 0.093$, data not shown). It is biologically plausible that the observed correlation is the result of higher circulating levels of the active estrogenic aglycone, which would be expected to produce concomitantly higher concentrations in tumor tissue (*21*) and stimulate tumor growth by an estrogen agonist mechanism (*3*, *10*−*12*). The correlation between circulating aglycon genistein, but not total genistein, and tumor growth suggests that soy components removed by processing can modify the metabolism of genistein to estrogenically inactive conjugates. It also suggests that comparisons based on total plasma genistein should be made with caution when other factors in the experimental design of isoflavone studies (e.g., diet composition) are changed.

Because the genistein aglycon content in all diets was quite low, and lowest in the genistin diet (0.2−2.1%, **Table 1**), differences in its absorption from stomach or intestine (*21*) cannot account for the observed tumor growth results; however, it is possible that the removal of soy components through the successive stages of processing could affect enzymatic deconjugation of isoflavone glucosides, a process important for the net uptake into blood of isoflavones from the intestine (*23*, *24*). Consistent with this hypothesis are the findings of Andlauer et al. (*25*) in which an isolated perfused rat small intestinal preparation was used to show that the absorption rate of genistein glycosides and the fraction secreted into vascular media as glucuronides were decreased in the presence of a tofu matrix as opposed to pure genistin. Although there is evidence for active transport-mediated processes for the absorption of flavonoid glycosides in the intestine (*26*), Andlauer et al. (*24*) concluded

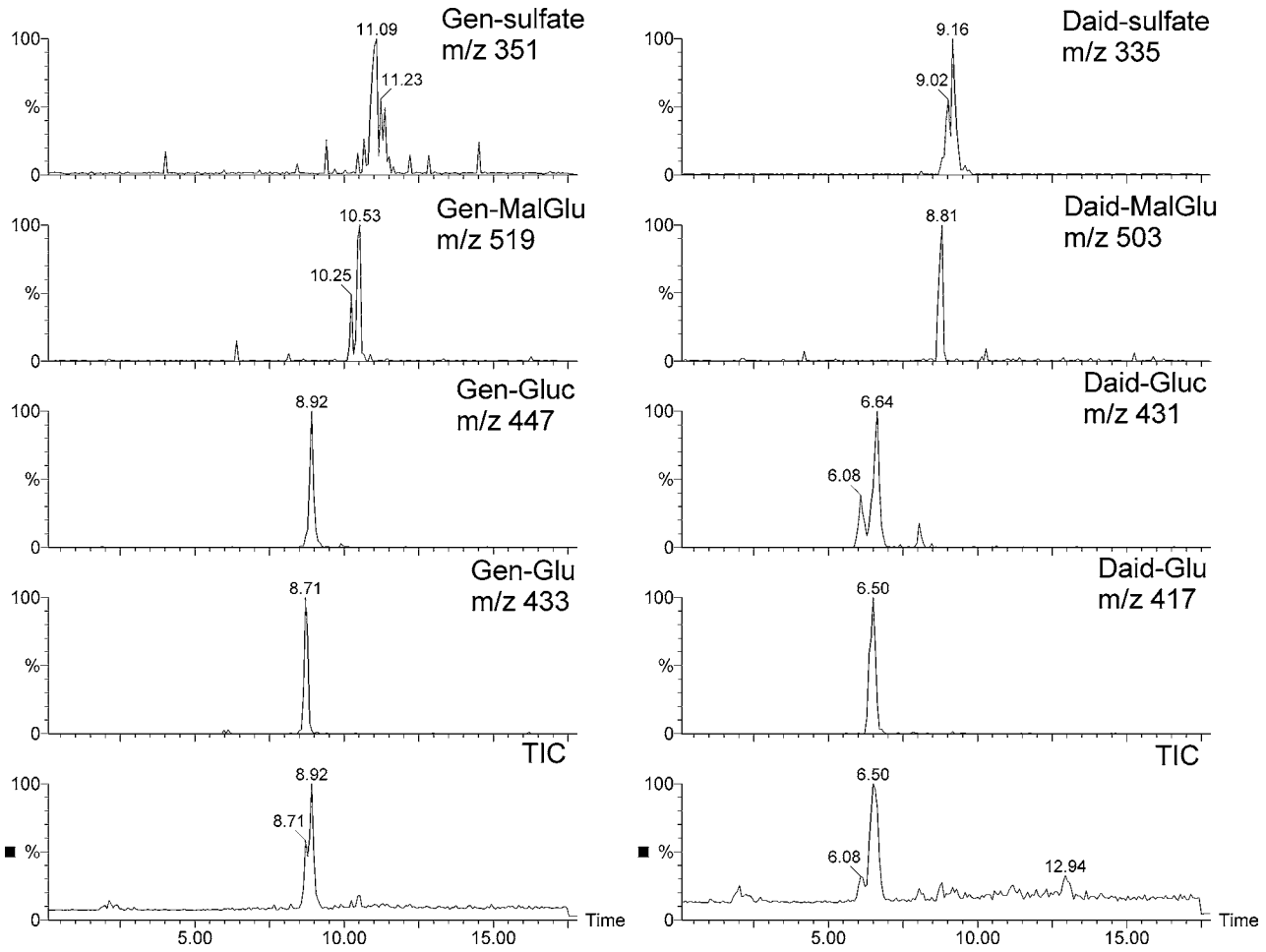

Figure 5. Detection of isoflavone conjugates using LC-ES/MS/MS with precursor ion scans. A pooled plasma sample (1 h SF + MI) was analyzed without enzymatic hydrolysis using LC separation with tandem mass spectrometric detection of conjugated forms following collision-induced dissociation to genistein (*m*/*z* 271) and daidzein (*m*/*z* 255) from precursor ion scans.

that transepithelial transport of genistin was more likely to occur by diffusion.

The degree of soy processing affected daidzein aglycon content similarly to genistein (**Figure 4**); however, the low affinity of daidzein for ER interactions (*27*−*29*) and the lack of effect of daidzein in a dimethylbenzanthracene (DMBA)-induced rat mammary carcinogenesis model (*30*) suggest that changes in plasma daidzein cannot account for ER-mediated stimulation of estrogen-dependent MCF-7 tumor growth in athymic mice.

Similar to previous studies on rats and monkeys, but in contrast to humans (*8*), Balb/c mice consistently produced significant levels of equol from diets with comparable daidzein contents (SF + MI, MOL, NS; **Table 1**). The relative production of total equol from daidzein by the gut microflora, measured as either AUCs (**Table 2**) or near-peak concentrations (**Figure 4**, top), was inversely correlated with the degree of soy processing. Although evidence has been reported that the addition of complex carbohydrates can affect the bioavailability of parent soy isoflavones in rats (*31*), alterations in dietary components have not been shown to affect the degree of equol production in humans (*32*).

A significant inverse correlation was observed between either total or aglycon equol concentrations in plasma and average tumor growth ($r^2 = 0.96$ or 0.98 and $p < 0.003$ or 0.002, respectively (*10*). Although these results are consistent with an unknown mechanism by which equol antagonizes the stimulatory estrogenic action of genistein on MCF-7 tumor growth in athymic mice, there is no plausible explanation for this notion. To the contrary, the dramatically lower fraction of circulating aglycon equol (<1%, see above and **Table 3**) relative to aglycon genistein (5−12%) suggests that comparably lower levels of the active estrogenic form would be present in target tissues, including the xenograft tumors. These data strongly support the notion that differences in phase II conjugation between genistein and equol are critical determinants of accumulation in tumor tissue and subsequent biological effects.

Direct evidence for differences in phase II metabolism between equol and the isoflavones was observed (**Table 3**). Glucuronides were the predominant form of genistein and daidzein, but not equol. Conversely, the fraction of equol sulfate was greater than that for either genistein or daidzein. The aglycon content of equol was 9-fold lower than that of genistein and 23-fold lower than that of daidzein. These differences in conjugation profiles suggest that differences in metabolism and disposition are important pharmacokinetic factors that distinguish the in vivo activity of genistein from the apparent inactivity of equol (see above).

There is evidence showing estrogen *agonistic* (but not *antagonistic*) activity for both equol and genistein in vitro (*27*−*29*, *33*−*35*) and for genistein in vivo (*11*); however, evidence for estrogenic activity of equol in vivo is quite limited (see below). Furthermore, because the ER α and ER β binding affinities and agonist-mediated transcriptional activation by either equol enantiomer or the racemate in vitro are comparable to or lower than those for genistein (*29*), it is implausible that

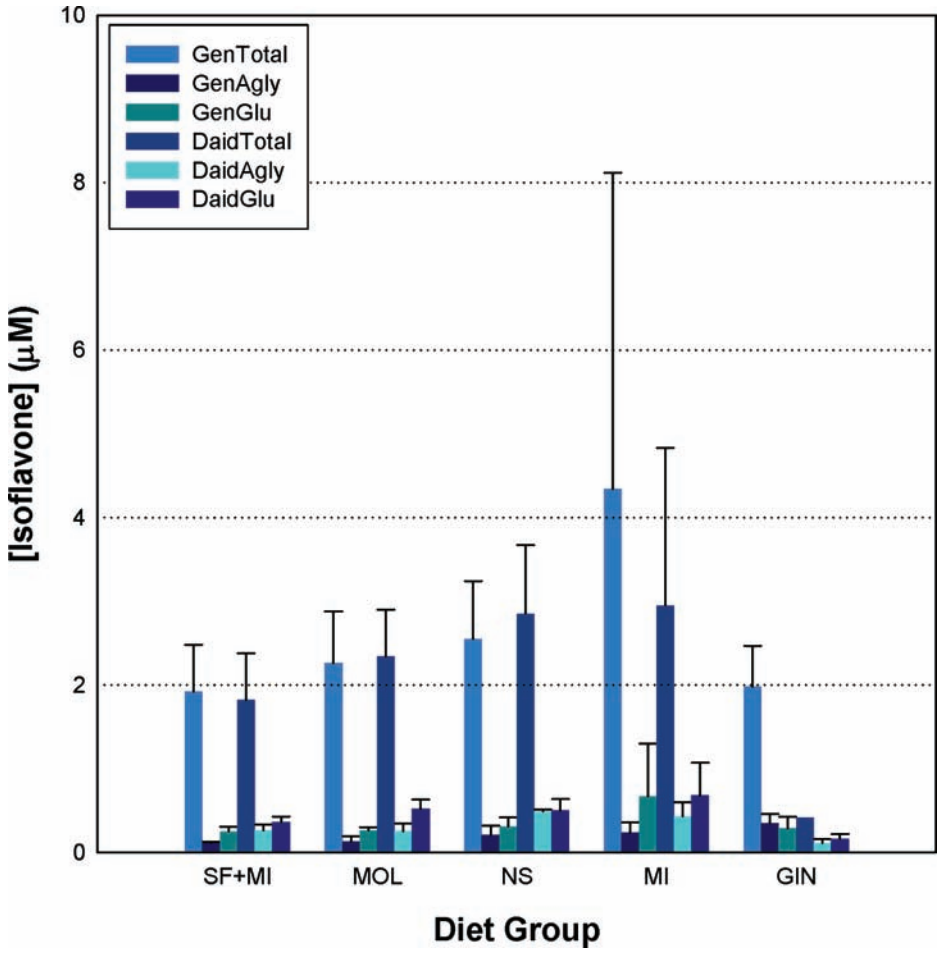

Figure 6. Distribution of isoflavones/equol in conjugated and aglycon forms following ingestion of different soy-containing diets. The plasma concentrations of genistein and daidzein were determined as total (glucuronidase + sulfatase + glucosidase treatment), aglycon (no enzyme treatment), and glucosides (glucosidase treatment). Mean values ± SD are shown ($n = 6$).

equol could produce ER-mediated responses in tissues at lower concentrations than genistein.

This conclusion is consistent with literature precedent on the equivocal activity of equol in estrogen-responsive tissues from experimental animals. Medlock et al. (*36*) reported that injected equol decreased uterine gland number without increasing uterine wet weight or luminal epithelial hypertrophy, effects that are inconsistent with either an estrogenic or antiestrogenic action in the uterus. Conversely, diethylstilbestrol, estradiol, and coumestrol all increased uterine weight and inhibited uterine gland genesis as expected for estrogen agonists (*36*). We recently reported that injected, but not dietary, equol produced estrogenic effects on uterine weight of C57BL/6 mice (*37*). These studies also showed that the fraction of aglycon equol present in serum was much higher from injections as opposed to dietary administration. These results suggested that the route of administration affects phase II conjugation of equol in the gastrointestinal tract and the circulating and target tissue levels of active aglycon species. Furthermore, this metabolic difference appeared to be a critical determinant of uterine pharmacodynamics.

Lamartiniere et al. (*30*) reported that DMBA-induced mammary tumor multiplicity was not affected in rats fed daidzein-containing diets that yielded high circulating levels of total daidzein and equol. The lack of effects on tumors and mammary gland differentiation from exposure to daidzein and equol was opposite the significant reduction in tumor multiplicity and increased gland differentiation caused by identical prepubertal treatment with genistein conducted earlier by the same research group (*38*). In the former study, the concentration of total equol in mammary tissue was 17% of that measured in blood; however, aglycon equol was not detected in mammary tissue (*29*). By comparison, Fritz et al. (*38*) showed that the concentration of total genistein in rat mammary tissue was 61% of the blood level and that genistein aglycon was prominent in mammary tissue (i.e., 72% of the total).

These findings confirm that differences in phase II conjugation between genistein and equol could be critical in determining effects in estrogen-responsive rat tissues such as the mammary gland. Genistein is clearly different from equol because there is evidence that equol does not affect the growth of DMBA-induced rat mammary tumors (*30*) and that genistein stimulates methylnitrosourea-induced rat mammary tumors (*39*).

It is possible that soy components such as phytosterols, which could be lost incrementally by successive processing steps, could directly inhibit the stimulatory estrogenic action of genistein on MCF-7 tumor growth (*40*). Alternatively, as stated previously, competition between genistin and other glycosidic soy constituents (e.g., saponins, fructooligosaccharides, or phytosterols) for cleavage by β-glucosidases in the gut or effects on phase II conjugation are possible modes of action for altering the intestinal secretion of aglycon and/or glucuronide forms of genistein into blood.

In addition to the sulfate and glucuronide conjugates of genistein and daidzein, the glucoside and malonyl glucoside conjugates were observed in Balb/c mouse plasma. The

structural assignments for glucoside and malonyl glucoside conjugates were made on the basis of full product ion mass spectra and concordance with retention times for commercially available glucoside standards. This is the first time that circulating isoflavone glucosides have been identified in vivo. Several examples of direct absorption of isoflavone glucosides have been reported using rodent intestinal preparations ex vivo (*24*, *25*) or human colon (Caco-2) cells in vitro (*41*). Furthermore, Paganga and Rice-Evans (*42*) reported the absorption of flavonoid glycosides in humans. However, when Setchell et al. (*43*) used LC-ES/MS with selected ion monitoring, they did not observe glucoside conjugates in human blood collected at 1, 2, and 8 h following administration of pure isoflavone glucosides or soy milk. It is not clear if higher analytical sensitivity/specificity was achieved in the present study by using precursor ion scans of the triple-quadrupole mass spectrometer or if a species difference in metabolic processing is responsible for the inability to detect isoflavone glucosides in humans.

The absorption profile for glucosides mirrored that for total isoflavones (not shown). At least some of these glucoside conjugates must have been absorbed without processing by microbial or brush border β-glucosidases because malonyl glucosides, which are produced only in plants (*44*), were observed in plasma from these mice. The glucosides were minor constituents in plasma (<10%) and were eliminated more quickly than total isoflavones (not shown), suggesting that metabolism occurs after absorption from the gut, first by deconjugation to aglycons (*23*) and then by glucuronidation and sulfation in the gut or liver (*45*). The low levels and faster clearance, coupled with the minimal estrogenic activity of conjugated isoflavones (*28*), suggest that the presence of glucoside conjugates per se should have little influence on estrogenic actions of dietary genistein.

In conclusion, our previous study (*10*) demonstrated that the degree of processing of the soy isoflavone components of test diets affected the stimulation of human MCF-7 tumor growth in an ovariectomized athymic mouse model for postmenopausal estrogen-dependent mammary carcinogenesis. In the present study, we have investigated the influence of these diets on the metabolism and disposition of isoflavones and metabolites in the parental Balb/c strain. As the soy components in the diets were successively more processed, peak plasma levels of the active estrogenic aglycon form of genistein increased regularly in a manner consistent with the stimulation of MCF-7 tumor growth previously observed in athymic mice consuming the same diets. Processing also affected the relative bioavailability of total serum genistein, but this endpoint did not correlate with tumor growth. Formation of equol from the metabolism of daidzein by the intestinal microflora was successively decreased by processing of the dietary isoflavones; however, the much lower levels of aglycon equol in plasma, relative to genistein and daidzein, suggest that equol formation is unrelated to estrogenic effects on tumor growth. These findings confirm the importance of the processed form of dietary isoflavones in determining circulating and, presumably, target tissue concentrations of active aglycon genistein that appear to be responsible for ER-mediated processes that can result in either beneficial or detrimental effects.

ABBREVIATIONS USED

AIN, American Institute for Nutrition; AUC, area under the time−plasma concentration curve; DMBA, 7,12-dimethylbenzanthracene; $t_{1/2elim}$, elimination half-time; ES, electrospray; ER, estrogen receptor; GIN, genistin; MS, mass spectrometry; MI, mixed isoflavones; MRM, multiple reaction monitoring; $(M + H)^+$, protonated molecule; C_{max}, peak serum concentration; NS, Novasoy; SF, soy flour; MOL, soy molasses.

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