

## Estrogenic Activity of Glycitein, a Soy Isoflavone

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Glycitein (4',7-dihydroxy-6-methoxyisoflavone) accounts for 5–10% of the total isoflavones in soy food products. The biological activity of this compound has not been reported to date, although numerous studies have been performed with the other soy isoflavones, daidzein and genistein. Glycitein was isolated from soy germ to 99% purity. Weaning female B6D2F1 mice were dosed with glycitein (3 mg/day), genistein (3 mg/day), and diethylstilbestrol (DES) (0.03  $\mu$ g/day) in 5% Tween 80 by gavage for 4 days. A control group received an equal volume of 5% Tween 80 solution daily. The uterine weight increased 150% with glycitein ( $p < 0.001$ ), 50% with genistein ( $p < 0.001$ ), and 60% with DES ( $p < 0.001$ ) compared with the control group. DES, 17 $\beta$ -estradiol, and three isoflavones (daidzein, genistein, and glycitein) were examined for their competitive binding abilities with 17 $\beta$ -(<sup>3</sup>H)estradiol to the estrogen receptor proteins of the B6D2F1 mouse uterine cytosol. The concentrations of each compound required to displace 50% of the (<sup>3</sup>H)estradiol at 5 nM in the competitive binding assay were 1.15 nM DES, 1.09 nM 17 $\beta$ -estradiol, 0.22  $\mu$ M genistein, 4.00  $\mu$ M daidzein, and 3.94  $\mu$ M glycitein. These data indicated that glycitein has weak estrogenic activity, comparable to that of the other soy isoflavones but much lower than that of DES and 17 $\beta$ -estradiol.

**Keywords:** Isoflavone; glycitein; genistein; daidzein; phytoestrogen; estrogenic activity

### INTRODUCTION

Estrogens play important hormonal roles among all vertebrates. Animal estrogens are exclusively steroidal compounds, and the principal physiological estrogen in most species is 17 $\beta$ -estradiol. Many plants produce compounds that possess estrogenic activity in animals and are, thus, called phytoestrogens. These compounds have some structural similarity to the mammalian estrogen, 17 $\beta$ -estradiol. A common structural characteristic of many of these compounds is the presence of a phenolic ring, a prerequisite for the binding to the estrogen receptor (Figure 1).

Soybeans contain the highest concentrations of isoflavones, at 1–3 mg/g, as daidzein, genistein, glycitein, and their corresponding glucosides, of foods consumed by humans. These soy isoflavones may have some important health-enhancing properties such as prevention of certain cancers (Barnes et al., 1991), lowering the risk of cardiovascular diseases (Anderson et al., 1995), and improvement of bone health (Bahram et al., 1996). Their estrogenic activities may play an important role in their health-enhancing properties. Genistein and daidzein account for the major portion of isoflavones in soy foods and have been the focus of numerous studies. The estrogenic activities of daidzein, genistein, and their glucoside forms, genistin and daidzin, were 10000–500000 times lower than that of diethylstilbestrol (DES) as assessed by the mouse uterotrophic assay (Farmakalidis and Murphy, 1985). Estrogen receptor binding studies with soy isoflavones, genistein and daidzein, demonstrated they had the abilities to bind to estrogen receptors from different species including mice, rats, and

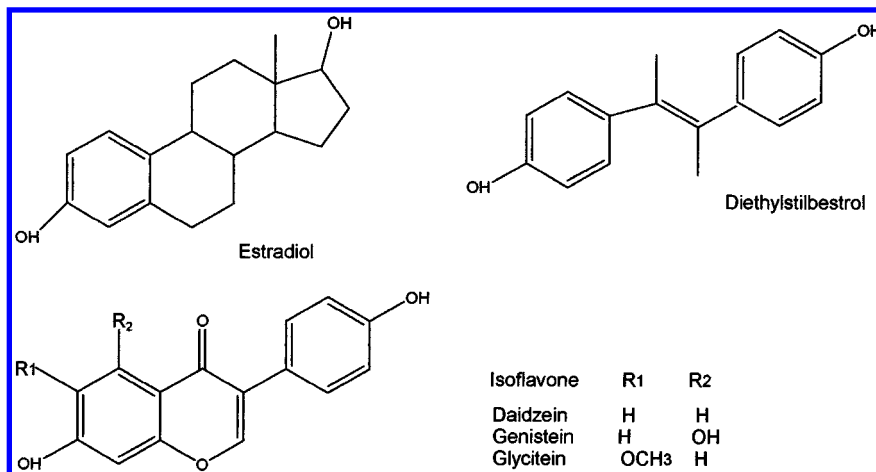
sheep (Verdeal et al., 1980). No biological studies on glycitein have been reported to date. However, glycitein accounts for 5–10% of the total isoflavones in soy foods and may be as high as 40% in some soy supplements composed of soy germ (Song et al., 1998; Tsukamoto et al., 1995). Therefore, it is important to evaluate the biological activity of glycitein. Because glycitein has a structure similar to those of genistein and daidzein, we hypothesize that it will have estrogenic activity.

There are a number of methods to assess estrogenic activity. Reel et al. (1996) proposed a test array for potential estrogenic activity. These methods can be grouped into four categories: (1) estrogen receptor binding; (2) estrogen receptor-dependent transcriptional expression; (3) reproductive tract response; and (4) nonreproductive tract target tissue response. It is preferable to perform more than one assay to confirm the results. The binding affinities of soybean isoflavones were much lower than those of estradiol and DES (Verdeal et al., 1980). Farmakalidis and Murphy (1985) evaluated the estrogenic activity of genistin and daidzin, the isoflavone glucosides, and showed that the glucosides had estrogenic activity equal to that of the aglycons on a molar basis. The mouse uterine enlargement assay has been the standard *in vivo* method to evaluate estrogenic activity (Bickoff et al., 1962), and, because it is performed in an intact animal, the effects of absorption, metabolism, serum binding, and pharmacokinetics are taken into account. These two methods were used to establish the estrogenic activity of glycitein.

### MATERIALS AND METHODS

**Chemicals.** DES, Tween 80, 17 $\beta$ -(2,4-<sup>3</sup>H)estradiol (23 mCi/mmol), 17 $\beta$ -estradiol, and dextran-coated charcoal (DCC) were obtained from Sigma Chemical Co. (St. Louis, MO). ACS

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**Figure 1.** Chemical structures of soy isoflavones, 17 $\beta$ -estradiol, and DES.

scintillation fluid was purchased from Fisher Scientific Inc. Glycitein was purified according to the following method: 10 g of soygerm (generously donated by Schouten USA, Inc., Minneapolis, MN) was hydrolyzed in 100 mL of 0.1 N HCl at 98 °C for 2 h, extracted with acetonitrile, and filtered through Whatman No. 42 filter paper. The filtrate was dried by a rotary evaporator at 50 °C. The residue was dissolved in 80% ethanol and applied to a Sephadex LH-20 column (2.5 cm  $\times$  50 cm) with 50% ethanol as the eluent. The glycitein peak was collected and freeze-dried. Genistein was purified from soybeans using the same procedure as glycitein. Daidzein was chemically synthesized according to the method of Song et al. (1998). The identification and purity of glycitein, daidzein, and genistein were confirmed by HPLC, ultraviolet (UV) spectral analysis, melting point, and mass spectrum analysis. Glycitein, daidzein, and genistein were analyzed by HPLC on a Beckman System Gold chromatography system consisting of a model 507 autosampler, a model 126 dual pump, a model 168 photodiode array detector, and an IBM 486 computer with Beckman Gold system HPLC data processing software (version 8, 1993) according to the procedure of Murphy et al. (1997). A YMC-pack ODS-AM-303 column (5  $\mu$ m, 25 cm  $\times$  4.6 mm) (YMC Inc., Wilmington, NC) was used. UV spectral analysis was performed according to the method of Mabry et al. (1970) using a Beckman DU 7400 spectrophotometer. The melting point was measured with a Perkin-Elmer 7 series differential scanning calorimeter (DSC) (Perkin-Elmer Inc., Norwalk, CT). Mass spectrum analysis using direct probe introduction and chemical ionization was performed on a Finnigan model TSQ-700 mass spectrometer (Finnigan Inc., Piscataway, NJ).

**Animals and Treatments.** The mice uterine enlargement assay was performed according to the method of Farmakalidis and Murphy (1985). Inbred B6D2F1 mice were obtained from Harlan Sprague-Dawley (Madison, WI). Each dam was housed separately with her female pups (14 days old on arrival), and the mice received AIN-93 M diet (Reeves et al., 1993) and water. Pups were weaned at 21 days of age. The mice were randomly assigned to treatment groups (control, DES, genistein, and glycitein) of 20 mice per group. An AIN 93-G diet and water were provided. This semipurified diet contained no soy isoflavones by our HPLC analysis (Song et al., 1998). Genistein, DES, and glycitein were suspended in 5% Tween 80 and were administered to the mice by gavage needle in four daily doses of 0.1 mL suspension/day beginning on the day after weaning for 4 days. The total doses were 0.12  $\mu$ g of DES/mouse or 12 mg of genistein or glycitein/mouse. The control group was given 0.1 mL of 5% Tween 80 for 4 days. At 24 h after the last dose, mice were weighed and sacrificed under CO<sub>2</sub>. Their uteri were dissected out, and the wet weights were obtained immediately. Mouse uteri were homogenized, and cytosols were prepared for estrogen receptor binding assay as described below. The blood from genistein and glycitein groups was collected by using cardiac puncture. Plasma was prepared by centrifugation at 5000g for 10 min at 4 °C and

kept frozen until analysis of plasma isoflavones. The animal experimental protocol was approved by Iowa State University Animal Use Committee.

**Tissue Handling and Estrogen Receptor Assay.** The estrogen-binding assay was modified from the procedure of Verdeal et al. (1980). Mouse uteri were homogenized (Brinkmann Instruments, Rexdale, ON, Canada) in 10 mM Tris and 1 mM EDTA, pH 7.4, buffer (1:10 w/v). The cytosol fractions were obtained by centrifugation of the homogenate at 100000g for 1 h. Total binding was determined by adding 0.2 mL each of 1 mM EDTA, pH 7.4, buffer, (<sup>3</sup>H)estradiol (1.5 ng/mL in 1 mM EDTA pH 7.4 buffer), and cytosol fraction. Nonspecific binding was determined by replacing the 1 mM EDTA buffer with 0.2 mL of 17 $\beta$ -estradiol (1500 ng/mL in the 1 mM EDTA, pH 7.4, buffer). Blanks contained 0.4 mL of 1 mM EDTA buffer and 0.2 mL of (<sup>3</sup>H)estradiol. Competitive binding was determined by replacing the 0.2 mL of buffer in the total binding mixture with 0.2 mL of solution containing the different estrogens at appropriate concentrations in 1 mM EDTA, pH 7.4, buffer. Duplicates were performed for each estrogen at each concentration. Samples were incubated at room temperature (23 °C) for 2 h. DCC (2.5%, 0.5 mL) in 1 mM EDTA buffer was added to all mixtures after incubation. The samples were mixed, incubated for 15 min, and centrifuged at 1000g for 10 min. After centrifugation, the supernatants were decanted into scintillation vials containing 10 mL of ACS scintillation fluid. The samples were counted for 10 min on a Packard liquid scintillation analyzer model 1900TR (Packard Instrument Co., Downers Grove, IL).

**Plasma Isoflavone Analysis.** Plasma samples of the genistein group and the glycitein group were combined to give one pooled sample for each treatment. Two milliliters of 0.2 M sodium acetate, pH 5.5, buffer, 100  $\mu$ L of  $\beta$ -glucuronidase/sulfatase (Sigma Chemical Co.), and 20  $\mu$ L of 4 mg/mL internal standard, 2,4,4'-trihydroxydeoxybenzoin (THB) (Song et al., 1998), were added to 2 mL of plasma sample. The mixture was incubated at 37 °C for 16 h. Six milliliters of methanol was added to the mixture, mixed well, and centrifuged at 10000g for 20 min. Eight milliliters of supernatant was brought to dryness and dissolved in 400  $\mu$ L of 80% methanol. After centrifugation, 20  $\mu$ L of sample was taken for isoflavone analysis by our HPLC system. The HPLC conditions were the same as in the identification of genistein and glycitein stated above. The minimal detection level by UV detector at 254 nm for genistein and glycitein was 0.5  $\mu$ g/mL or 1.9  $\mu$ M in injection solution.

**Statistical Analysis.** The uterine enlargement data were analyzed by using a one-way classification analysis of a completely randomized design (SAS version 6, SAS Institute Inc., Cary, NC) at  $\alpha = 0.05$ .

**Table 1. Estrogenic Activities of Soy Isoflavones in Mice**

estrogen	treatment		uterine wt <sup>a</sup> (mg, mean ± SEM)	body wt (g)		relative potency <sup>b</sup>
	total dose	no. of mice		initial	final	
control	0 mg	20	17.7 ± 0.9 <sup>c</sup>	8.1	11.6	
DES	12 μg	20	28.5 ± 1.1 <sup>b</sup>	8.1	11.7	100000
glycitein	12 mg	20	44.6 ± 3.1 <sup>a</sup>	8.3	12.1	3
genistein	12 mg	20	26.4 ± 1.0 <sup>b</sup>	8.2	11.7	1

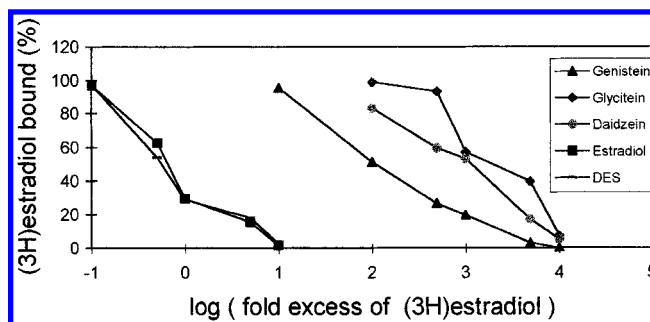
<sup>a</sup> Uterine weight means bearing different letters are significantly different,  $p < 0.05$ . <sup>b</sup> The relative potency was calculated on the basis of the doses of estrogen required to produce a 10-mg increase of uterine weight.

## RESULTS AND DISCUSSION

The purity of glycitein and genistein was confirmed by HPLC, UV spectral analysis, melting point, and mass spectrum analysis. The HPLC chromatography of glycitein showed one single peak. The purity of glycitein was confirmed by peak area percentage and peak purity program to be >99%. Naim et al. (1973) first isolated glycitein from soybeans and reported UV spectral data of glycitein. The UV spectral data for our glycitein were  $\lambda_{\max}$  (methanol) 257 ( $\epsilon = 31622$ ), 319 nm;  $\lambda_{\max}$  (sodium methoxide) 259, 344 nm;  $\lambda_{\max}$  (AlCl<sub>3</sub>) 257, 317 nm;  $\lambda_{\max}$  (AlCl<sub>3</sub> - HCl) 257, 317 nm;  $\lambda_{\max}$  (sodium acetate) 255, 346 nm; and  $\lambda_{\max}$  (sodium acetate/H<sub>3</sub>BO<sub>3</sub>) 255, 320 nm. These data were the same as reported by Naim et al. (1973). The molecular weight of our glycitein from the mass spectrum analysis was 284, which matched the mass reported by Naim et al. (1973) and the calculated molecular weight of glycitein. The melting point of glycitein measured by DSC was 337–339 °C and gave a very sharp thermogram. This melting point differed from that reported by Naim et al. (1973) of 311–313 °C and that reported by Kelly et al. (1993) of 290 °C (dec). Recently, Nogradi and Szollosy (1996) chemically synthesized glycitein and reported a melting point of 337–339 °C. The purity of genistein was confirmed to be >99% by using the similar method for glycitein.

In a preliminary study, we used chemically synthesized genistein (Chang et al., 1994) as one of the positive controls and did not obtain any estrogenic response from the mice uterine enlargement assay. However, estrogenic responses from glycitein and DES were apparent. We evaluated the purity of the synthesized genistein and found that it contained a contaminant coeluting in our analytical HPLC system with authentic genistein. A broad melting point thermogram indicating a mixture of genistein and the contaminant was detected by DSC analysis. To avoid this problem, we purified the genistein from soybeans for the present study.

The uterine enlargement assay yielded significant differences ( $p < 0.001$ ) in uterine weights among treatments (Table 1). The glycitein, genistein, and DES groups had 150, 50, and 60% greater uterine weight, respectively, compared with the control. The relative estrogenic potencies of DES, genistein, and glycitein were 100000, 1, and 3, respectively (Table 1). The relative estrogenic potencies of DES, glycitein, and genistein were estimated on the basis of the doses required to produce the same increase of uterine weights. The relative estrogenic potencies of genistein and DES from the present study were similar to the results of Farmakalidis and Murphy (1985), who found the relative molar potencies of DES, genistein, genistin, and daidzin to be 100000, 1, 1, and 0.26, respectively. The present study demonstrated that the estrogenic potency



**Figure 2.** Competitive binding analysis of isoflavones. Cytosol from mouse uteri was incubated with 1.5 ng/mL 17 $\beta$ -(<sup>3</sup>H)-estradiol in the presence of increasing concentrations of unlabeled estrogens.  $n = 2$ .

**Table 2. Relative Affinities of Estrogens for Estrogen Receptors<sup>a</sup>**

compound	CB <sub>50</sub> (μM)	relative affinity
17 $\beta$ -estradiol	0.00109	100
DES	0.00115	95
genistein	0.223	0.49
daidzein	4.00	0.027
glycitein	3.94	0.028

<sup>a</sup> Based on the molar concentrations (CB<sub>50</sub>) required to displace 50% of the (<sup>3</sup>H) estradiol.  $n = 2$ .

of glycitein in mice uterine enlargement assay was 3 times higher than that of genistein, but, as hypothesized, was much lower than that of DES.

It is generally considered that nonsteroidal estrogens exert their stimulatory effect on the estrogen receptor by binding to the same site as that occupied by steroidal estrogens such as 17 $\beta$ -estradiol. Our competitive binding study results are shown in Figure 2, in which increasing concentrations of unlabeled competitors displace bound (<sup>3</sup>H)estradiol. These results confirm that glycitein has the ability to bind to the estrogen receptor. DES had a much higher binding affinity compared with that of the three isoflavones. The relative affinities of these compounds for the mice estrogen receptor were calculated by dividing the CB<sub>50</sub> of unlabeled 17 $\beta$ -estradiol by the CB<sub>50</sub> of a competitor and then multiplying by 100 (Table 2). These data, once again, confirmed that both DES and estradiol had a much greater binding affinity to estrogen receptor than the three isoflavones. Among the three soybean isoflavones, genistein had the greatest estrogen receptor binding affinity. The in vitro estrogen receptor binding data in this study were comparable to the previous results summarized by Verdeal et al. (1980). They reported that genistein had a binding affinity roughly 100 times lower than that of 17 $\beta$ -estradiol, and daidzein had a binding affinity 10–15 times lower than that of genistein.

The plasma extraction and analysis recovery was 85% for internal standard THB. The recovery-corrected plasma isoflavone concentrations for genistein-treated and glycitein-treated group pooled sample were 0.42 and 0.72 μM, respectively. Xu et al. (1994) and King et al. (1995) have reported in humans and in rats that 4–6 h after isoflavone dosing, daidzein and genistein reached maximal concentrations in plasma and then decreased thereafter. At 24 h after dosing, there were only trace concentrations of daidzein and genistein in the plasmas. Because we collected blood 24 h after the last dose of isoflavones, the low plasma isoflavone concentrations may be the result of rapid metabolism or excretion of isoflavones after dosing.



Glycitein gave a much lower *in vitro* binding affinity compared to genistein in the estrogen receptor-binding assay. However, it gave higher estrogenic response in the *in vivo* mouse uterine enlargement assay. This may be the result of a higher bioavailability of glycitein compared to that of genistein in mice. In a human isoflavone metabolic study, a higher bioavailability of glycitein compared with that of genistein was demonstrated (Zhang et al., 1999). Shutt et al. (1970) reported that genistein could be metabolized to *p*-ethylphenol, which is not an estrogenic compound. It may be possible that glycitein is metabolized to compounds with greater estrogenic potency than that of glycitein.

Daidzein has been reported to be a less effective estrogen than genistein when fed to mice (Bickoff et al., 1962). Our present study demonstrated that glycitein is a stronger estrogen in mice uterine enlargement assay compared to genistein. Of the three soy isoflavone aglycons, it appears that glycitein has the highest estrogenic potency in the *in vivo* estrogen assay.

This study has demonstrated the *in vivo* estrogenic activity and the *in vitro* binding affinity to estrogen receptors of glycitein. These data reveal that, although glycitein accounts for ~10% of the total isoflavones in soy foods, its biological potency is comparable to those of the other soy isoflavones. Development of soybean and soy food isoflavone databases needs to include glycitein and its glucosides. Additionally, the biological activity of glycitein and its glucosides in humans needs to be explored.

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