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Cancer Res 2000;60:5704-5709.

[CANCER RESEARCH 60, 5704–5709, October 15, 2000]

Estrogenic and Antiproliferative Properties of Glabridin from Licorice in Human Breast Cancer Cells

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ABSTRACT

There is an increasing demand for natural compounds that improve women's health by mimicking the critical benefits of estrogen to the bones and the cardiovascular system but avoiding its deleterious effects on the breast and uterus. The estrogenic properties of glabridin, the major isoflavan in licorice root, were tested in view of the resemblance of its structure and lipophilicity to those of estradiol. The results indicate that glabridin is a phytoestrogen, binding to the human estrogen receptor and stimulating creatine kinase activity in rat uterus, epiphyseal cartilage, diaphyseal bone, aorta, and left ventricle of the heart. The stimulatory effects of 2.5–25 μg/animal glabridin were similar to those of 5 μg/animal estradiol. Chemical modification of glabridin showed that the position of the hydroxyl groups has a significant role in binding to the human estrogen receptor and in proliferation-inducing activity. Glabridin was found to be three to four times more active than 2′-*O*-methylglabridin and 4′-*O*-methylglabridin, and both derivatives were more active than 2′,4′-*O*-methylglabridin. The effect of increasing concentrations of glabridin on the growth of breast tumor cells was biphasic. Glabridin showed an estrogen receptor-dependent, growth-promoting effect at low concentrations (10 nM–10 μM) and estrogen receptor-independent antiproliferative activity at concentrations of >15 μM. This is the first study to indicate that isoflavans have estrogen-like activities. Glabridin and its derivatives exhibited varying degrees of estrogen receptor agonism in different tests and demonstrated growth-inhibitory actions on breast cancer cells.

INTRODUCTION

The importance of estrogens in homeostatic regulation of many cellular and biochemical events is well illustrated by the pathophysiological changes that occur with estrogen deficiency (1, 2). Estrogen is active in the development of the mammary gland and the uterus, in maintaining pregnancy and bone density, in protecting from cardiovascular diseases, and in relieving menopausal symptoms (2). However, estrogen can also stimulate malignant growths and thus contributes to the development of estrogen-dependent tumors, such as breast cancer and hyperplasia of the uterus (3).

Breast cancer is the most common malignancy among women in Western society, and over the past decades its incidence rates have increased steadily (4). It is estimated that approximately one of nine women in the United States will develop breast cancer during their lifetime, and it is the leading cause of death among American women 40–55 years of age (5). Experimental, clinical, and epidemiological evidence indicates that ovarian hormones play a major role in the growth and differentiation of normal breast tissues and the development and progression of breast cancer (6). Estrogens can support growth in estrogen-responsive target tissues, including the breast (7), and thus can influence the risk of developing cancer. In addition to estradiol (the natural ligand), a wide variety of nonsteroidal compounds, including tamoxifen (8), have been studied, which have varying effects as agonists or antagonists, depending on the particular organ system or gene examined (5, 3, 9). Hence, identifying natural compounds that act as antagonists of estrogen in breast tissue and as agonists in bone and cardiovascular tissues would be beneficial.

Phytoestrogens are natural compounds derived from plants, which exhibit estrogen-like activities (10, 11). They can be divided into the subclasses lignans, isoflavonoids, and coumestans. They are widely distributed in oil seeds, vegetables, and soybeans and hence are part of the normal human diet. Studies show a correlation between diet and major cancers (12). Epidemiological evidence indicates that soy intake is associated with lower breast cancer risk in women (13, 14) and prolonged menstrual cycle length (11). Soybeans contain high amounts of the two isoflavonoids daidzein and genistein (100–300 mg/100 g), which, like lignans, have been found to possess weak estrogenic activity, ranging from 500 to 15,000 times less than that of estradiol (15–17). Japanese women whose diet is rich in isoflavonoids showed a very low incidence of breast cancer (18). *In vivo* experiments in rats have demonstrated that genistein can prevent breast cancer (19).

There is also a good correlation between diet and diseases of the bone and heart (10, 11, 20). Osteoporosis affects >25 million women, causing some 250,000 hip fractures yearly (21). Genistein is reported to prevent cancellous bone loss and to maintain or to increase bone density in postmenopausal women (22). Estrogen is also beneficial in reducing the risk of cardiovascular disease (1, 23). The incidence of heart diseases among premenopausal women is low compared with that in males, whereas among postmenopausal women incidence approaches that of males. Isoflavones reduced low-density lipoprotein and very low-density lipoprotein cholesterol concentrations and caused an increase in high-density lipoprotein cholesterol in females (24).

Isoflavans are a subclass of the flavonoid compounds, containing ring A fused to ring C, which is connected to ring B through carbon 3 (Fig. 1). Several functional groups may be attached to this basic skeleton, mainly hydroxyl groups. In the isoflavan subclass, the heterocyclic ring C does not contain a double bond between carbons 2 and 3 or a carbonyl group attached to carbon 4. This structure does not allow conjugation of the double bonds between rings A and B.

Several isoflavans from the licorice root that presented antioxidant activity have been isolated in our laboratory. Of these, glabridin is the major constituent (11%) of the alcohol extract (25). Its lipophilicity and its structural similarity to estradiol led us to test it for estrogenic-like activities. In the present study, the properties of newly identified phytoestrogenic compounds, the isoflavans, were investigated by comparing their ability to bind to the human ER[2] and their effect on estrogen-responsive human breast cancer cells over a broad range of concentrations. *In vivo* studies included the effects of glabridin on rat uterus wet weight and on the induction of the immediate early "estrogen-induced protein" creatine kinase B in rat skeletal and cardiovascular tissues as well as uterus. Chemical modifications were performed to shed some light on the binding and antiproliferation mechanisms involved. Our results indicate that glabridin bound to the human ER exhibited varying degrees of ER agonism *in vitro* and *in*

Received 1/26/00; accepted 8/16/00.

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[2] The abbreviations used are: ER, estrogen receptor; CK, creatine kinase; 2′-*O*-MG, 2′-*O*-methylglabridin; 4′-*O*-MG, 4′-*O*-methylglabridin; 2,4′-*O*-MG, 2,4′-*O*-dimethylglabridin; C-SFCS, charcoal-stripped FCS; XTT, 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxanilide inner salt.

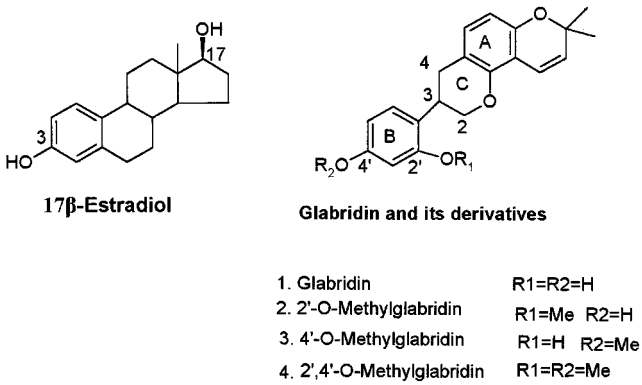

Fig. 1. Structures of 17β-estradiol, the isoflavan glabridin, and its derivatives.

vivo, and that it demonstrated estrogen-independent inhibitory activity on the growth of breast cancer cells.

MATERIALS AND METHODS

Chemicals and Reagents. 17β-Estradiol was purchased from Sigma (St. Louis, MO), and [^{3}H]17β-estradiol for competition assay was from New England Nuclear (Boston, MA). Leibovitz L-15, FCS, RPMI 1640, trypsin-EDTA, L-glutamine, HEPES buffer, penicillin-streptomycin, sodium piruvate solutions, and the XTT reagent cell proliferation kit were all purchased from Biological Industries (Beth Haemek, Israel).

Glabridin and Its Derivatives. Glabridin and 4′-*O*-MG were isolated from the acetone extract of the roots of *Glycyrrhiza glabra*. 2′-*O*-MG and 2′,4′-*O*-MG were synthesized from glabridin (26, 27).

Human Breast Cancer Cells. Different lines of human breast cancer cells (T-47D, MCF-7, and MDA-MB-468) were purchased from the American Type Culture Collection (Manassas, VA). The cells were grown in DMEM supplemented with 2 μg/ml insulin, 1 mM sodium pyruvate, 1 mM nonessential amino acids, 4 mM glutamine, 10% FCS, and antibiotics (penicillin-streptomycin). One week before experiments, cells were transferred to phenol red-free medium supplemented with 5% C-SFCS.

Cell Proliferation. Cells were seeded into 96-well tissue culture plates (5000 cells/well) in 5% C-SFCS-supplemented RPMI 1640 phenol red-free medium (T-47D cells) or 5% C-SFCS-supplemented Leibovitz L-15 medium (MDA-MB-468 cells) and incubated at 37°C for 48 h. The medium was then removed, and fresh media with test compounds were added (control contained 0.1% ethanol). The medium was changed every 3 days. To evaluate relative cell concentration, XTT reagent was used. Absorbance was measured at 450 nm using a Spectra II spectrophotometer (SLT-Labinstrument, Austria).

Colony Formation in Soft Agar. MCF-7 cells were plated onto soft agar plates in the presence of various concentrations of the test compounds for 3 weeks and assayed for colony formation. Cells (10^3) were first suspended in 0.15 ml of medium (MEM supplemented with 2 μg/ml insulin and 5% C-SFCS) containing 0.3% agar. The mixture was added over a layer of 0.5% agar in MEM on a 24-well plate. Plates were fed weekly and after 3 weeks were stained with vital stain 2-(*p*-isodiphenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride hydrate. Colonies >0.15 mm diameter were scored.

Estrogen Receptor Binding Assay. Test compounds were prepared in 100% ethanol, and the stock solutions were diluted in 1% C-SFBS in RPMI 1640. Control tubes contained 0.4% ethanol (0.1% final concentration in incubation). Triplicate 50-μl aliquots of test compounds were added to 50 μl of [^{3}H]17β-estradiol (100 Ci/mmol) diluted in 1% C-SFBS to a concentration of 0.4 nM. The test tubes were equilibrated at 37°C while the cells were prepared. T47D cells were fed with 1% C-SFBS in RPMI 1640 (containing 0.2 ng/ml insulin), without phenol red, at least 2 days before assay. Cells were removed with trypsin-EDTA and diluted in 1% C-SFBS RPMI 1640 to 3×10^6 cells/ml. One hundred μl of diluted cells and [^{3}H]estradiol were added to the test compounds. The tubes were mixed gently and incubated at 37°C for 1 h. After incubation the cells were sedimented by centrifugation at 3000 rpm for 5 min at 4°C. After removal of the supernatant, the cells were washed once with ice-cold TPSG (0.2% Triton X-100 and PBS containing 0.1 M sucrose and 10% glycerol). Tubes were vigorously vortexed in fresh TPSG and incubated for 5 min. Intact nuclei were sedimented by centrifugation at 3000 rpm for 5 min at 4°C. The supernatant was aspirated, and [^{3}H]estradiol remaining in the nuclei was measured by a beta counter (16). Results are presented as percent [^{3}H]estradiol binding to ER in the nucleus in the absence (control = 100%) or presence of increasing concentrations of test compounds.

***In Vivo* Experiments.** Twenty-five-day-old Wistar-derived female rats at a weight of ~60 g were housed in metal cages in groups of five per cage and maintained on a 14-h light, 10-h dark cycle at 23°C. Access to food and tap water was *ad libitum*.

The animals were injected with 0.5 ml of PBS containing test compounds dissolved in ethanol or ethanol as a control. The final concentration of ethanol in PBS was 1%. After 24 h, the animals were killed, and the uterus was removed through a midline incision. The wet uterine weight was determined. In addition, the aorta, left ventricle, diaphysis, and epiphysis of the femur were removed, and all organs were frozen at −20°C for later analysis of CK activity.

CK Activity. Frozen organs were collected in cold isotonic extraction buffer (0.25 M sucrose, 0.05 M Tris, 0.4 mM EDTA, 2.5 mM DTT, and 5 mM sodium acetate) and homogenized in a Polytron homogenizer (Kinematica, Lucerne, Switzerland) Homogenates were centrifuged at 14,000 × *g* for 5 min at 4°C. The supernatant was tested for CK activity in a Kontron 922 Uvicon spectrophotometer at 340 nm, using a coupled assay for ATP, as described by Somjen *et al.* (28). Protein was determined by Coomassie brilliant blue.

Statistical Analysis. Statistical significance was determined by ANOVA.

RESULTS

Glabridin Binds to the Human ER. The structures of glabridin and estradiol are shown in Fig. 1. Several features are common to both: an aromatic ring substituted with hydroxyl group at the *para* (glabridin) or 3 position (estradiol), with additional three fused rings of phenanthrene shape. Both molecules are relatively lipophilic, containing a second hydroxyl group, although not at the same position (17β in estradiol and 2′ in glabridin). These structural similarities prompted us to test whether glabridin can interact with the ER. Competition binding studies were performed by using extracts of T47D cells, known to contain the ER. Glabridin competed for binding of a single saturating concentration of [^{3}H]estradiol to ER (Fig. 2). The degree of inhibition is dose dependent, related to glabridin concentration. The IC_{50} for glabridin was ~5 μM, indicating that it is a relatively weak ligand for the receptor. Nevertheless, this IC_{50} value is similar to values of other known phytoestrogens, such as genistein (16), which is ~10^4 lower than that of estradiol.

Biphasic Effects of Glabridin on Proliferation of Breast Cancer Cells. The effects of increasing concentrations of glabridin on cell growth are shown in Fig. 3*A*. Cell growth was found to be biphasic.

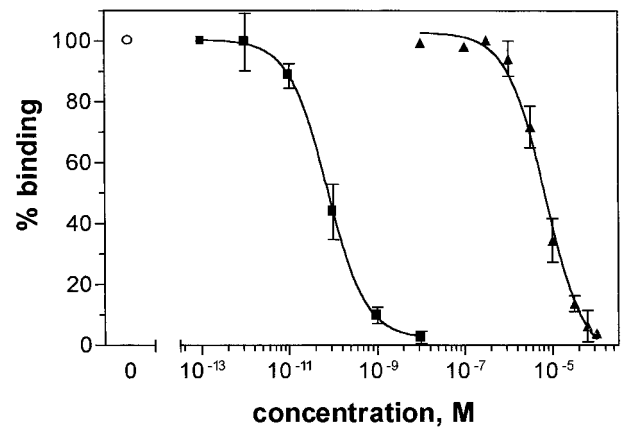

Fig. 2. Competition of glabridin for ER with [^{3}H]17β-estradiol in T47D cells. Cells were incubated with [^{3}H]17β-estradiol and increasing concentrations of glabridin (▲), 17β-estradiol (■), or 0.1% of ethanol as a control (○). Radioactivity in cell nuclei was counted and plotted as the percentage of control. Values are means of three or more experiments; *bars*, SD.

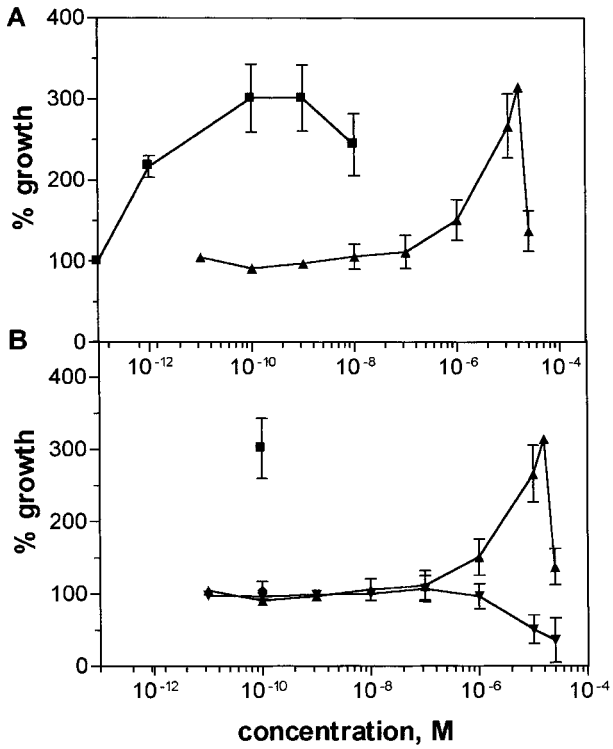

Fig. 3. *A*, effect of 17β-estradiol and glabridin on the growth of estrogen-responsive human breast cancer cells. T47D (ER+) cells were incubated with increasing concentrations of 17β-estradiol (■) or glabridin (▲) for 7 days. Proliferation was tested using the XTT cell proliferation reagent. Results are presented as the percentage of controls (0.1% ethanol). Values are means of three or more experiments; *bars*, SD. *B*, dose-response curves of T47D (ER+; ▲) and MDA (ER−; ▼) human breast cells to glabridin. Cells were exposed to glabridin for 7 days. Proliferation was tested using the XTT cell proliferation reagent. Values are means expressed as the percentage of control in three or more experiments; *bars*, SD.

Glabridin stimulated growth over a range of 0.1–10 μM, reaching maximum levels at ~10 μM. The maximum growth stimulation by glabridin was equal to that of estradiol at 0.1–10 nM. In contrast to its growth-promoting effects at lower concentrations (<10 μM), glabridin inhibited cell growth at concentrations of >15 μM. To differentiate the estrogenic agonist activities of glabridin from its antiproliferative effects, a dose-response experiment of glabridin with ER− (MDA-MB-468) and ER+ (T47D) human breast cancer cell lines was performed. The proliferation rate of T47D cells is known to be sensitive to estrogens (16), and our results confirm that in ER+ cells, the growth of cells increased above control with ~0.1–10 μM glabridin and then was abruptly inhibited at ~25 μM glabridin. In the ER− breast cancer cell line MDA-MB-468, glabridin did not increase cell growth, but at 25 μM its inhibitory effect appeared, as in the ER+ cells (Fig. 3*B*).

Effect of Glabridin on Growth of Estradiol-stimulated Breast Cancer Cells. The effect of glabridin on estradiol-stimulated breast cancer cells was tested in T47D ER+ cells over 7 days. Cells were treated with a single growth-promoting concentration (100 pM) of estradiol and with different concentrations of glabridin. Fig. 4 shows that glabridin alone, tested over a broad concentration range (1 nM–25 μM), had a biphasic effect on TD47 cell growth (as also shown in Fig. 3, *A* and *B*). Glabridin had no effect on the growth-promoting activity of 100 pM estradiol over a concentration range of 1 nM–0.1 μM, but the pronounced growth-inhibiting action of glabridin (250–100%) over 15 μM was not modified by the presence of estradiol.

Effect of Glabridin on Growth of Tamoxifen-inhibited Breast Cancer Cells. The possible antiestrogen effect of glabridin was tested on tamoxifen-arrested proliferation of breast tumor cells. Different concentrations of glabridin were tested on T47D ER+ cells treated with a single growth-inhibiting concentration (1 μM) of tamoxifen, over 7 days, in comparison with estradiol. The dose of tamoxifen was chosen on the basis of the levels reported in women receiving tamoxifen for the prevention or treatment of breast cancer. Tamoxifen at 1 μM inhibited the growth of 0.1 nM estrogen-treated ER+ breast cancer cells to the level of the control cells (Fig. 5). Glabridin alone, over a broad concentration range of 10 nM–25 μM, had a biphasic effect on T47D cell growth. When glabridin was added to the tamoxifen-treated cells, the dose-response curve seen with glabridin alone shifted approximately by 1 log to the right. This response explains the higher amounts of glabridin required for displacing tamoxifen from the ER sites and activating cell proliferation. Thus, 1 μM tamoxifen inhibited the maximum growth of ER+ breast cancer cells treated with glabridin (10 μM) by ~50%. An increase in tamoxifen concentration to 5 μM inhibited the proliferative effect of glabridin to control levels (data not shown). Tamoxifen did not block the growth-inhibiting effect of a high dose (25 μM) of glabridin.

Effect of Glabridin on Anchorage-independent Growth of MCF-7 Cells. The effects of increasing concentrations of glabridin on colony formation were also tested. Its effect was biphasic, like its

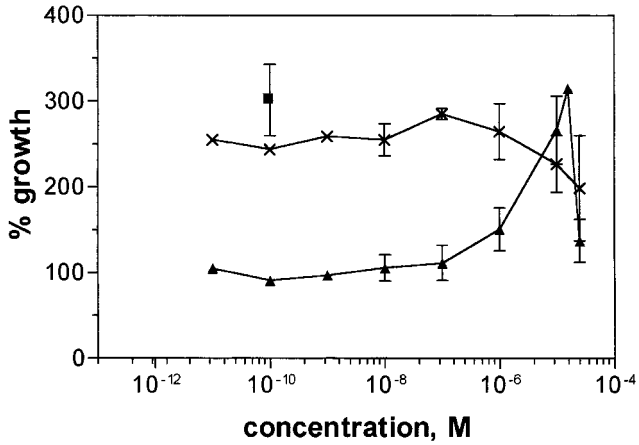

Fig. 4. Effect of glabridin on growth of T47D cells treated with 17β-estradiol. Cells were exposed to increasing concentrations of glabridin in the absence (▲) or presence (×) of 100 pM 17β-estradiol for 10 days. Proliferation was tested using the XTT cell proliferation reagent. ■, growth of cells treated by 100 pM 17β-estradiol alone. Results are presented as the percentage of control (0.1% ethanol; means; $n \geq 3$); *bars*, SD.

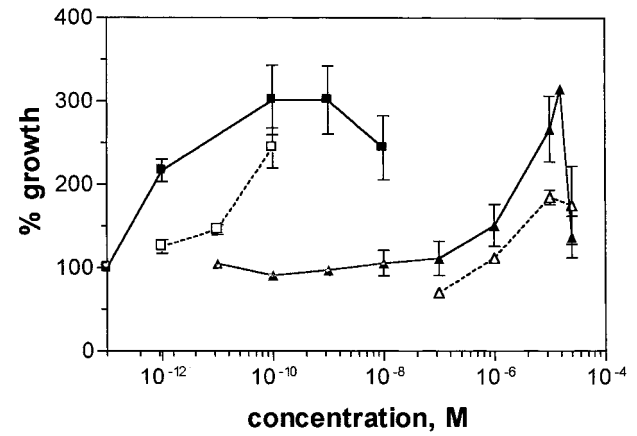

Fig. 5. Effect of glabridin on tamoxifen-treated T47D cells. Cells were exposed for 7 days to increasing concentrations of test compound in the absence (▲, glabridin; ■, 17β-estradiol) or presence (△, glabridin; □, 17β-estradiol) of tamoxifen (1 μM). Proliferation was tested using the XTT cell proliferation reagent. Results are presented as the percentage of control (0.1% of ethanol; means; $n = 3$); *bars*, SD.

effect on cell proliferation. When grown in suspension in 0.3% agar in complete medium, cells formed large colonies in the presence of 10 μM glabridin (Fig. 6 and Table 1) or 10 nM estradiol. In contrast to its promotion of colony formation at lower concentrations, glabridin inhibited anchorage independent growth at concentrations of 25 μM. When glabridin was tested in the presence of 10 nM estradiol, it had no effect on the anchorage-independent, growth-promoting effects of estradiol. The pronounced growth-inhibiting action of glabridin at concentrations of ≥25 μM reached control levels and was not modified by estradiol (Fig. 6 and Table 1).

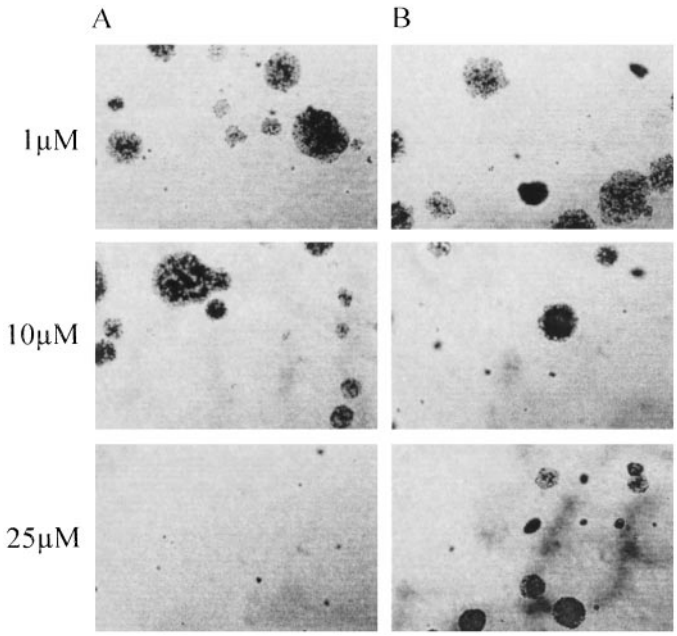

Fig. 6. Effect of glabridin on anchorage-independent growth of MCF-7 cells. MCF-7 cells were plated onto soft agar plates in the presence of increasing concentrations of glabridin with and without 10 nM estradiol. Colony formation was observed after 3 weeks. *A*, increasing concentrations of glabridin (1, 10, and 25 μM). *B*, increasing concentrations of glabridin (1, 10, and 25 μM) in the presence of 1 nM estradiol.

Table 1 *Effect of increasing concentrations of glabridin on anchorage-independent growth of MCF-7 cells*

MCF-7 cells were plated onto soft agar plates in the presence of increasing concentrations of glabridin, with and without 10 nM estradiol. Colonies >0.15 mm were counted after 3 weeks.

Treatment	Colonies
Estradiol, 10 nM	11 ± 4
Glabridin, 10 μM	7 ± 3
Glabridin, 25 μM	3 ± 1
Glabridin, 35 μM	1 ± 0.8
Estradiol, 10 nM + glabridin, 10 μM	8 ± 2
Estradiol, 10 nM + glabridin, 25 μM	4 ± 2
Estradiol, 10 nM + glabridin, 35 μM	0

Table 3 *Glabridin stimulation of uterus wet weight in female rats*

Rats were killed 24 h after injection with 5 μg of estradiol or 200 μg of glabridin, and wet uterus weight was determined. Results are presented as wet uterus weight ± SD.

Treatment	Wet uterus weight (mg)
Control	57.80 ± 4.97
Estradiol (5 μg/animal)	90.52 ± 19.45[a]
Glabridin (200 μg/animal)	78.60 ± 19.42[a]

[a] One-way ANOVA ($P < 0.05$).

Tissue-selective Action of Glabridin *in Vivo*. Injection of estradiol (5 μg) or glabridin (2.5, 25, 200, and 250 μg) into prepubertal female rats resulted in a significant increase in CK activity in rat uterus, epiphyseal cartilage, diaphyseal bone, and cardiovascular tissues, measured after 24 h (Table 2). CK activity is known to be induced by estrogens *in vivo* and *in vitro* (29, 30). Our results showed that estradiol, at 5 μg/rat, stimulated CK activity to the same level as glabridin at 2.5 μg/rat in the diaphysis and aorta and at 25 μg/rat in the uterus and left ventricle. Glabridin had a weaker effect on the stimulation of CK activity in the left ventricle (1.43 ± 0.13 experimental/control) than estradiol (3.36 ± 0.7 E/C), which may be attributable to tissue specificity. Glabridin (200 μg/animal) and estradiol (5 μg/animal) caused an increase in uterus wet weight to 78.6 ± 19 and 90.5 ± 19 mg, respectively, compared with 57.8 ± 5 mg in control (Table 3).

Structure-Activity Relationship Studies. The influence of modifications to the structure of glabridin on its estrogen-like activities was studied. The binding and proliferation properties of natural and semisynthetic glabridin derivatives were tested. The structure of 4′-*O*-MG resembles that of glabridin, with one hydroxyl at position 4′ blocked with a methyl group, leaving the second hydroxyl group at position 2′ free. Both 2′-*O*-MG and 2′,4′-*O*-MG are semisynthetic products, synthesized from glabridin (25), one with the hydroxyl at position 2′ blocked and that at position 4′ free and the other with both hydroxyl groups blocked. Using these derivatives, the influence of the hydroxyl groups of glabridin was examined. The binding of a single subsaturating concentration (0.1 nM) of radiolabeled estradiol to ER in intact human breast cancer cells is shown in Fig. 7*A*. Competition studies were performed using extracts of T47D cells (ER+). The binding affinities of 2′-*O*-MG and 4′-*O*-MG to ER were ~10 times lower than those of glabridin. 2′,4′-*O*-MG, with both hydroxyl groups blocked, did not bind to the human ER. These results indicate that both hydroxyl groups contribute to the binding capacity, and when both are blocked, binding to the human ER significantly diminishes.

The effects of increasing concentrations of glabridin derivatives on cell growth were compared with those of glabridin. Cell growth was tripled by 10 μM glabridin, but 10 μM 4′-*O*-MG and 50 μM 2′-*O*-MG were not as effective, causing only a 50% increase in growth. No effect on cell proliferation was observed by 2′,4′-*O*-MG. Glabridin at 25 μM markedly inhibited growth, whereas 2′-*O*-MG and 4′-*O*-MG inhibited the growth of the human breast cancer cells only at ~100 μM (Fig. 7*B*).

Table 2 *Glabridin induction of creatine kinase activity in various female rat tissues*

Rats were killed 24 h after injection with 5 μg of estradiol or 2.5, 5, 200, or 250 μg of glabridin. CK activity was assayed as described in "Materials and Methods." Results are presented as increase fold of enzyme activity (experimental/control).

Tissue	Control	17β-Estradiol (5 μg/animal)	Glabridin (2.5 μg/animal)	Glabridin (25 μg/animal)	Glabridin (250 μg/animal)
Uterus	1.0 ± 0.20	1.55 ± 0.15[a]	1.32 ± 0.70[a]	1.53 ± 0.09[a]	1.63 ± 0.06[b]
Diaphysis	1.0 ± 0.13	1.61 ± 0.18[b]	1.75 ± 0.17[a]	2.29 ± 0.23[b]	2.12 ± 0.13[b]
Epiphysis	1.0 ± 0.90	1.37 ± 0.16[a]	1.27 ± 0.10[a]	2.02 ± 0.12[b]	1.79 ± 0.03[b]
Aorta	1.0 ± 0.04	1.36 ± 0.18[a]	2.32 ± 0.08[b]	2.63 ± 0.18[b]	2.35 ± 0.11[b]
Pituitary	1.0 ± 0.07	2.00 ± 0.14[b]	1.34 ± 0.15[a]	1.31 ± 0.08[a]	1.56 ± 0.05[b]
Left ventricle	1.0 ± 0.11	3.36 ± 0.70[b]	1.43 ± 0.13[a]	2.19 ± 0.07[b]	1.91 ± 0.12[b]

[a] One-way ANOVA ($P < 0.05$).
[b] One-way ANOVA ($P < 0.01$).

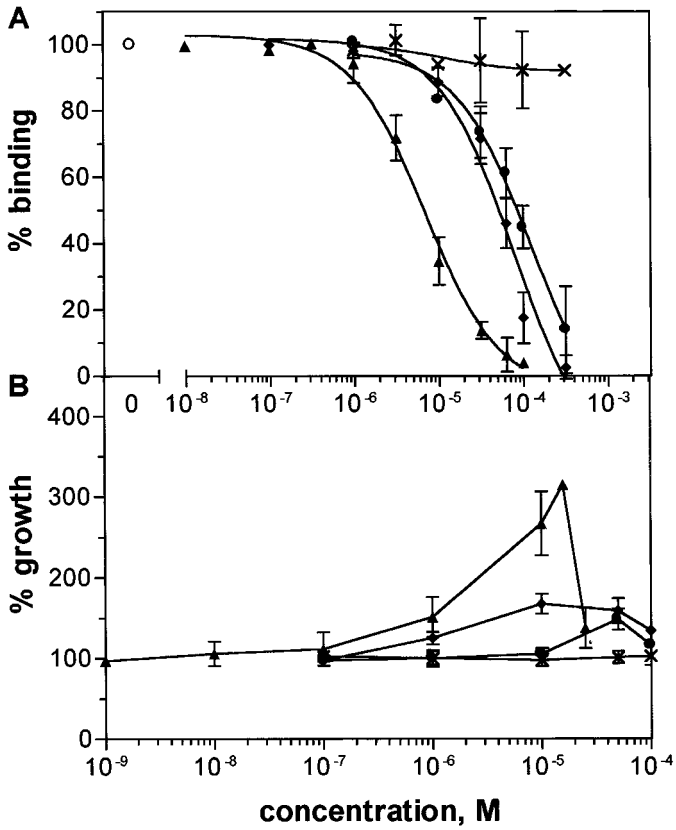

Fig. 7. *A*, competition of glabridin derivatives for ER with [^{3}H]17β-estradiol in T47D cells. Cells were incubated with [^{3}H]17β-estradiol and increasing concentrations of glabridin derivatives (○, control; ▲, glabridin; ♦, 4′-*O*- MG; ●, 2′-*O*-MG; ×, 2,4′-*O*-MG). Radioactivity in cell nuclei was counted and plotted as the percentage of control. Values are means ($n \geq 3$); *bars*, SD. *B*, effect of glabridin derivatives on the growth of estrogen-responsive breast cancer cells. T47D (ER+) cells were incubated with increasing concentrations of test compound (○, control, 0.1% ethanol; ▲, glabridin; ♦, 4′-*O*- MG; ●, 2′-*O*-MG; ×, 2,4′-*O*-MG) for 7 days. Proliferation was estimated using the XTT cell proliferation reagent. Results are presented as the percentage of control (0.1% ethanol; means; $n \geq 3$); *bars*, SD.

DISCUSSION

In the present study we characterized glabridin, a novel phytoestrogen isolated from licorice extract. Glabridin and its derivatives bind to the human ER and were found to act as an estrogen agonist in the induction of an estrogen response marker, such as CK activity, *in vivo*, to induce uterus wet weight, and to stimulate human breast cancer cell growth.

Glabridin bound to the human ER with about the same affinity as genistein, the best known phytoestrogen, 10^4 times lower than estradiol (16, 31). It not only competed with ^{3}H-labeled estradiol in binding the human ER but also enhanced the proliferation of estrogen-dependent human breast cancer cells *in vitro*. Growth stimulation of ER+ cells by glabridin closely correlated to its binding affinity to the ER. Stimulation of cell proliferation was optimal at a concentration at which about half of the ER sites were saturated. The concentrations in which we observed the proliferative effects of glabridin (100 nM–10 μM) are well within the reported *in vitro* range of other phytoestrogens, such as genistein, diadezein, and resveratrol from grapes (32–35).

To provide some more insight into what effect glabridin has on breast tumor cells stimulated by estradiol and cell proliferation arrested by antiestrogen, we treated cells with glabridin in the presence of estradiol or tamoxifen. We found that glabridin, like genistein, had little effect on the growth-promoting effect of estradiol in the range of 0.1 nM–1.0 μM. Tamoxifen at 1 μM inhibited the optimal growth of cells treated with glabridin by 50%, and at 5 μM the effect of glabridin was blocked completely. This suggests that the growth-promoting effect of glabridin, like that of other phytoestrogens, is ER mediated (15, 36).

To further confirm that glabridin is a phytoestrogen acting via an ER mechanism, we evaluated *in vivo* the stimulation of CK activity in estrogen-responsive tissues. This specific activity, as a sensitive and rapid postreceptor response marker, was used in other ER-containing cells, such as skeletal cells, containing a low concentration of steroid hormone receptors (30). The brain type isoenzyme of CK, the major component of the estrogen-induced protein of rat uterus, is part of the energy buffer system that regenerates ATP from ADP and has been a useful marker for the action of steroids and their analogues (30). Our results demonstrated that the administration of 25 μg/rat glabridin doubled CK activity in skeletal and cardiovascular tissues. These results not only confirm that glabridin acts through the ER but also suggest that it has the potential to mimic the beneficial activities of estrogen in bone and cardiovascular tissues.

It was also shown *in vivo* that glabridin acts as estrogen agonist in the uterus. Two hundred μg/rat glabridin increased the uterine wet weight to the same extent as 5 μg of estradiol. The determination of uterine wet and/or dry weights has also been used to demonstrate estrogenic activity by other phytoestrogens (37, 38). Markaverich *et al.* (39) reported that an increase in uterine wet and dry weight in ovariectomized animals induced by coumesterol is not indicative of uterine hyperplasia, as determined by a doubling in DNA content, but reflects an increase in water and protein content. Therefore, the potential estrogenicity of glabridin requires reassessment before defining the relationships between glabridin exposure and neoplasia in uterine endometrium.

In contrast to the ER-regulated, growth-promoting effects of glabridin at concentrations ranging from 100 nM to 10 μM, higher concentrations (>10 μM) abruptly inhibited the proliferation of ER+ and ER− breast cancer cells. The same biphasic effect was demonstrated in the anchorage-independent growth of human breast cancer cells in soft agar. Interestingly, neither estradiol nor tamoxifen reversed the antiproliferative effect of glabridin. These results are consistent with those previously reported (32, 33), observing growth stimulation by genistein in a concentration-dependent manner between 10 nM and 1 μM and growth inhibition of MCF7 cells at concentrations of >10 μM. Fioravanti *et al.* (40) and Shao *et al.* (41) reported that genistein-treated cells accumulated in S and G_2-M and underwent apoptosis. On the other hand, in preliminary results, glabridin treatment using two different methods suggested that apoptosis may not be involved (data not shown). The most plausible explanation for this biphasic effect of glabridin on human breast cancer cells is not only that it mediates its actions not only via the ER as an estrogen agonist but also that at higher concentrations it interacts with other ER-independent cellular mechanisms to inhibit cell proliferation induced by glabridin via ER pathways. Recent studies have observed antiproliferative effects of genistein in other, non-breast carcinoma cell lines (42). In the present study, glabridin inhibited the growth of ER− cells (MDA-MB-468), supporting the hypothesis that the actions of phytoestrogens on ER and on cell growth inhibition occur via different molecular mechanisms (36, 41, 43). Some studies suggest that high concentrations of phytoestrogens may function as estrogen antagonists and inhibit cell growth by competing with estradiol on binding to the ER site (44). In the present study, glabridin overrode the growth-inhibitory effects of tamoxifen, demonstrating that the inhibitory action of glabridin on tumor growth is different from that of other known antagonists, such as tamoxifen, because the mechanism

of its action is not ER dependent. Further studies for understanding the mechanism are required.

To shed some light on the role of the two hydroxyl groups attached to the glabridin molecule in its ability to bind to the human ER and in its growth-promoting effect, natural and semisynthetic glabridin derivatives were tested. Our results showed that, among the isoflavans examined, glabridin has higher affinity to ER and also showed optimal cell growth stimulation. Weaker estrogen agonists than glabridin, 2′-*O*-MG and 4′-*O*-MG, were nearly as potent as glabridin as growth inhibitors. However, 2′,4′-*O*-MG did not bind to the human ER and demonstrated no proliferative activity. This suggests that the two hydroxyl groups in the glabridin are essential to binding and to promoting cell growth. The agonist effects were higher when both hydroxyl groups were present than with a single group. The data also demonstrate that the growth-inhibitory effects of these compounds are not related to their binding or proliferative capability. Previous reports on the involvement of the two hydroxyl groups of estradiol in binding to the human ER demonstrated that both hydroxyl groups 3 and 17β are required for binding (45, 46). Our data suggest that hydroxyl 4′ of glabridin may have the same role as hydroxyl 3 of estradiol. Glabridin lacks the additional hydroxyl group of estradiol at position 17β, but it has an ether oxygen on a parallel position, which could contribute a weaker hydrogen bond to histidine 524 at the ligand-binding domain.

The present study demonstrates for the first time that the isoflavan glabridin is a new phytoestrogen. It bound to human ER and activated CK in estrogen-responsive tissues *in vivo*. The results also reveal that glabridin inhibits the growth of breast cancer cells independently of ERs. This suggests that isoflavans may serve as natural estrogen agonists in preventing the symptoms and diseases associated with estrogen deficiency.

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