Interactions of Phytoestrogens with Estrogen Receptors α and β (III). Estrogenic Activities of Soy Isoflavone Aglycones and Their Metabolites Isolated from Human Urine

Junei KINJO,*[,a] Ryota TSUCHIHASHI,[b] Keiko MORITO,[c] Toshiharu HIROSE,[c] Tohru AOMORI,[c] Tsuneatsu NAGAO,[a] Hikaru OKABE,[a] Toshihiro NOHARA,[b] and Yukito MASAMUNE[c]

[a] *Faculty of Pharmaceutical Sciences, Fukuoka University; 8–19–1 Nanakuma, Jonan-ku, Fukuoka 814–0180, Japan:* [b] *Faculty of Pharmaceutical Sciences, Kumamoto University; 5–1 Oe-honmachi, Kumamoto 862–0973, Japan: and* [c] *Faculty of Pharmaceutical Sciences, Kanazawa University; 13–1 Takara-machi, Kanazawa, Ishikawa 920–0934, Japan.*
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Two glucuronides (4′-*O*-, and 7-*O*-) and a glucuronyl (7-*O*-) sulfate (4′-*O*-) of genistein, two glucuronides (4′-*O*-, and 7-*O*-) and a glucuronyl (7-*O*-) sulfate (4′-*O*-) of daidzein, 7-*O*-glucuronides of glycitein, dihydrodaidzein and *O*-desmethylangolensin were isolated from the urine of volunteer subjects fed soy bean curds (Tofu). The estrogenic activities, *i.e.*, i) the effect on the estrogen-dependent growth of MCF-7 cells, ii) the binding ability to human estrogen receptors (hERs) α and β, and iii) the effect on hER-dependent β-galactosidase induction, of these isoflavone metabolites were examined. Two synthetic isoflavone aglycones (dihydrodaidzein and *O*-desmethylangolensin) and four synthetic sulfates (4′-*O*- and 4′-, 7-di-*O*-) of genistein and daidzein were also studied for their estrogenic activities for the purpose of comparison. With respect to estrogenic acivity, the tested isoflavone metabolites were classified into three groups. The first group shows a very poor stimulatory effect toward the growth of MCF-7 cells, binding activity, and β-galactosidase induction. The sulfates belong to this group. The second group shows a moderate binding activity but poor stimulation and β-galactosidase induction. Some glucuronyl conjugates belong to this group. The last group shows a moderate stimulation and β-galactosidase induction but poor binding activity. A mixed type of conjugates having glucuronyl and sulfony moieties belong to this group.

Key words phytoestrogen; isoflavone metabolite; human estrogen receptor (hER); hER binding; hER-dependent β-galactosidase induction; hER-dependent MCF-7 cell growth

The intake of weakly estrogenic isoflavonoids (phytoestrogens) is high in countries with a low incidence of estrogen-related cancers, such as breast and prostate cancers.[1] Two ERs (α and β) have been identified to date and the physiological responses of estrogens are known to be mediated within specific tissues by at least these two receptors.[2,3] The ERs are a 3A member of the nuclear hormone receptor family and act as a ligand-activated nuclear transcription factor.[4] In a previous paper, we reported the results of systematic examination of the estrogenic activities of soy isoflavones (*e.g.* daidzin, genistin and glycitin) and their metabolites (*e.g.* daidzein, genistein, glycitein, equol, dihydrogenistein and dihydroglycitein) by enteric bacteria,[5] and the activities of several other isoflavone derivatives isolated from *Pueralia lobata* and *P. thomsonii*.[6]

Usually, when soy foods are consumed, the soy isoflavones are metabolized into their aglycones and the related compounds by enteric bacteria. We have proved in *in vitro* experiments that the soy isoflavone glycosides are less estrogenic than the metabolites, *i.e.*, they are activated by enteric bacteria. The metabolites produced by enteric bacteria are absorbed through the intestinal membranes and transported to the liver where they undergo re-metabolization by hepatic enzymes. To evaluate the bioactive compounds in soy products, investigation of the compounds which are actually absorbed within the body is necessary.[7,8]

Herein, we describe the results of an investigation on the estrogenic activities of soy isoflavone metabolites isolated from human urine samples. Their effects on the estrogen dependent growth of MCF-7 cells,[9,10] on human ERs (hERs α and β)-dependent β-galactosidase induction, and their binding behavior to hERs were studied. We also examined the estrogenic activities of some synthetic isoflavone sulfates for the purpose of comparison.

MATERIALS AND METHODS

Chemicals [2,4,6,7-^{3}H(N)]-17β-Estradiol (72 Ci/mmol) was purchased from Dai-Ichi Pure Chemicals Co. Ltd. RPMI 1640 medium (with or without phenol red), fetal bovine serum (FBS), trypsin/EDTA, Dulbecco's phosphate-bufferd saline (PBS), and kanamycin sulfate were purchased from Lifetech (Rockville, MD, U.S.A.).

Isoflavone Metabolites Isoflavone metabolites examined in this work are shown in Fig. 1. **M-1**—**9** were isolated from human urine samples collected for 12 h after feeding soy bean curds.[11] Dihydrodaidzein and *O*-desmethylangolensin (*O*-DMA) were synthesized following the method described by Wahara *et al.*[12] Genistein 4′-*O*-sulfate (**S-1**), 4′,7-di-*O*-sulfate (**S-2**), daidzein 4′-*O*-sulfate (**S-3**), and 4′,7-di-*O*-sulfate (**S-4**) were synthesized following the method described by Peterson *et al.*[13]

Cells MCF-7 cells were supplied by the Cell Resource Center for the Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University.

Growth of MCF-7 Cells MCF-7 cells were cultured in phenol red-free RPMI 1640 medium supplemented with 10% (v/v) FBS and kanamycin sulfate (100 μg/ml) in 25 cm^3 flasks at 37 °C in a humidified atmosphere of 5% CO_2/95% air. Cell suspensions were prepared by rinsing the confluent monolayer with PBS and treating it with trypsin/EDTA for 3 min. After inactivation of trypsin by FBS, the resulting cell

* To whom correspondence should be addressed. e-mail: kinjojun@fukuoka-u.ac.jp

suspension was centrifuged at 150×***g*** for 5 min. The cell suspension (2×10^3 cells per well) in phenol red-free DMEM supplemented with 5% heat-inactivated dextran/charcoal-stripped FBS was plated and incubated at 37 °C for 24 h. After cultivation, the dimethyl sulfoxide (DMSO) solution of the isoflavone samples at various concentrations were added to the wells. After 4 d incubation under the above-mentioned conditions, cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay method described by Mosmann.[14]

Preparation of the Extract of hERs α and β hER α and β were prepared according to the method described in a previous paper.[5] The concentrations of hERs α and β were 0.6 and 0.3% of the total protein, respectively.

Competition Assay The binding of isoflavones to hER α or β was examined by the method described in a previous paper.[5] Binding of estrogen to hER α or β was determined by incubation of hER α or β (5 μl in 250 μl TKE reaction mixture) with [^{3}H]-17β-estradiol (1.25 pmol) at 0 °C in the presence of various concentrations of the isoflavone samples. The TKE reaction mixture is composed of 20 mM Tris–HCl, pH 7.4, 1 mM EDTA, and 20 mM KCl.

Yeast Strain Carrying Full-Length hER α or β The preparation of *Saccharomyces cerevisiae* Y190 (*MATa*, *ura3-52*, *his3-D200*, *ade2-101*, *trp1-901*, *leu2+3*, *112*, *gal4Dgal80D*, *URA3:::GAL-lacZ*, *cyhr2*, *LYS2:::GAL-HIS3*) carrying pGBT9-hER α or pGBT9-hER and pGAD424-hTIF2 was described in a previous paper.[5]

ER-Dependent β-Galactosidase Induction The effects of isoflavone samples on ER-dependent β-galactosidase induction in yeast were examined according to the method described by Morito *et al.*[5]

RESULTS AND DISCUSSION

Estrogens are critical for proper maintenance and function of a diverse array of tissues and physiological systems in mammals. The physiological responses to estrogens are known to be mediated within specific tissues by at least two ERs, ERs α and β. Isoflavones are known to have estrogenic activity,[15] and we are interested in this activity on hERs α and β. In a previous paper,[5] we examined the estrogenic activities of isoflavones isolated after the digestion of soybeans by enteric bacteria. The estrogenic activities were examined with respect to their binding to hERs α and β, ER-dependent β-galactosidase induction, and growth of MCF-7 cells. The binding and gene expressional effects of genistein were the strongest among the isoflavones examined.[5] In another paper,[6] we examined the estrogenic activities of isoflavones and related compounds isolated from *Pueraria* spp. The most active compound was coumestrol, which is known to induce infertility in sheep.[16] It bound almost as strongly as E_2 to both hERs. The β-galactosidase induction by coumestrol was very similar to genistein, however, the binding to hER α was much stronger than that of genistein.

In this study, we examined the estrogenic activities of soy isoflavone metabolites isolated from human urine sample in order to evaluate the actual compounds active in the body. We tested the estrogenic activities of genistein, daidzein, glycitein, dihydrodaidzein, *O*-desmethylangolensin (*O*-DMA), and nine isoflavone metabolites (**M-1**—**9**) which

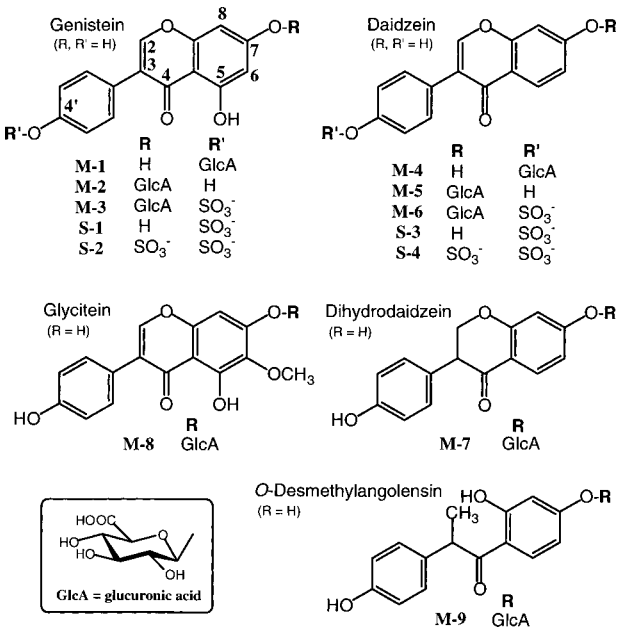

Fig. 1. Structures of Isoflavones Examined for the Estrogenic Activities

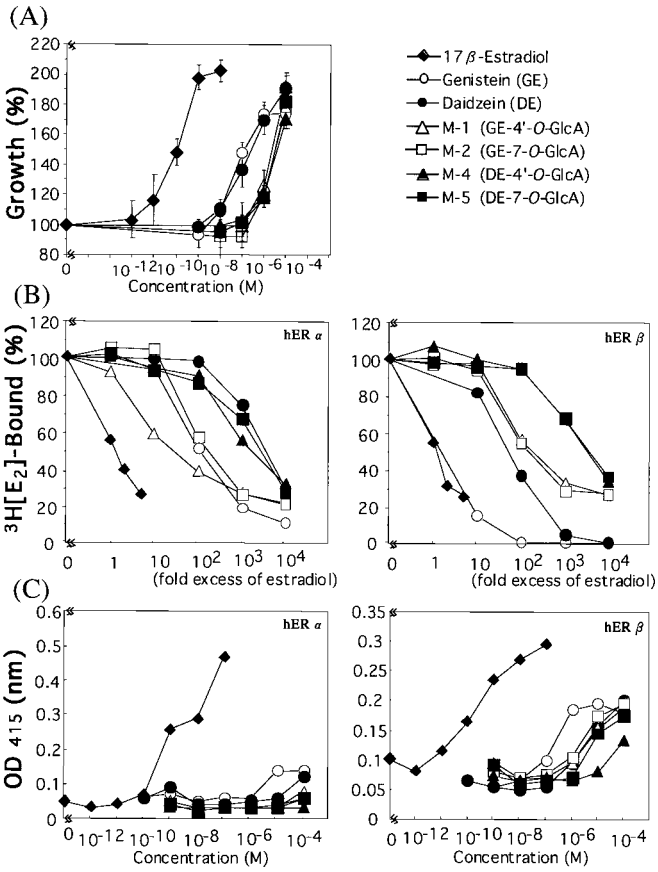

Fig. 2. Assays of (A) Growth of MCF-7 Cells, (B) Binding to hERs, and (C) hER-Dependent β-Galactosidase Induction

◆, 17β-estradiol; ○, genistein; ●, daidzein; △, **M-1**; □, **M-2**; ▲, **M-4**; ■, **M-5**. The growth of MCF-7 cells was measured by an MTT assay and the results are shown as percent increase. The bar at each point is the standard deviation. Binding to the hERs was examined by competitive incorporation of [^{3}H]-17β-estradiol (E_2) as described in Materials and Methods. Estrogen receptor-dependent β-galactosidase induction was measured by the increase of $OD_{415\,nm}$ by *p*-nitrophenol produced by the enzymatic hydrolysis of *p*-nitrophenyl-β-D-galactoside.

were isolated from human urine samples collected for 12 h after giving soy bean curds to the volunteer subjects,[11] and four synthetic isoflavone sulfates (**S-1**—**4**). The structures are shown in Fig. 1.

Effect of Isoflavones on MCF-7 Cell Growth The stimulatory effect of genistein and daidzein on MCF-7 cell growth was lowered by conjugation with glucuronic acid (**M-1**, **2**, **4**, **5**) as shown in Fig. 2A, and similarly, the effect of the glucuronides (**M-7**, **9**) of dihydrodaidzein and *O*-DMA was also less than that of their aglycones (Fig. 3A). The effect of glycitein and its glucuronide (**M-8**) was very poor. On the contrary, **M-3** and **6**, the respective glucuronyl and sulfonyl conjugates of genistein and daidzein, showed a stimulatory effect on the growth of MCF-7 cells equal to that of their aglycones (Fig. 4A). However, the synthetic sulfates (**S-1**—**4**) of genistein and daidzein did not exhibit a stimulatory effect.

Binding of Isoflavones to hERs Next, we examined the binding activity to hERs α and β of several conjugates which stimulated the growth of MCF-7 cells, except for **M-3** owing to the small amounts of the sample. The glucuronides (**M-1**, **2**) of genistein bound more strongly to both hERs than daidzein glucuronides (**M-4**, **5**) (Fig. 2B), as was seen in the case of the aglycones. Moreover, the binding activity to hER α of **M-1** and **4** was stronger than that of genistein and daidzein, respectively. Further, the 4′-*O*-glucuronide (**M-1**) of genistein bound to hER α better than 7-*O*-glucuronide (**M-2**). Since the binding effects of **M-1** and **2** to hER β were almost comparable to each other, the position of glucuronic acid would affect the binding effect to hER α. The glucuronides (**M-7**, **8**) of dihydrodaidzein and glycitein bound poorly to both hERs compared to the glucuronides (**M-1**, **2**, **4**, **5**) of genistein and daidzein (Fig. 3B). However, the binding activity of *O*-DMA glucuronide (**M-9**) to hER β was comparable to that of the daidzein glucuronides (**M-4**, **5**). Further, *O*-DMA itself showed the strongest binding activity to hER β, which was as strong as E_2. The synthetic sulfates (**S-1**, **2**) of genistein bound very poorly to both hERs, but 4′-*O*-sulfate (**S-1**) bound slightly to hER β (Fig. 4B). On the other hand, daidzein glucuronyl sulfate (**M-6**) exhibited binding activity as potent as that of daidzein glucuronides (**M-4**, **5**).

ER-Dependent β-Galactosidase Induction by Isoflavones The glucuronides (**M-1**, **2**, **4**, **5**) of genistein and daidzein showed slight hER β-dependent β-galactosidase induction, while, their hER α-dependent induction was very poor (Fig. 2C). Glucuronides (**M-7**—**9**) of dihydrodaidzein, glycitein and *O*-DMA did not induce hER α-dependent induction (Fig. 3C), however, *O*-DMA induced the most potent hER β-dependent induction among all compounds tested. *O*-DMA glucuronide (**M-9**) exhibited activity equal to that of **M-1**, **2** and **5**. The synthetic sulfates (**S-1**, **2**) of genistein did not induce either hER-dependent β-galactosidase induction (Fig. 4C). On the other hand, daidzein glucuronyl sulfate (**M-6**) showed potent hER α and β-dependent induction.

Among the tested compounds, *O*-DMA showed the strongest stimulatory effect toward the growth of MCF-7 cells (Fig. 3A).[5] *O*-DMA, a metabolite of daidzein, is pro-

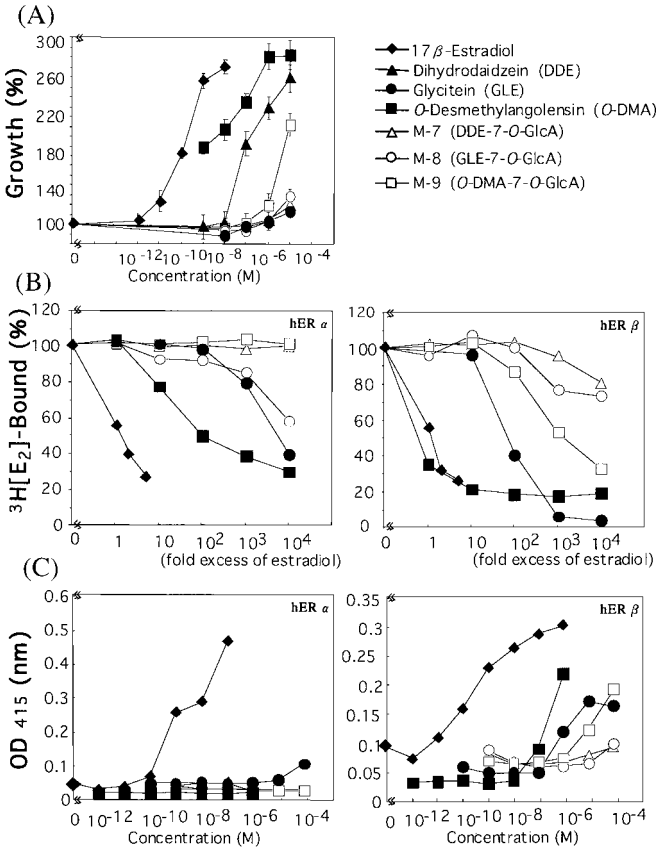

Fig. 3. The Same Assays as Fig. 2 for **M-7** (△), **M-8** (○), **M-9** (□), Dihydrodaidzein (▲), Glycitein (●), and *O*-Desmethylangolensin (■)

17β-Estradiol (◆) was assayed as a control.

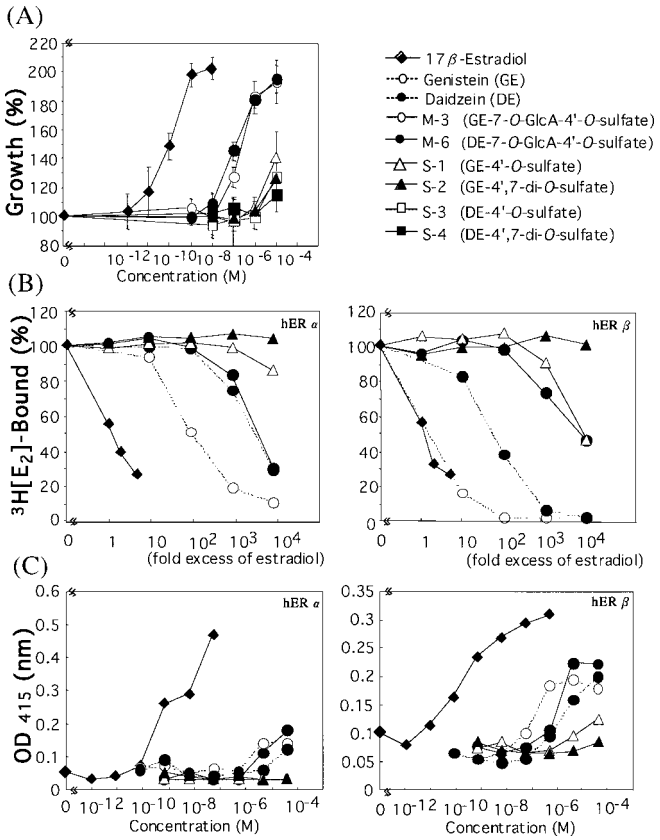

Fig. 4. The Same Assays as Fig. 2 for **M-3** (○), **M-6** (●), **S-1** (△), **S-2** (▲), **S-3** (□), and **S-4** (■)

17β-Estradiol (◆) was assayed as a control.

duced by human enteric bacteria.[17] It is noteworthy that *O*-DMA has an acyclic structure similar to that of diethylstilbestrol (DES). The binding activity to both hERs of *O*-DMA was comparable to that of genistein (Fig. 3B).[5] In particular, the binding activity to hER β was the strongest and was as strong as E_2. However, *O*-DMA did not show any β-galactosidase induction with hER α (Fig. 3C), although the induction with hER β was 10^3 times less than that of E_2. Since DES showed potent β-galactosidase induction together with potent binding to both hERs and a stimulatory effect toward the growth of MCF-7 cells,[5] the structural difference between DES and *O*-DMA must affect β-galactosidase induction.

The genistein and daidzein metabolites tested were classified into three groups with respect to estrogenic activity. The first group shows a very poor stimulatory effect toward the growth of MCF-7 cells, binding activity, and β-galactosidase induction. The sulfates (**S-1**, **2**) belong to this group. The second group shows moderate binding activity but a poor stimulatory effect toward the growth of MCF-7 cells and β-galactosidase induction. Some glucuronyl conjugates (**M-1**, **2**, **4**, **5**) belong to this group. The third group shows moderate stimulation and β-galactosidase induction but poor binding activity. A mixed type conjugate (**M-6**) having glucuronyl and sulfonyl moieties belongs to this group. In previous papers, we have clarified the estrogenic activity of isoflavone aglycones or related compounds, including the metabolites by enteric bacteria.[5,6] This is the first report, to the best of our knowledge which suggests that the metabolism of isoflavones by hepatic enzymes affects estrogenic acivity. Therefore, the differences in estrogenic acivity among individuals consuming soy foods would be influenced by not only enteric bacteria but also drug metabolizing enzymes.

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