Anti-Estrogenic Activity of Prenylated Isoflavones from *Millettia pachycarpa*: Implications for Pharmacophores and Unique Mechanisms

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Phytoestrogens containing isoflavonoids are thought to exhibit preventative effects on estrogen-responsive diseases. Chemical modifications, such as prenylation, in biosynthetic processes enhance the structural variety of isoflavonoids and prompted us to carry out a structure-activity relationship study. We determined the estrogenic/anti-estrogenic activities and estrogen receptor (ER)-binding affinities of eight kinds of prenylated isoflavones isolated from *Millettia pachycarpa* (Leguminosae), and those of two kinds of non-prenylated compounds (genistein and daidzein). By comparing these compounds, the pharmacophores for estrogenic/anti-estrogenic activities were elucidated. None of the tested compounds (except genistein) were estrogenic on ligand-dependent yeast-two hybrid assay. On the other hand, 5 isoflavones showed distinct anti-estrogenic activity. Unexpectedly, the most potent antagonists, isoerysenegalensein E and 6,8-diprenylorobol, showed anti-estrogenic activity comparable to that of 4-hydroxytamoxifen, a typical ER antagonist. This suggests that genistein became an antagonist after prenylation and hydroxylation. The pharmacophores providing genistein with strong anti-estrogenic activity were as follows: prenyl groups of the 6- and 8-positions on the A-ring, hydroxyl group of the 6-prenyl moiety or the B-ring (catechol form), non-cyclization of the prenyl group with the A-ring, and non-hydroxylation of the 8-prenyl group on the A-ring. The ER-binding affinities of the isoflavonoids were not sufficiently high to explain their potent antagonistic activities, thus suggesting 17β-estradiol-non-competitive mechanisms.

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INTRODUCTION

Isoflavonoids have a very limited distribution in the plant kingdom, and soy plants, particularly the family Leguminosae, are the major source of these compounds. Japanese have traditionally consumed soy foods such as *tofu*, *miso*, *natto* and *soy sauce*, and these traditional foods are thought to be closely linked with their health.[1)] For example, consumption of certain soy foods was inversely associated with the risk of breast cancer, and this inverse association was stronger in postmenopausal women.[1,2)] The reduction of cancer risk is probably associated with the estrogenic/anti-estrogenic actions of soy isoflavones. Previously, Nishihara *et al*. developed a ligand-dependent yeast two-hybrid assay to determine the estrogenic activity of endocrine disrupting chemicals.[3)] Using this assay, newly isolated prenylated isoflavones from the leaves of *Millettia pachycarpa* (Leguminosae) were found to exhibit anti-estrogenic activity.[4)] In addition, isoflavones from *Moghania philippinensis* (Leguminosae) exhibited estrogenic/anti-estrogenic activities.[5)] These results suggest that prenylated isoflavones are useful in estrogen-responsive cancer prevention.

The American Cancer Society has reported that breast cancer incidence rates in women continued to increase between 1980 and 2001.[6)] Breast cancer is the leading cause of cancer death in women at ages 20 to 59 years and is second overall. In general, tamoxifen is used as first-line drug in breast

cancer therapy. Tamoxifen acts as an antagonist of estrogen receptor (ER) in breast tissue and reduces proliferation of ER-positive breast cancer cells. However, tamoxifen is an agonist in the uterus and increases the risk of endometrial cancer.[7,8] In clinical practice, tamoxifen-resistance is occurring, thus complicating the medication of this condition.[9,10] Therefore, development of novel selective estrogen receptor modulators is desirable. In this study, the estrogenic/anti-estrogenic activities of prenylated isoflavones[4,11] from the stem and leaves of *Millettia pachycarpa* were determined using a ligand-dependent yeast two-hybrid assay, and the pharmacophores for these activities were elucidated. Moreover, the anti-estrogenic mechanism of action of prenylated isoflavones is discussed.

MATERIALS AND METHODS

Chemicals —— 17β-Estradiol (E2) and chlorophenolred-β-D-galactopyranoside (CPRG) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), genistein and daidzein were obtained from Fujicco Co., Ltd. (Kobe, Japan), 4-hydroxytamoxifen (OHT) was purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.), and zymolyase-20T was obtained from Seikagaku Corp. (Tokyo, Japan). Other chemicals were of the highest purity available.

Assay for Estrogenic/Anti-Estrogenic Activities —— Estrogenic/anti-estrogenic activities were determined by assay using yeast cells, in which two plasmids that express the GAL4DBD-ERα ligand-binding domain and GAL4AD-TIF2 receptor interaction domain were introduced.[3,12] In this assay, the ligand-dependent interaction of ERα and coactivator TIF2 is determined by the expression of a reporter gene, β-galactosidase (β-gal). To measure estrogenic activity, cells were grown overnight at 30°C with agitation in synthetic defined (SD) medium lacking tryptophan and leucine. Test compounds, OHT and E2, were dissolved in dimethylsulfoxide (DMSO) and the solution was diluted with SD medium to appropriate concentrations in order to obtain sample solutions. Yeast cell suspension [optical density at 630 nm (OD_{630}) = 0.1] and the sample solution (DMSO less than 10%) were added to each well of a 96-well microtiter plate, which was then incubated at 30°C for 18 hr. The growth of yeast cells was monitored by measuring the OD_{630} using a microplate reader (model 550, Bio-Rad Laboratories Inc., Hercules, CA, U.S.A.). Next, 4 × Z-buffer containing zymolyase-20T (4 mg/ml) was added to each well, and the plate was incubated at 30°C for 30 min. Then, 0.5 mg/ml CPRG was added, followed by incubation at 30°C for 1 hr. After incubation, 2 M Na_2CO_3 was added to stop the reaction. Absorbance of each well was measured at 540 and 630 nm (A_{540} and A_{630}). β-Gal activity was calculated using the following formula:

$$\text{Relative } \beta\text{-gal activity} = (A_{540} - A_{630})/OD_{630}.$$

In order to measure anti-estrogenic activity, test compounds were assayed in the presence of 1 nM E2. The transcription inhibition curve was obtained from the reduction of β-gal activity induced by E2. The IC_{50} value of each compound was obtained based on the concentration providing 50% activity of that of E2 alone.

ER-Binding Assay —— Binding affinities for ERα were measured using a commercially available kit (Estrogen-R α Competitor Screening Kit, Wako Pure Chemical Industries, Ltd.). Test chemicals were dissolved in DMSO to give a concentration range from 10^{-3} to 10^{-8} M. Sample solutions and fluorescent-labeled E2 were added to each well of a 96-well microtiter plate, which was pre-coated with human ERα, and the plate was incubated competitively for 2 hr at room temperature. After incubation, wells were washed twice with the provided washing solution, and the provided measurement solution was added each well. The concentration of fluorescent-labeled E2 bound to ER was estimated based on fluorescence intensity (Ex. 485 nm, Em. 535 nm) using a microplate reader (Wallac 1420 ARVOsx, PerkinElmer Inc., Wellesley, MA, U.S.A.). The inhibition curve of fluorescent-labeled E2 binding was obtained based on the reduction in fluorescence intensity. IC_{50} value, *i.e.*, the concentration to elicit 50% replacement of fluorescent-labeled E2, was calculated using the binding inhibition curve.

RESULTS

Estrogenic/Anti-Estrogenic Activities of Isoflavones

The chemical structures of the isoflavones used in this study are shown in Fig. 1. Genistein exhibited clear estrogenic activity in the yeast two-hybrid assay, while other isoflavones showed no detectable activity (Fig. 2). The inhibitory effects of isoflavones on β-gal activity induced by E2 (1 nM) were then

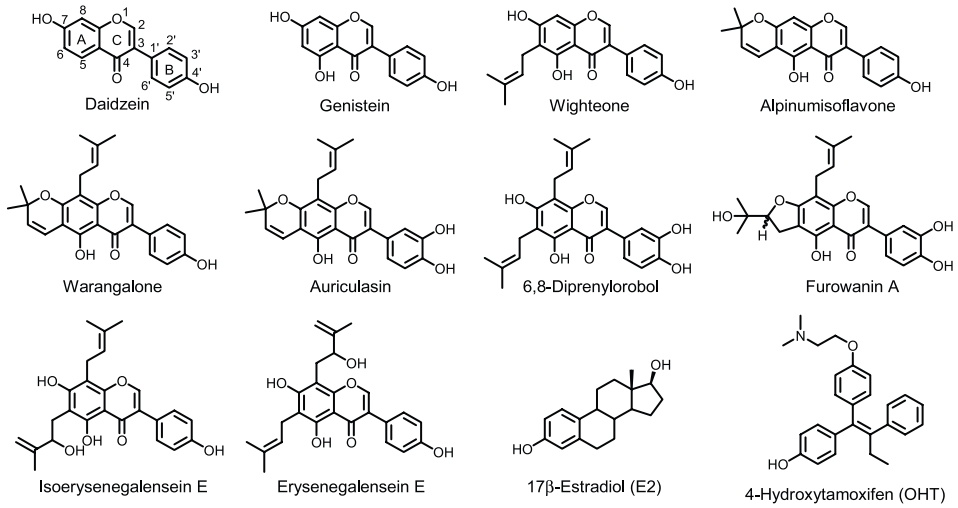

Fig. 1. Chemical Structures of E2, OHT and Isoflavonoids Used in this Study

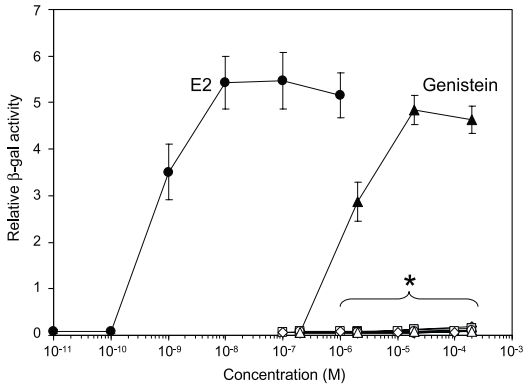

Fig. 2. Estrogenic Activities of Isolated Compounds on Yeast Two-Hybrid Assay

*Daidzein, OHT and prenylated isoflavones. The bar at each point indicates the standard error of three independent experiments (n = 5).

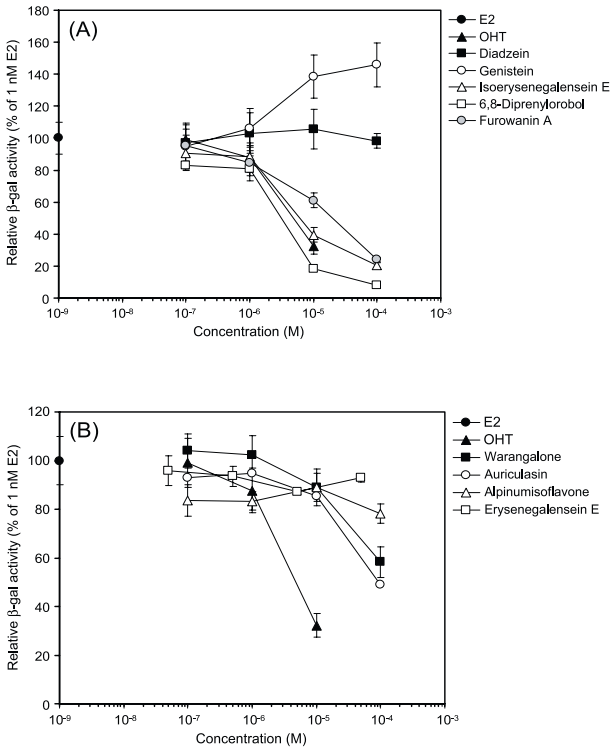

Fig. 3. Inhibitory Effects of Isoflavonoids and OHT on β-Gal Activity Induced by E2 in Yeast Two-Hybrid Assay

Yeast strain was incubated with 1 nM E2 in the presence or absence of tested compounds. The bar at each point indicates the standard error of three independent experiments (n = 5). Prenylated isoflavonoids with strong inhibitory effect (A) and weak or no inhibitory effect (B).

determined (Fig. 3). OHT, a typical ER antagonist, exhibited strong inhibitory effects on β-gal expression induced by E2 (Fig. 3). Daidzein did not inhibit β-gal expression, and genistein stimulated β-gal expression. Most prenylated isoflavones exhibited inhibitory effects on β-gal expression induced by E2. The inhibitory effects of prenylated isoflavones were classified into 3 groups based on degree of inhibitory effect (Fig. 3A and 3B). Isoerysenegalensein E and 6,8-diprenylorobol were the most potent inhibitors tested (Fig. 3A), and the inhibitory effects were comparable to that of OHT. The IC_{50} values (concentration required for 50% inhibition of 1 nM E2 activity) of 6,8-diprenylorobol, isoerysenegalensein E and furowanin A were 3.2, 6.1 and 20 μM, respectively. Warangalone and auriculasin were weak ER inhibitors, and alpinumisoflavone and erysenegalensein E were non-ER inhibitors (Fig. 3B). The inhibitory effects of wighteone could not be determined because of its

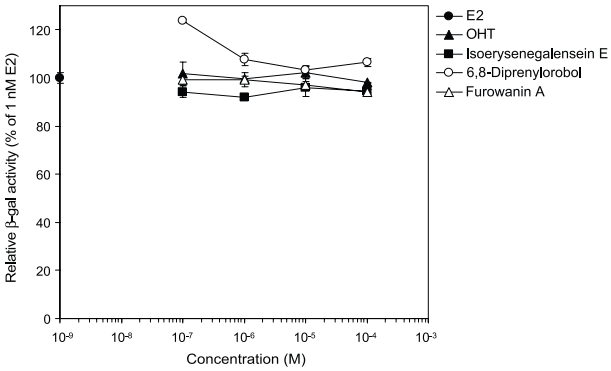

Fig. 4. Effects of Prenylated Isoflavones on β-Gal Enzyme Activity Induced by 1 nM of E2

The bar at each point indicates the standard error of three independent experiments (n = 3).

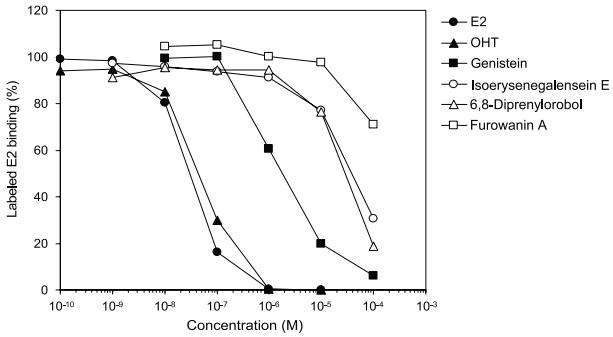

Fig. 5. ER-Binding Affinities of E2, OHT and Isoflavonoids

strong cytotoxic effects.

Mechanism for Anti-Estrogenic Effects of Prenylated Isoflavones

The observed inhibitory action of isoflavones could also result from ER-independent mechanisms; for example, the inhibition of β-gal enzyme activity or yeast cell proliferation. In order to confirm whether isoflavones inhibit β-gal enzyme activity, isoflavones were added to zymolyase-lysate of yeast cells after incubation, during which β-gal was expressed by 1 nM E2. None of the isoflavones inhibited colorimetric reactions of β-gal (Fig. 4). The isoflavons did not affect yeast cell growth even at the highest concentration tested in the assay for anti-estrogenic activity. Given these observations, isoflavones are considered to be an ER antagonist acting on ER transcriptional processes.

In order to better understand the inhibitory action of isoflavones, ER-binding affinity was determined as described in MATERIALS AND METHODS. Tested isoflavones bound to ER in a dose-dependent manner and at similar levels as observed for anti-estrogenic activity assays (Fig. 5). Among these, isoerysenegalensein E and 6,8-diprenylorobol exhibited higher binding affinities, with IC_{50} values of 6.1 and 3.2 μM, respectively. These affinities were several hundred times lower than that of OHT, although the anti-estrogenic activities of the isoflavones and OHT were comparable, thus suggesting unique anti-estrogenic mechanisms for those isoflavones.

DISCUSSION

Pharmacophores of Isoflavones for Anti-Estrogenic Activity

In this study, the estrogenic/anti-estrogenic activities of ten kinds of isoflavones were determined. Only genistein exhibited estrogenic activity (Fig. 2). In contrast, almost all isoflavones except genistein inhibited the β-gal activity induced by 1 nM E2 at approximately three levels of potency (Fig. 3). Structure-activity relationship analysis revealed four essential structures for anti-estrogenic activity: prenyl groups at the 6- and 8-positions of the A-ring (based on non-inhibitory effects of alpinumisoflavone and wighteone), non-cyclization of the 6-prenyl group with the A-ring (lower potency of auriculasin and furowanin A relative to 6,8-diprenylorobol), hydroxyl group of the 6-prenyl moiety or the 3′-position of the B-ring (highest potency of isoerysenegalensein E and 6,8-diprenylorobol), and non-hydroxylation of the 8-prenyl group on the A-ring (loss of anti-estrogenic activity of erysenegalensein E). Wighteone (6-prenylated genistein) was cytotoxic, but 8-prenylated genistein was reported to have potent anti-estrogen activity.[5] Moreover, 6,8-diprenylated isoflavones, such as isoerysenegalensein E, 6,8-diprenylorobol and erythenegalensein E, did not inhibit yeast cell growth. These data suggest that the presence of the prenyl group at the 8-position on the A-ring counteracts the cytotoxicity of 6-prenylated analogue.

E2-Non-Competitive ER Inhibition of Prenylated Isoflavones

Isoerysenegalensein E and 6,8-diprenylorobol were the most potent antagonists tested, and their effects were comparable to that of OHT, a typical ER antagonist. However, the underlying mechanism

for this strong antagonism is expected to be different from that of OHT. The expected mechanism is probably ER-mediated from the experiments which exclude other possibilities (Fig. 4). The ER-binding affinities of isoerysenegalensein E and 6,8-diprenylorobol were several hundred times lower than that of OHT, despite their comparable anti-estrogenic activities (Fig. 5). Therefore, ER inhibition by isoerysenegalensein E and 6,8-diprenylorobol would involve certain mechanisms in addition to direct competition with E2. Tyulmenkov and Klinge suggested that a non-competitive second binding site in ER may be present, as tetrahydrocrysene ketone was found to bind with E2-liganded ER.[13] Considering the low affinity of prenylated isoflavones to the E2-competitive binding site in ER, these isoflavones may bind to this second binding site. Typical ER antagonists, such as OHT and raloxifene, disrupt the normal helix 12 positioning, thus preventing recruitment of coactivators, such as TIF2, onto ER.[14,15] However, our preliminary experiments showed that prenylated isoflavones did not inhibit TIF2 recruitment. Therefore, other mechanisms, for example, inhibition of the ER-dimerization process or disruption of DNA sequence recognition, should be examined. It has been reported that crude extracts of *Millettia pachycarpa* inhibit DNA polymerase activity *in vitro*.[16] In our experiment using yeast, none of extracted compounds except wighteone from the same plant interfered with yeast cell growth, thus also with the DNA replication which is essential for growth. Because DNA recognition by RNA polymerase is also critical for cell growth, the antagonism of prenylated isoflavons would not be due to the inhibition of general transcriptional machinery.

Food Chemical Significance of Prenylated Isoflavones

Soy extracts are often used as a supplement for menopausal syndrome, and natural products are believed to be safe. However, excess ingestion may cause adverse effects by additive and/or synergistic action of the isoflavones, including the strong antagonist we have demonstrated in the present study.

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REFERENCES

1) Yamamoto, S., Sobue, T., Kobayashi, M., Sasaki, S. and Tsugane, S. (2003) Soy, isoflavones, and breast cancer risk in Japan. *J. Natl. Cancer Inst*., **95**, 906–913.
2) Hirose, K., Imaeda, N., Tokudome, Y., Goto, C., Wakai, K., Matsuo, K., Ito, H., Toyama, T., Iwata, H., Tokudome, S. and Tajima, K. (2005) Soybean products and reduction of breast cancer risk: a case-control study in Japan. *Br. J. Cancer*, **93**, 15–22.
3) Nishihara, T., Nishikawa, J., Kanayama, T., Dakeyama, F., Saito, K., Imagawa, M., Takatori, S., Kitagawa, Y., Hori, S. and Utsumi, H. (2000) Estrogenic activities of 517 chemicals by yeast two-hybrid assay. *J. Health Sci*., **46**, 282–298.
4) Ito, C., Itoigawa, M., Kumagaya, M., Okamoto, Y., Ueda, K., Nishihara, T., Kojima, N. and Furukawa, H. (2006) Isoflavonoids with antiestrogenic activity from Millettia pachycarpa. *J. Nat. Prod*., **69**, 138–141.
5) Ahn, E. M., Nakamura, N., Akao, T., Nishihara, T. and Hattori, M. (2004) Estrogenic and antiestrogenic activities of the roots of *Moghania philippinensis* and their constituents. *Biol. Pharm. Bull*., **27**, 548–553.
6) Jemal, A., Murray, T., Ward, E., Samuels, A., Tiwari, R. C., Ghafoor, A., Feuer, E. J. and Thun, M. J. (2005) Cancer statistics, 2005. *CA Cancer J. Clin*., **55**, 10–30.
7) Swerdlow, A. J. and Jones, M. E. (2005) Tamoxifen treatment for breast cancer and risk of endometrial cancer: a case-control study. *J. Natl. Cancer Inst*., **97**, 375–384.
8) IARC Working Group (1996) Tamoxifen. In *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*, IARC Press, Lyon, vol. 66, pp. 253–365.
9) Katzenellenbogen, B. S., Montano, M. M., Ekena, K., Herman, M. E. and McInerney, E. M. (1997) William L. McGuire Memorial Lecture. Antiestrogens: mechanisms of action and resistance in breast cancer. *Breast Cancer Res. Treat*., **44**, 23–38.
10) McDonnell, D. P., Connor, C. E., Wijayaratne, A., Chang, C. Y. and Norris, J. D. (2002) Definition of the molecular and cellular mechanisms underlying the tissue-selective agonist/antagonist activities of selective estrogen receptor modulators. *Recent Prog. Horm. Res*., **57**, 295–316.
11) Ito, C., Itoigawa, M., Kojima, N., Tokuda, H., Hirata, T., Nishino, H. and Furukawa, H. (2004) Chemical constituents of *Millettia taiwaniana*: structure elucidation of five new isoflavonoids and their cancer chemopreventive activity. *J. Nat. Prod*., **67**,

1125–1130.
12) Nishikawa, J., Saito, K., Goto, J., Dakeyama, F., Matsuo, M. and Nishihara, T. (1999) New screening methods for chemicals with hormonal activities using interaction of nuclear hormone receptor with coactivator. *Toxicol. Appl. Pharmacol*., **154**, 76–83.
13) Tyulmenkov, V. V. and Klinge, C. M. (2000) Interaction of tetrahydrocrysene ketone with estrogen receptors alpha and beta indicates conformational differences in the receptor subtypes. *Arch. Biochem. Biophys*., **381**, 135–142.
14) Brzozowski, A. M., Pike, A. C. W., Dauter, Z., Hubbard, R. E., Bonn, T., Engström, O., Öhman, L., Greene, G. L., Gustafsson, J. Å. and Carlquist, M. (1997) Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature* (London), **389**, 753–758.
15) Pike, A. C. W., Brzozowski, A. M., Hubbard, R. E., Bonn, T., Thorsell, A. G., Engström, O., Ljunggren, J., Gustafsson, J. Å. and Carlquist, M. (1999) Structure of the ligand-binding domain of oestrogen receptor beta in the presence of a partial agonist and a full antagonist. *EMBO J*., **18**, 4608–4618.
16) Ono, K., Nakane, H., Meng, Z. M., Ose, Y., Sakai, Y. and Mizuno, M. (1989) Differential inhibitory effects of various herb extracts on the activities of reverse transcriptase and various deoxyribonucleic acid (DNA) polymerases. *Chem. Pharm. Bull*., **37**, 1810–1812.