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Diverse Effects of Phytoestrogen Biochanin A on Rat Pituitary Tumor Cells

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ABSTRACT

Biochanin A, a nonsteroidal estrogen present in plants, has been suggested in epidemiological studies to be associated with a reduced risk of hormone-related cancers. In this study, we examined the effects of biochanin A on the cell growth and gene expression of an estrogen-responsive pituitary cell line GH_3. Results showed that biochanin A inhibited growth of GH_3 cells in a dose-dependent manner and at physiologically relevant concentrations. At concentrations of 1 and 5 μg/mL, inhibition of cell growth was observed. The gene expressions of estrogen receptor (ER) mRNA and an estrogen responsive gene, the progesterone receptor (PR), were down-regulated by the biochanin A treatment. In addition, co-administration of antiestrogen ICI182,780 blocked this biochanin A induced down-regulation of the ER and PR mRNA level. However, the expression level of rat prolactin (rPRL) gene, one of the typical estrogen responsive genes, was not regulated by biochanin A. These results indicated that biochanin A at physiologically achieveable concentrations was able to inhibit cell growth of estrogen-responsive rat pituitary cells. In addition, dietary-derived biochanin A compounds exhibit diverse estrogenic and antiestrogenic effects on estrogen-related gene expression, suggesting gene-specific regulation.

Key words: dietary estrogen, pituitary cells, cell growth, gene expression

INTRODUCTION

Dietary phytoestrogens are naturally occurring constituents of plants that are known to elicit endocrine-modulation effects in animals[1,2]. The richest sources of phytoestrogens among foodstuffs are legumes and grains, and the amount of phytoestrogens in the soybean is on the order of 0.5 to 3 mg/g of soy proteins[3]. Phytoestrogens have been suspected to contribute to clover disease in grazing animals that results in high abortion rates and permanent sterility[4]. Recently, epidemiological studies showed that the high contents of phytoestrogens present in traditional Asian diets are associated with reduced risk of heart disease and hormone-dependent cancers, including breast and prostate cancers, and a decrease in menopausal symptoms[5,6]. *In vitro* studies indicated that phytoestrogens were able to regulate cell growth of breast tumor cells and induce cell death through apoptosis[7,8]. The mechanisms of action of phytoestrogens, especially in non-reproductive tissues, remain unclear despite that fact that their biological effects on humans and animals have been studied extensively in the recent years.

The effects of phytoestrogens have been investigated in several model systems including human breast cancer cells, endothelial cells, prostate cells and rat uterus. Phytoestrogens have been found to increase proliferation of human breast cancer cells at low concentrations but inhibit cell growth at high concentrations[8,9]. Stimulation of estrogen responsive gene expression, increase in uterine weight and regulation of cell cycle progression have also been observed[3,10,11]. Reports also showed that phytoestrogens exhibit inhibitory effects on the cell proliferation of endothelial, ovary and prostate cells[12-14]. However, concentrations higher than that found in serum after normal consumption of phytoestrogen-containing food were often required to demonstrate the effects of phytoestrogens on the cell lines studied[15-17].

Little attention has been focused on the pituitary whose function can be regulated by estrogen binding to its receptor (estrogen receptor; ER). Using ER knockout mice, it has been demonstrated that estrogen and its receptor play an important role in lactotroph function in the pituitary[18]. The regulation of rat prolactin (rPRL) gene expression and secretion has been found to involve estrogen[19,20]. Upon binding to its receptor estrogen can interact with the pituitary specific transcription factor Pit-1 to modulate rPRL expression. To examine whether phytoestrogens that exhibit characteristics of estrogen and anti-estrogen affect pituitary cells, we analyzed the rat pituitary GH_3 cell line's response to phytoestrogen on cell growth and gene activation. The results showed that biochanin A was able to regulate cell growth of GH_3 cells at physiologically achievable concentrations. In addition, differential effects on estrogen-related gene expression were mediated by biochanin A in these pituitary cells, suggesting a complex functional mechanism and tissue specific regulation of phytoestrogens.

MATERIALS AND METHODS

I. *Cells and Culture Condition*

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GH_3 cells, derived from rat pituitary tumors, were obtained from American Type Cell Culture (ATCC). They were grown in phenol red-free DMEM containing a 1X antibiotic/antimycotic mixture, 5 mM N-(2-hydroxyethyl) piperazine-N'-2-ethanesulfonic acid, and 0.37% sodium bicarbonate. The medium was supplemented with either 10% FBS (fetal bovine serum) or 3X dextran/charcoal-stripped FBS.

II. *Cell Proliferation Assay*

Cells were grown to confluence and subjected to serum free DMEM for 24 hr. After reseeding into culture flasks in DMEM containing 3X dextran/charcoal-stripped FBS, cells were incubated with biochanin A for the indicated time and cell proliferation was determined by the trypan blue dye exclusion method.

III. *Reverse Transcription-PCR (RT-PCR)*

Total RNA was prepared from cells and first reverse-transcribed into cDNA, which in turn was subjected to PCR amplification using primers specific for ER-alpha, rPRL and PR. PCR amplification of the constitutively expressed RPL19 (ribosomal protein L19) was used as a measure of input RNA[21]. Controls containing water instead of reverse transcribed RNA were used to demonstrate the absence of contaminating DNA. The PCR products were analyzed by electrophoresis in agarose gels and visualized using ethidium bromide staining and UV light. The sequences for ER-alpha, rPRL and PR were 5'-GGAGATTCTGATGATTGGTCT-3' and 5'-CATCTCCAGGAGCAGGCTAT-3'; 5'-TCAGGAACTTGAGATAATTGT-3' and 5'-CTGAAGACAAGGAACAAGCCCA-3'; 5'-CCCACAGGAGTTTGTCAAACTC-3' and 5'-TAACTTCAGACATCATTTCCGG-3', respectively[22]. The sequences for RPL19 primers were 5'-CTGAAGGTCAAAGGGAATGTG-3' and 5'-GGACAGAGTCTTGATGATCTC-3'[21].

IV. *Immunoprecipitation and Western Analysis*

Cell lysates were prepared from cells treated with biochanin A for 4 days as described[23]. In brief, cells were washed three times with ice-cold HBSS before the addition of lysis buffer (0.1% Triton X-100, 1 mM iodoacetamide, 1% bovine hemoglobin, 1 mM PMSF, 0.002 U/mL aprotinin, 20 mM Tris-HCl, 0.14 M NaCl) and then incubated at 4˚C for 1 hr. Following centrifugation at 3000 xg for 10 min at 4˚C, the pellets were removed and the protein concentrations of the cell lysates were determined by Bradford assays with bovine serum albumin as the standard. Samples containing equal amounts of cellular proteins were incubated with ER-alpha antibody at 4˚C. Following gentle agitation overnight, 50 μL of protein A agarose previously equilibrated with lysis buffer was added to the reaction mixture and incubated for 12 hr at 4˚C. The reaction mixture was then centrifuged and washed with ice-cold dilution buffer (0.1% Triton X-100, 1% bovine hemoglobin, 20 mM Tris-HCl, 0.14 M NaCl) for three times, and with TSA buffer (20 mM Tris-HCl, 0.14 M NaCl) and 0.005 M Tris-HCl (pH 6.8) once each at 4˚C. The resulting precipitated immune complexes were solubilized at 100˚C for 3-5 min in 20 μL of laemmli sample buffer. The solubilized proteins were fractionated by 12% SDS-PAGE and transblotted onto nitrocellulose membrane. After blocking overnight at 4˚C in 5% skim milk in TBS buffer (20 mM Tris-HCl, pH 7.5, 500 mM NaCl), the presence of ER-alpha proteins in each sample was detected with ER175, an antibody against ER-alpha protein. After washing with TBS buffer, ER proteins present in the immune complexes by immunoprecipitation were detected using alkaline phosphatase-conjugated goat anti-rabbit IgG antibodies and an enhanced chemiluminescence (ECL) Western Blotting System (Amersham).

V. *Statistical Analysis*

Data were analyzed by the orthogonal contrast test to detect differences[24]. Only p values < 0.01 were considered significant.

RESULTS

The potential effects of biochanin A on cell growth of pituitary cells were measured (Figure 1A). The results showed that biochanin A decreased the number of viable cells in a dose-dependent manner and started to show growth inhibitory effects at the concentration of 1 μg/mL at 4-day treatment. Biochanin A appeared to induce cell growth arrest rather than cell death, since the percentage of cell death was not significant at all concentrations used. When the effects of biochanin A on GH_3 cells were analyzed with WST-1 assay, similar observation was obtained (data not shown). The WST-1 assay was based on the quantitative detection of the formation of formazan derived from the tetrazolium salt "WST-1" by the succinate-tetrazolium reductase system that beloned to the respiratory chain of the mitochondria and is active only in viable cells. Changes in cell morphology were observed in cells treated with high concentrations of biochanin A (Figure 1B). Condensation of the cellular components and decrease in cell size were noticed. These cells remained attached to the surface of the culture flasks and appeared transparent when treated with trypan blue dye suggesting the possibility of still being enclosed with cell membranes. Acridine orange stain also suggested the occurrence of apparent condensed chromosomes in these treated cells (data not shown). The concentrations required for biochanin A to inhibit cell growth might have physiological relevance since 11% and 22% inhibition was achieved at concentrations of 1 and 5 μg/mL, respectively. The reported plasma concentrations of phytoestrogens after consumption of phytoestrogen-rich

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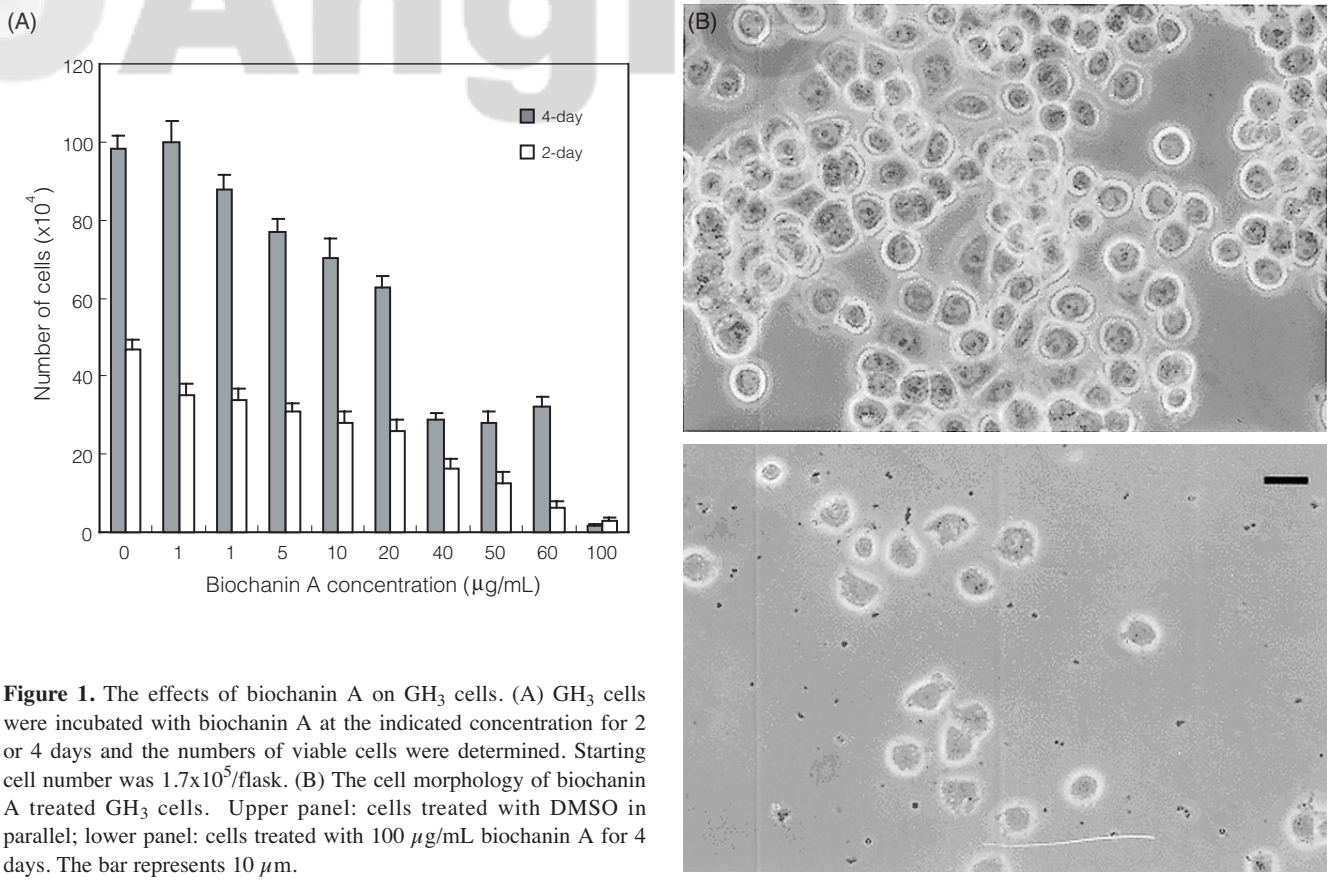

Figure 1. The effects of biochanin A on GH_3 cells. (A) GH_3 cells were incubated with biochanin A at the indicated concentration for 2 or 4 days and the numbers of viable cells were determined. Starting cell number was $1.7x10^5$/flask. (B) The cell morphology of biochanin A treated GH_3 cells. Upper panel: cells treated with DMSO in parallel; lower panel: cells treated with 100 μg/mL biochanin A for 4 days. The bar represents 10 μm.

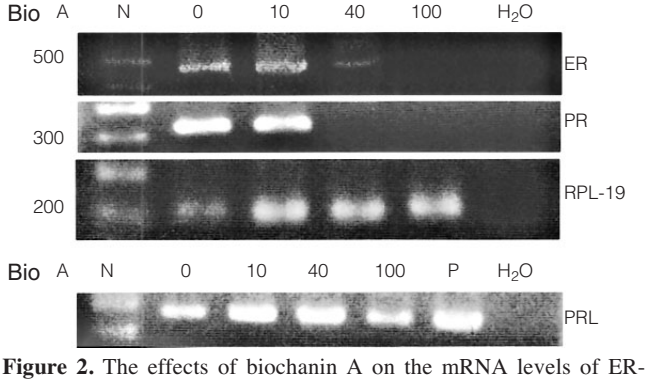

Figure 2. The effects of biochanin A on the mRNA levels of ER-alpha, rPRL and PR in biochanin A treated GH_3 cells. GH_3 cells were treated with biochanin A at the concentration of 0, 10, 40 or 100 μg/mL for 4 days and the level of ER-alpha mRNA, rPRL and PR was individually detected using RT-PCR. The level of RPL19, measured in parallel, was also determined as a control. H_2O: water substituted for DNA template in the PCR reaction.

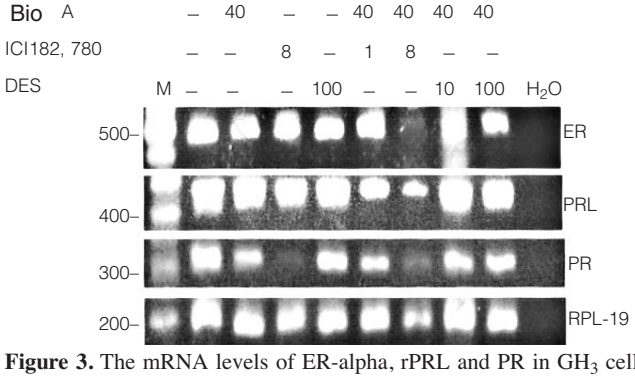

Figure 3. The mRNA levels of ER-alpha, rPRL and PR in GH_3 cells after biochanin A was co-administrated with ICI182,780 or estrogen. GH_3 cells were incubated with 40 μg/mL biochanin A alone or simultaneously with antiestrogen ICI182,780 (1 or 8 μg/mL) or the synthetic estrogen DES (100nM) for 4 days. The level of ER-alpha, rPRL and PR mRNA was measured individually with RT-PCR assays. H_2O: water substituted for DNA template in the PCR reaction.

food were in the range of 0.1-6 μg/mL.

Previous studies showed that estrogen exhibits self-regulation on its receptor-alpha (ER-alpha) at the levels of mRNA and protein. In addition, estrogen has been shown to activate the gene expression of rat prolactin (rPRL) and progesterone receptor (PR) in pituitary cells. To investigate whether the effects of biochanin A on cell growth is also associated with ER-alpha, rPRL and PR in GH_3 cells, we measured the mRNA levels of these three genes in GH_3 cells by RT-PCR assays (Figure 2). The identity of these amplified DNA products was confirmed by Southern blot analysis using cloned DNA fragments as the probe. The results show that the mRNA level of ER-alpha and PR was down-regulated by 40 and 100 μg/mL biochanin A. The levels of rPRL mRNA, on the other hand, did not appear to be affected by the same treatment. Western analysis of the rPRL protein levels in biochanin A treated-GH_3 cells showed similar results, in that rPRL protein level was unchanged in the presence of biochanin A (data not shown). Lower concentrations of biochanin A, although exhibiting

cell growth inhibitory activity, did not cause detectable changes in the level of ER and PR gene expression.

Pure antiestrogen ICI 182,790 or synthetic estrogen DES was simultaneously added with biochanin A to analyze the combined effect on ER-alpha gene expression in GH_3 cells and to examine the receptor specificity of this regulation by biochanin A (Figure 3). Cells treated with 40 μg/mL biochanin A and 8 μg/mL ICI182,790 simultaneously contained lower levels of ER-alpha mRNA than cells treated with either compound alone. The presence of both DES and biochanin A also resulted in decrease of the ER-alpha mRNA levels in GH_3 cells. Co-administration of DES and biochanin A exhibited different effects on PR mRNA. The level of PR mRNA in DES-treated cells was decreased by the addition of biochanin A. The antiestrgoen ICI 182,780 alone interestingly caused significant down-regulation of PR mRNA. It implies that biochanin A exhibits antiestrogenic activity toward PR, especially at the basal expression level. Administration of DES or ICI182,780 at the concentrations used in this study showed little, if there was any effects on the number of viable GH_3 cells.

DISCUSSION

Phytoestrogens have been shown to mediate multiple estrogenic and antiestrogenic effects in different cell and tissue types. The effects of phytoestrogens reported previously also appear to be concentration-dependent, in that lower concentrations of phytoestrogens may stimulate cell growth while high concentrations inhibit growth of the same cells. In this study, we examined the effects of biochanin A on GH_3 a pituitary cell line to investigate the potential roles of phytoestrogens on pituitary cell growth. Our results showed that biochanin A inhibited the growth of rat pituitary cells and down-regulated the gene expression of ER and PR. The expression of rPRL, on the other hand, was not significantly affected by biochanin A. Biochanin A was able to inhibit GH_3 cell growth at concentrations of 1 and 5 μg/mL, which are within the range of detected serum concentrations previously reported[16,17,25]. The vast majority of publications that describe the effects of phytoestrogens *in vitro* have reported significant results at concentrations higher than those achievable *in vivo* after oral consumption of soy-based foods or products enriched with phytoestrogens. For instance, Wang and Kurzer[8] showed that the IC_{50} for biochanin A was 13 μg/mL and the IC_{50} for genistein for cell growth was 10 μg/mL in MDA-468 cells[26]. Singhal and co-workers showed an IC_{50} value of 18 μg/mL for quercetin in MDA-486 cells[27]. Although the physiological relevance of phytoestrogens at these high concentrations has been argued, little is known about the bioavailable concentrations of phytoestrogens in tissues after consumption of soy-rich diet or the exact targets of phytoestrogens *in vivo*[17,28,29]. Nevertheless, in GH_3 cells, we have observed that biochanin A at the physiological achievable concentrations was able to affect the cell viability in a does-dependent manner.

At the concentrations that induced growth inhibition, biochanin A showed little effect on the gene expression of ER and PR. Down-regulation started to take place at higher concentrations and resulted in 50-100% inhibition of cell growth. This apparent disparity between the cell growth and gene regulation has been reported in other estrogen responsive pituitary cells by estrogen. The current hypothesis is that growth and gene regulation are controlled by different percentages of "active" factors. Only a small pool of these factors is required for growth regulation. At lower concentrations, cell growth is preferentially affected while gene regulation requires a higher concentration threshold.

Biochanin A induced down-regulation on mRNA levels in GH_3 cells was detected only in the ER and PR, but not in PRL. Similar down-regulation on ER-alpha mRNA levels induced by phytoestrogens was observed in human breast cancer cells[9]. However, previous studies showed that other phytoestrogens were able to stimulate the secretion and expression of PRL in a rat pituitary cell line PR1[30]. This discrepancy might be due to different phytoestrogens used or the difference in the cell lines. Indeed, their studies showed that only stimulation of the cell growth was observed in this PR1 cell line at all phytoestrogen concentrations tested. The PR1 cells were originally desived from an estrogen-induced pituitary tumor of F344 rats that have been used as a model to study the impact of their high sensitivity to estrogen. The extra high sensitivity toward estrogen of this cell line compared to the GH_3 cell line used in this study might contribute to the difference in our results.

The concentrations of biochanin A that are required for growth inhibition were physiologically achievable and no significant cell death was detected at the highest concentration below 50 μg/mL. However, morphological changes, including condensation of the cell contents and apparent decrease in cell volume, were noted after treatment with high concentrations of biochanin A. It would be interesting to analyze and compare the molecular mechanisms in pituitary cells with other cancer cell types such as breast cancer cells. Several studies on breast and prostate cancer cells indicate that phytoestrogens exhibit cytotoxic and/or apoptotic effects and induce cell death at high concentrations. Our previous results showed that the level of released lactate dehydrogenase (LDH) was induced by the treatment of biochanin A in human breast cancer cells, suggesting biochanin A's effects on cell membrane permeability[9]. Although the LDH level was not examined fully in GH_3 cells upon exposure to biochanin A, was similar observation was expected since some cell death was detected when GH_3 cells were treated with 100 μg/mL biochanin A. In the rat hypothalamus, a phytoestrogen, coumestrol, increased the ER-beta mRNA signal by 47.5% while 17-beta estradiol decreased this signal[31]. The biological functions of phytoestrogens are probably mediated through multiple pathways. Cell, as well as tissue-specific regulation, might also be involved. In this study, the data

indicated that the mRNA levels of ER-alpha and PR were influenced by DES and biochanin A. However, the cell growth was not inhibited by DES to the same degree as by biochanin A. It thus appeared that the cell growth regulation by biochanin A in GH_3 cells probably is not mediated through the classical ER-alpha pathways. It is evident that isoflavones are able to inhibit the cell growth in numerous types of cells via action on the cell cycle. Biochanin A may exhibit a regulation on cell cycle in GH_3 cells as well. Further investigation to identify the action target(s) in GH_3 cells would certainly facilitate the understanding of the effects of biochanin A on GH_3 cells. Evidently, the neuroendocrine effects of phytoestrogens also require further investigation since they have been suggested as an alternative or supplement to estrogen replacement therapy.

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REFERENCES

1. Adlercreut, H., Mazur, W., Stumpf, K., Kilkkinen, A., Pietinen, P., Hulten, K. and Hallmans, G. 2000. Food containing phytoestrogens and breast cancer. Biofactors 12: 89-93.
2. Knight, D. C. and Eden, J. A. 1996. A review of the clinical effects of phytoestrogens. Obs. Gynecol. 87: 897-904.
3. Chapin, R., Stevens, J., Hughes, C., Kelces, W., Hess, R. and Daston, G. 1996. Endocrine modulation of reproduction. F. Appl. Toxicol. 29: 1-27.
4. Adams, N. R. 1995. Detection of the effects of phytoestrogens on sheep and cattle. J. Anim. Sci. 73: 1509-1515.
5. Price, K. R. and Fenwick, G. R. 1984. Soyasaponin I, a compound possessing undesirable taste characteristics isolated from the dried pea (Pisum sativum L.). J. Sci. Food Agri. 35: 887-892.
6. Setchell, K. D. and Cassidy, A. 1999. Dietary isoflavones: biological effects and relevance to human health. J. Nutr. 129: 758S-767S.
7. Constantinou, A., Kamath, N. and Murley, J. 1998. Genistein inactivates bcl-2, delays the G2/M phase of the cell cycle, and induces apoptosis of human breast adenocarcinoma MCF-7 cells. Eur. J. Cancer 34: 1972-1934.
8. Wang, C. and Kurzer, M. 1997. Phytoestrogen concentration determines effects on DNA synthesis in human breast cancer cells. Nutr. Cancer 28: 236-247.
9. Hsu, J., Hung, H., Chen, C., Hsu, W. and Ying, C. 1999. Effects of the dietary phytoestrogen biochanin A on cell growth in the mammary carcinoma cell line MCF-7. J. Nutr. Biochem. 10: 510-517.
10. Delcols, K. B., Bucci, T., Lomax, L., Latendresse, J., Warbritton, A., Weis, C. and Newbold, R. 2001. Effects of dietary genistein exposure during development on male and female CD (Sprague-Dawley)rats. Repro. Toxicol. 15: 647-663.
11. Sathyamoorthy, N., Wang, T. T. Y. and Phang, J. M. 1994. Stimulation of pS2 expression by diet-derived compounds. Cancer Res. 79: 957-961.
12. Ying, C., Hsu, J. and Shieh, S. 2001. Growth inhibition of human endothelial cells by the phytoestrogen biochanin A, a metabolite of genistein. Br. J. Nutr. 85: 615-620.
13. Schmitt, E., Dekant, W. and Stopper, H. 2001. Assaying the estrogenicity of phytoestrogens in cells of different estrogen sensitive tissues. Toxicol. *in vitro* 15: 433-439.
14. Weber, K. S., Setchell, K. D. R., Stocco, D. M. and Lephart, E. R. 2001. Dietary soy-phytoestrogens decrease testosterone levels and prostate weight without altering LH, prostate 5 alpha-reductase or testicular steroidogenic acute regulatory peptide levels in adult male Sprague-Dawley rats. J. Endocrinol. 170: 591-599.
15. Peterson, G. 1995. Evaluation of the biochemical targets of genistein in tumor cells. J. Nutr. 125: 784s-770s.
16. Adlercreutz, C. H. T., Goldin, B. R., Gorbach, S. L., Hockerstedt, K. A. V. and Watanabe, S. 1995. Soy phytoestrogen intake and cancer risk. J. Nutr. 125: 757s-770s.
17. Morton, M. S., Wilcox, G., Wahlqvist, M. L. and Griffiths, K. 1994. Determination of lignans and isoflavonoids in human female plasma following dietary supplementation. J. Endocrinol. 142: 251-259.
18. Scully, K. M., Gleiberman, A. S., Lindzey, J., Lubahn, D.B., Korach, K. S. and Rosenfeld, M. G. 1997. Role of estrogen receptor-alpha in the anterior pituitary gland. Mol. Endocrinol. 11: 674-681.
19. Day, R. N., Koike, S., Sakai, M., Muramatsu, M. and Maurer, R. A. 1990. Both Pit-1 and the estrogen receptor are required for estrogen responsiveness of the rat prolactin gene. Mol. Endocrinol. 4: 1964-1971.
20. Seyfred, M. A. and Gorksi, J. 1990. An interaction between the 5' flanking distal and proximal regulatory domains of the rat prolactin gene is required for transcriptional activation by estrogens. Mol. Endocrinol. 4: 1226-1234.
21. Chan, Y., Lin, A., McNally, J., Peleg, D., Meyuhas, O. and Wool, I. 1987. The primary structure of rat ribosomal protein L19: A determination from the sequence of nucleotides in a cDNA and from the sequence of amino acids in the protein. J. Biol. Chem. 262: 1111-1115.
22. Kaneko, K., Gélinas, C. and Gorski, J. 1993. Activation of the silent progesterone receptor gene by ectopic expression of estrogen receptor in a rat fibroblast cell line. Biochemistry 32: 8348-8359.
23. Ying, C. and Lin, D. H. 2000. Estrogen modulated

estrogen receptor-Pit-1 protein complex formation and prolactin gene activation requires novel protein synthesis. J. Biol. Chem. 275: 15407-15412.

24. SAS (1990): "SAS/STAT? User's Guide (Release 6.04) Vol.2". Cary, NC: SAS Inst. Inc. U. S. A.
25. Franke, A. A., Custer, L. J. and Tanaka, Y. 1999. Isoflavones in human breast milk and other biological fluids. A. J. Clin. Nutr. 68: 1446S-1473S.
26. Peterson, G. and Barnes, S. 1991. Genistein inhibition of the growth of human breast cancer cells; independence from estrogen receptors and the multi-drug resistance gene. Biochem. Biophys Res. Comm. 179: 661-667.
27. Singhal, R. L., Yeh, Y. A., Praja, N., Olah, E., Sledge, G. W. Jr. et al. 1995. Quercetin down regulates signal transduction in human breastcarcinoma cells. Biochem. Biophys Res. Comm. 208: 425-431.
28. Goldin, B. R., Adlercreutz, H., Gorbach, S. L., Woods, M. N., Dwyer, J. T. et al. 1986. The relationship between estrogen levels and diets of Caucasian American and oriental immigrant women. Amer. J. Clin. Nutr. 44: 945-953.
29. Adlercreutz, H., Markkanen, H. and Watanabe, S. 1993. Plasma concentrations of phytoestrogens in Japanese men. Lancet 342: 1209-1210.
30. Stahl, S., Chun, T. Y. and Gray, W. G. 1998. Phytoestrogens act as estrogen agonists in an estrogen-responsive pituitary cell line. Toxicol. App. Pharmacol. 152: 41-48.
31. Patisaul, H. B., Whitten, P. L. and Young, L. J. 1999. Regulation of estrogen receptor beta mRNA in the brain: opposite effects of 17 beta-estradiol and the phytoestrogen, coumestrol. Brain Res. Mol. Brain Res. 67: 165-171.