In Vitro Cell. Dev. Biol.—Animal 37:275–282, May 2001
© 2001 Society for In Vitro Biology
1071-2690/01 $10.00+0.00

INHIBITION OF ENVIRONMENTAL ESTROGEN–INDUCED PROLIFERATION OF HUMAN BREAST CARCINOMA MCF-7 CELLS BY FLAVONOIDS

DAL-HO HAN, HIROFUMI TACHIBANA, AND KOJI YAMADA[1]

Laboratory of Food Chemistry, Department of Bioscience and Biotechnology, Division of Bioresource and Bioenvironmental Science, Graduate School, Kyushu University, Fukuoka 812-8581, Japan (D.-H. H., H. T., K. Y.) and CREST, Japan Science and Technology, Japan (K. Y.)

(Received 31 August 2000; accepted 6 March 2001)

Summary

In the present study, we evaluated the individual and combined effects of environmental estrogens and flavonoids on the proliferation of human breast carcinoma MCF-7 cells. These compounds are as follows: (1) pharmaceutical chemicals such as diethylstilbestrol, 17α-ethynylestradiol (17ES), tamoxifen, mestranol, and clomiphene, (2) industrial chemicals such as bisphenol A (BisA), 4-octylphenol (OP), 4-nonylphenol (NP), and *p,p′*-biphenol, and (3) flavonoids such as daidzein (D), genistein (G), quercetin (Q), and luteolin (L). We found that nanomolar concentrations of 17ES, BisA, OP, and NP were sufficient to stimulate the proliferation of MCF-7 cells. Among them, 1 μ*M* BisA exhibited cell proliferation–stimulating activity as strong as 10 n*M* 17β-estradiol; and D and G exhibited cell proliferation–stimulating activity at 10 n*M*. On the other hand, Q and L exhibited cell proliferation–inhibiting activity. We also found that 10 n*M* flavonoids, such as D, G, Q, and L, were able to inhibit the proliferation-stimulating activity in MCF-7 cells by 1 μ*M* environmental estrogens.

Key words: 17β-estradiol; environmental estrogens; flavonoids.

Introduction

In the last 50 yr, many reports on environmental estrogens have been published, and various types of environmental estrogens have been shown to modify the endocrine system in wildlife animals and human beings (Guillette, 1995). In these studies, various types of natural and synthetic chemicals have been shown to exert estrogenic activity in both in vivo and in vitro assay systems (Colborn et al., 1993; Sharpe and Skakkenback, 1993; Safe, 1995).

The list of environmental estrogens contains polychlorinated biphenols, polycyclic aromatic hydrocarbons, polychlorinated dibenzodioxins, and alkylphenolic compounds, pesticides, herbicides, some phytoestrogens, and mycoestrogens. In addition, natural estrogens, such as 17β-estradiol (E2), play pivotal roles not only in controlling reproduction in females and, to a lesser extent, in males, but also in the development and growth of some types of cancers (Soto et al., 1995; Colborn et al., 1996).

The majority of these environmental estrogens are quite different in structure from the natural estrogens, so it is not possible at present to assess whether a compound is to be estrogenic, based on the knowledge of its chemical structure. Among these environmental estrogens, phytoestrogens have been linked to infertility in animals (Setchell et al., 1984). However, there is mounting evidence that they can relieve menopausal symptoms and be a safeguard against breast and prostate cancers (Cook and Samman, 1996; Mitchell et al., 1998).

There are at least 20 groups of phytoestrogens, which were derived from various plants, and some of them exhibit estrogenic activities (Cheng et al., 1954; Farnsworth et al., 1975). When taken by animals, such substances influence the synthesis and metabolism of estrogen, and exhibit estrogenic or antiestrogenic effects (Stob, 1983). In addition to their weak estrogenic and antiestrogenic activities, phytoestrogens exhibit antioxidative, antiviral, antibacterial activities, as well as proliferation-inhibiting and differentiation-inducing activities, in mammalian cells (Leclerg and Heuson, 1979; Price and Fenwick, 1985; Setchell and Fenwick, 1985; Setchell, 1998).

Correlative studies have shown that the intake of flavonoids is significantly higher in countries where the incidence of breast and prostate cancer is low, suggesting that they may act as chemopreventive agents (Adlercrentz, 1990). These results suggest that their biological and physiological activities are useful for cancer prevention as well as modification of estrogenic activity.

Though approximately 70 chemicals are suspected already of having potential endocrine-disrupting activity, it is still necessary to clarify whether these chemicals have the potential to be endocrine disruptors (Murphy, 1982; Wang et al., 1994). However, data on the combined effect of environmental estrogens and flavonoids, a representative of plant-derived natural estrogens in food, is so far limited. Thus, it is necessary to evaluate how strong their effects are, and what combined effects they may have when they coexist with other environmental estrogens. In addition, the human breast carcinoma cell line, MCF-7 cells, is a well-established in vitro sys-

[1] To whom correspondence should be addressed at Laboratory of Food Chemistry, Department of Bioscience and Biotechnology, Division of Bioresource and Bioenvironmental Science, Graduate School, Kyushu University, Fukuoka 812-8581, Japan. E-mail: yamadako@agr.kyushu-ac.jp

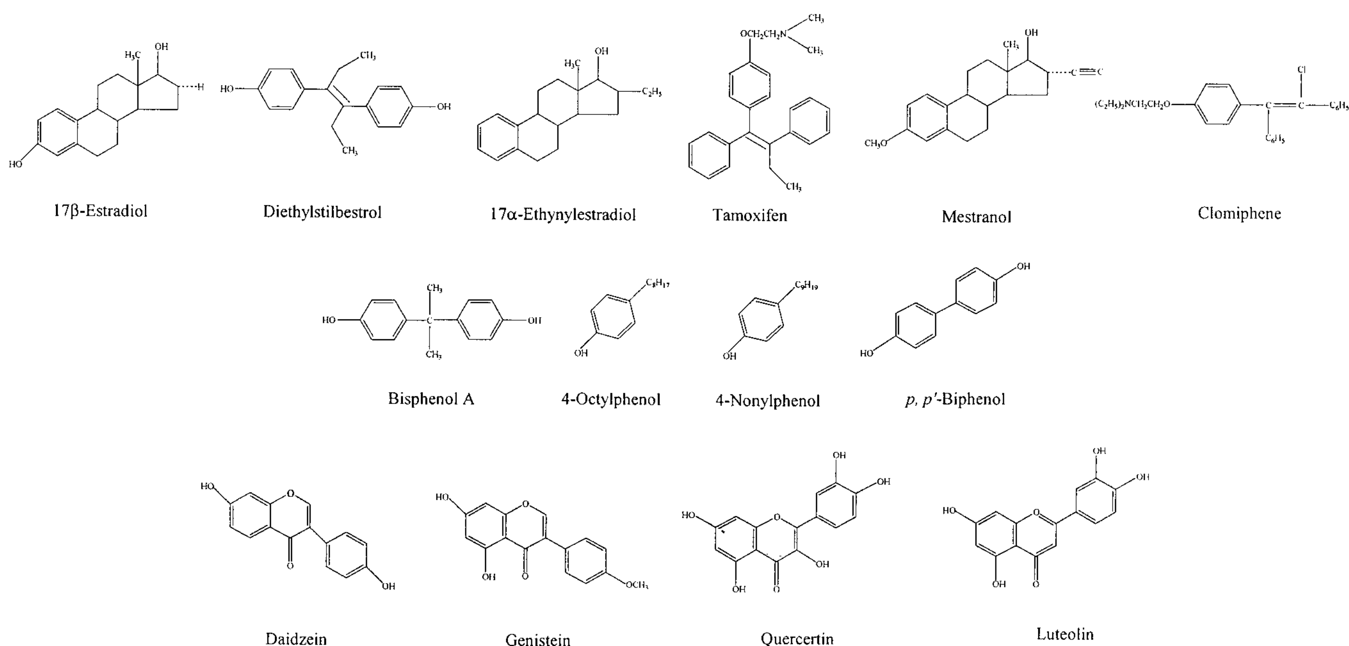


FIG. 1. Chemical structure of environmental estrogens and flavonoids used in this study.

tem characterized by its estrogen responsiveness through expression of the estrogen receptor (Levenson and Jordan, 1997).

Therefore, the proliferative effect of natural estrogens on the target cells, which have estrogen receptor, remains the hallmark of estrogen action. As one method to determine the estrogenicity of environmental estrogens and flavonoids, we tested the proliferative effect of these compounds on MCF-7 cells. Furthermore, we investigated the inhibition of environmental estrogen–induced proliferation of MCF-7 cells by flavonoids.

MATERIALS AND METHODS

Materials. Natural steroid estrogen E2 and activated-charcoal were obtained from Sigma Chemical Co. (St. Louis, MO). Pharmaceutical chemicals, such as diethylstilbestrol (DES), 17α-ethynylestradiol (17ES), tamoxifen (Tam), mestranol (Mes), and clomiphene (Clo), were also obtained from Sigma. A plastic component bisphenol A (BisA) and alkylphenols, such as 4-octylphenol (OP), 4-nonylphenol (NP), and *p,p′*-biphenol (BiP), were obtained from Aldrich (Milwaukee, WI). Flavonoids, such as daidzein (D), genistein (G), quercetin (Q), and luteolin (L), were obtained from Fujicco Co. (Kyoto, Japan). Fetal bovine serum (FBS) was purchased from BioWhittaker (Walkersville, MD). The chemical structures of the compounds used in this study are shown in Fig. 1. All the environmental estrogens were dissolved by a 7:3 mixture of ethanol and phosphate-buffered saline (EtOH/PBS, pH 7.4), and the final concentration in culture medium did not exceed 0.1%; this concentration did not affect the cell yields. The other chemicals used here were all analytical grade.

Charcoal treatment of serum. To remove the endogenous estrogens, 100 ml of FBS was treated twice with 5 g each of activated-charcoal at 56° C for 30 min. Then, the activated charcoal was removed by centrifugation at 450 × *g* and 4° C for 20 min. This procedure was repeated twice, and the supernatant was filtered through a 0.22-μm cellulose acetate filter. Then, the charcoal-treated FBS (cFBS) was stored at −20° C until use.

Cells and cell culture. Human breast carcinoma MCF-7 cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 2.3 mg/ml *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid, 100 U/ml penicillin G, 0.1 mg/ml streptomycin, 2.08 mg/ml sodium bicarbonate, and 5% FBS. The cells were cultured using 100-mm plastic tissue-culture dishes (Nalge Nunc International, Roskilde, Denmark) at 37° C with 5% CO_2 in a humidified atmosphere.

MCF-7 cells from stock cultures were washed twice with PBS. Then, the cells were treated with 0.2% trypsin/PBS, and washed once with RPMI 1640 medium by centrifuging at 150 × *g* at 4° C for 3 min. The cell pellet was resuspended in RPMI 1640 medium and washed twice with the medium. Finally, the cell pellet was resuspended in RPMI 1640 medium, and the cell number was counted using a Coulter Counter (model Z1; Coulter Electronics, Hialeah, FL).

MCF-7 cells (1×10^5 cells/ml) were seeded into 24-well plates (Limbro, McLean, VA) and allowed to attach for 24 h. The medium was then replaced with phenol red–free RPMI 1640 medium containing 1% cFBS with environmental estrogens and flavonoids. Cell number was determined on 5 or 6 d by counting the number of cells in each well using a Coulter counter (model Z1; Coulter Electronics).

Statistical. Results are expressed as the mean ± standard deviation of three or four independent assays performed in duplicate. Mean cell numbers from each experiment were normalized to the hormone-free control culture cell (100%) to correct for the difference in the initial plating density. Differences between the different chemical treatment groups were determined by analysis of variance and Student's *t*-tests using Sigmaplot software (SPSS Inc., Chicago, IL). A *P*-value of ≤ 0.01 was regarded as significant.

RESULTS

Dose-dependent effect of environmental estrogens and flavonoids on the proliferation of MCF-7 cells. The MCF-7 cells were cultured in different concentrations of environmental estrogens and flavonoids in phenol red–free RPMI 1640 containing 1% cFBS, and cell proliferation was determined on 5 d during the logarithmic growth phase (Figs. 2–4). For controls, we used EtOH/PBS. This solvent was tested for its ability to affect the proliferation of MCF-7 cells. This solvent was unable to stimulate the proliferation of MCF-7 cells (data not shown). As EtOH/PBS was the primary solvent used for all the environmental estrogens, we used it as the negative control in our assay. As shown in Figs. 2–4, E2 exhibited strong proliferation-stimulating activity, about threefold in comparison with the hormone-free control cell. In the pharmaceutical chemicals (Fig. 2), DES exerted proliferation-stimulating activity above two- or threefold compared with the hormone-free control culture cell at all

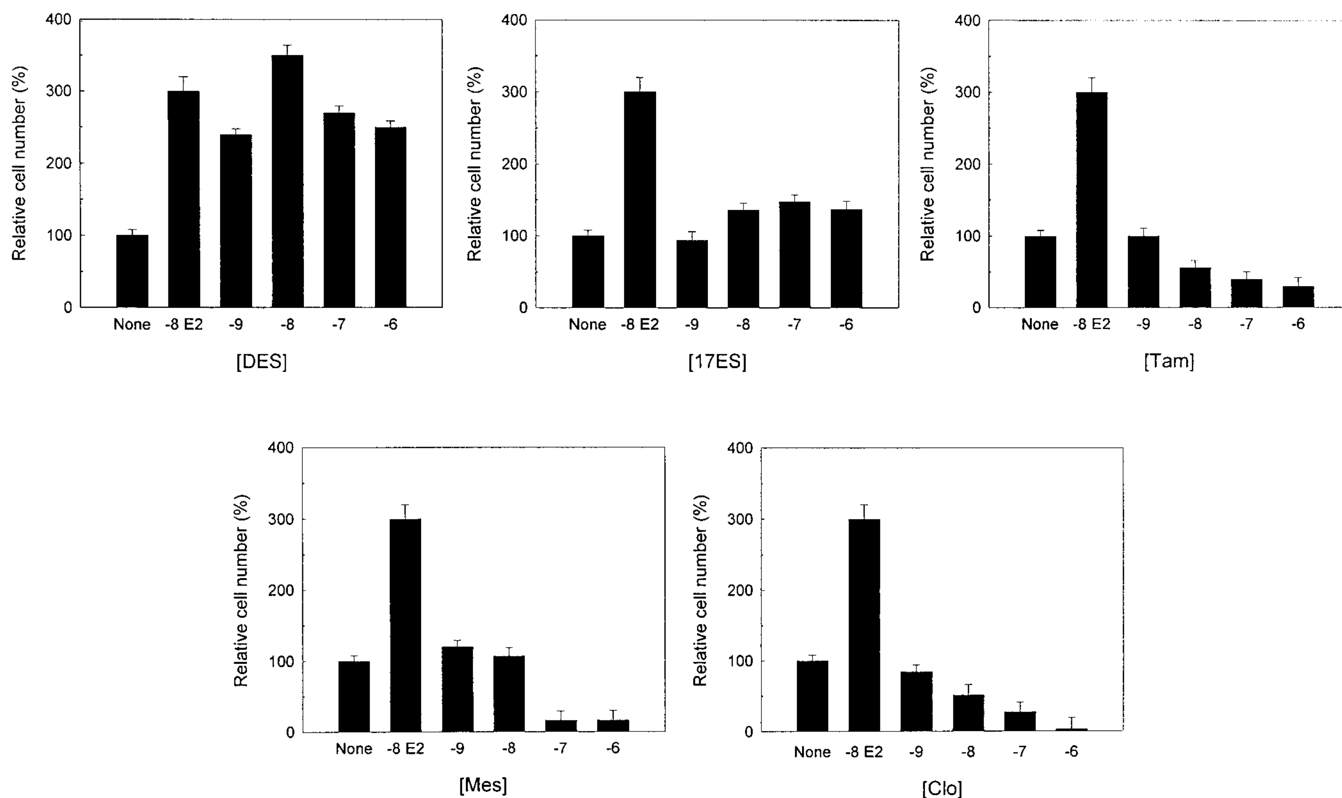


FIG. 2. Dose-dependent effect of pharmaceutical chemicals on the proliferation of MCF-7 cells. The pharmaceutical chemicals were tested at concentrations ranging from 1 n*M* (10^{-9} *M*, −9) to 1 μ*M* (10^{-6} *M*, −6) for 5 d. Results are expressed as the percentage for each compound tested over the hormone-free control. E2 (10 n*M*) was used as the positive control. Data are mean ± SD (n = 4). Abbreviations: none, hormone-free control; E2, 17β-estradiol; −8 E2, 10 n*M* 17β-estradiol; DES, diethylstilbestrol; 17ES, 17α-ethynylestradiol; Tam, tamoxifen; Mes, mestranol; Clo, clomiphene.

concentrations between 1 n*M* and 1 μ*M*. In particular, 10 n*M* DES exhibited proliferation-stimulating activity as strong as E2; and 17ES exhibited proliferation-stimulating activity at concentrations between 10 n*M* and 1 μ*M*. On the other hand, Tam and Clo exhibited strong cell proliferation–inhibiting activity at concentrations between 10 n*M* and 1 μ*M*. Likewise, Mes also inhibited the proliferation of MCF-7 cells at concentrations between 100 n*M* and 1 μ*M*. Industrial chemicals, such as BisA, OP, NP, and BiP, exhibited a strong dose–response trend toward cell proliferation–stimulating activity with increasing concentrations (Fig. 3). Among them, 1 μ*M* BisA exhibited cell proliferation–stimulating activity as strong as 10 n*M* E2. In addition, 10 n*M* flavonoids, such as D and G, exhibited proliferation-stimulating activity in MCF-7 cells, while Q and L exhibited a strong dose–response trend toward cell proliferation–stimulating activity at all concentrations between 1 n*M* and 1 μ*M*. In this study, we found that the proliferation-stimulating effect on MCF-7 cells was induced by 17ES, BisA, OP, NP, D, and G at the indicated concentrations.

Time-dependent effect of environmental estrogens and flavonoids on the proliferation of MCF-7 cells. In order to test the proliferative pattern of MCF-7 cells, cells were cultured with 1 μ*M* environmental estrogens and 10 n*M* flavonoids or 10 n*M* E2 in phenol red–free RPMI 1640 medium containing 1% cFBS for 6 d. After the addition of Tam and Clo at 1 μ*M*, the MCF-7 cells exhibited a strong proliferation-inhibiting activity (Fig. 5). On the other hand, 1 μ*M* 17ES exhibited a cell proliferation–stimulating activity as strong as 10 n*M* E2. The strongest proliferation activity of 17ES was detectable at 3 d and reached a maximum level at 4 d, whereas that of 10 n*M* E2 was detectable by 5 d of treatment. In addition, DES exhibited a weak proliferation-stimulating activity at 4 and 5 d, and Mes exhibited a proliferation-inhibiting activity at 5 and 6 d. In the case of industrial chemicals (Fig. 6), BisA, OP, NP, and BiP, at 1 μ*M*, exhibited a strong proliferation-stimulating activity in MCF-7 cells by 5 d of treatment. Among them, 1 μ*M* BisA exerted cell proliferation–stimulating activity as strong as 10 n*M* E2. In the results of 10 n*M* flavonoids (Fig. 7), D and G exhibited a weak proliferation-stimulating activity at 5 d of treatment, whereas Q and L did not.

Inhibition of environmental estrogen–stimulated proliferation of MCF-7 cells by flavonoids. To investigate the inhibition of environmental estrogen–stimulated proliferation of MCF-7 cells by flavonoids such as D, G, Q, and L, MCF-7 cells were cultured with 1 μ*M* environmental estrogens in the absence or presence of 10 n*M* flavonoids for 5 d of treatment; and the individual proliferation effects of the environmental estrogens were compared with their effects in combination with flavonoids. Since our initial results showed that D and G at 10 n*M* exhibited proliferation-stimulating activity in MCF-7 cells, we examined whether these flavonoids could inhibit the proliferation of MCF-7 cells stimulated by environmental estrogens. When 10 n*M* flavonoids were combined with 17ES, Tam, and Clo (1 μ*M*), they inhibited the environmental estrogen–stimulated proliferation of MCF-7 cells (Fig. 8). Among

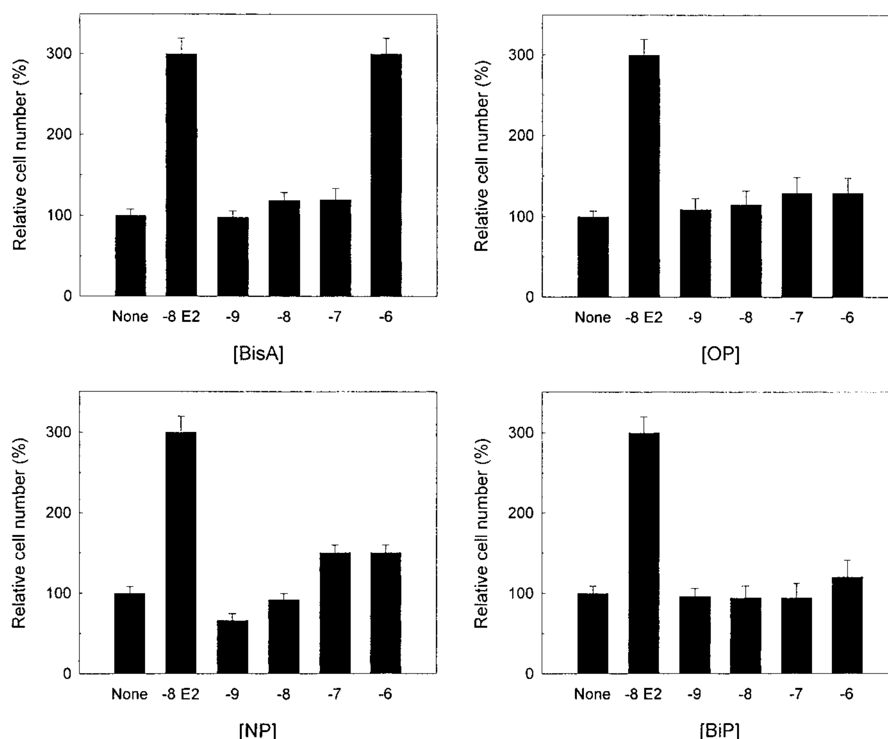


FIG. 3. Dose-dependent effect of industrial chemicals on the proliferation of MCF-7 cells. The industrial chemicals were tested at concentrations ranging from 1 n*M* (10^{-9} *M*, −9) to 1 μ*M* (10^{-6} *M*, −6) for 5 d. Results are expressed as the percentage for each compound tested over the hormone-free control. E2 (10 n*M*) was used as the positive control. Data are mean ± SD (n = 4). Abbreviations: none, hormone-free control; E2, 17β-estradiol; −8 E2, 10 n*M* 17β-estradiol; BisA, bisphenol A; OP, 4-octylphenol; NP, 4-nonylphenol; BiP, *p,p′*-biphenol.

them, Tam and Clo were able to inhibit the environmental estrogen–stimulated proliferation activity down to basal levels. In combination with 1 μ*M* Mes, these flavonoids had little effect on proliferation-inhibiting activity in MCF-7 cells stimulated by Mes. To investigate further the inhibition of industrial chemicals–stimulated proliferation of MCF-7 cells by flavonoids, we combined 10 n*M* flavonoids with 1 μ*M* industrial chemicals (Fig. 9). When 10 n*M* flavonoids were combined with BisA, OP, and BiP (1 μ*M*), the flavonoids exhibited a strongly inhibited proliferation activity in MCF-7 cells stimulated by these chemicals. On the other hand, L combined with BisA had little effect on proliferation-inhibiting activity. Moreover, when 10 n*M* flavonoids were combined with 1 μ*M* NP, the flavonoids exhibited a weakly inhibited proliferation-stimulating activity. However, the inhibition levels of flavonoids were shown to be higher than those of hormone-free control cells.

DISCUSSION

The endocrine and reproductive impairment of environmental estrogen has been extensively documented in wildlife animals and in humans in relation to the incidence of breast cancer. These effects of environmental estrogens are believed to be due to their: (1) mimicking the effects of endogenous hormones such as estrogens and androgens, (2) antagonizing the effects of normal, endogenous hormones, (3) altering the pattern of synthesis and metabolism of natural hormones, and (4) modifying the hormone receptor levels (Soto et al., 1995). As endocrine disruptors, many kinds of environmental estrogen have been studied for their estrogenic activity (Wang et al., 1997).

In the present study, we evaluated the estrogenicity of several environmental estrogens as the proliferative effect of MCF-7 cells. We investigated three groups of environmental estrogens: (1) pharmaceutical chemicals such as DES, 17ES, Tam, Mes, and Clo, (2) industrial chemicals such as BisA, OP, NP, and BiP, and (3) flavonoids such as D, G, Q, and L.

D, G, Q, and L are the major flavonoids in soybean and plant foods (Bradbury and White, 1954). These compounds have been historically used to enhance or reduce fertility (Farnsworth et al., 1975), so it is not surprising that the compounds derived from them have been found to have estrogenic properties. Also, these compounds have antiestrogenic properties in rats and humans (Lee et al., 1991, Cook and Samman, 1996). Antiestrogenic and antioxidant activities are proposed to be anticarcinogenic (Fisher et al., 1988; Setchell and Aldercreutz, 1988). G also inhibits tyrosine kinases and blocks angiogenesis in vitro (Cook and Samman, 1996), and these actions are also the proposed mechanisms of anticarcinogenesis. Although the proliferation-inhibiting activity of flavonoids has been reported in MCF-7 cells, most of these studies were conducted at a high dose of D and G (Katsuze et al., 1975; Barkly et al., 1981; Richard and Benita, 1981). Studies of dose-dependent effects were conducted with each compound to determine the minimum concentration necessary to stimulate the proliferation of MCF-7 cells. In the present study, we found that flavonoids such as D and G, which

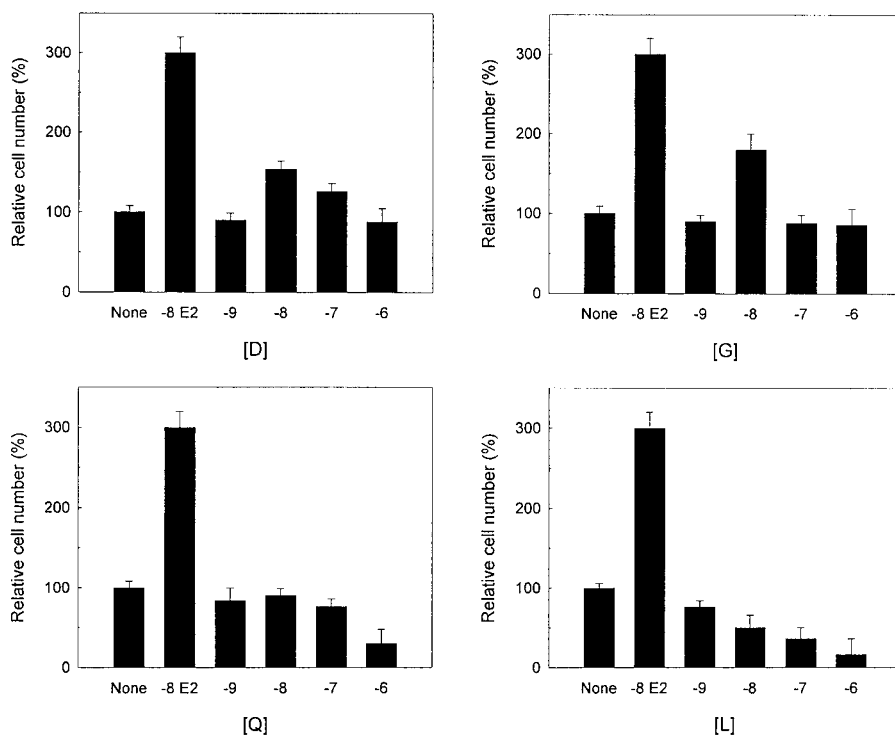


FIG. 4. Dose-dependent effect of flavonoids on the proliferation of MCF-7 cells. The flavonoids were tested at concentrations ranging from 1 n*M* (10^{-9} *M*, −9) to 1 μ*M* (10^{-6} *M*, −6) for 5 d. Results are expressed as the percentage for each compound tested over the hormone-free control. E2 (10 n*M*) was used as the positive control. Data are mean ± SD (n = 4). Abbreviations: none, hormone-free control; E2, 17β-estradiol; −8 E2, 10 n*M* 17β-estradiol; D, daidzein; G, genistein; Q, quercetin; L, luteolin.

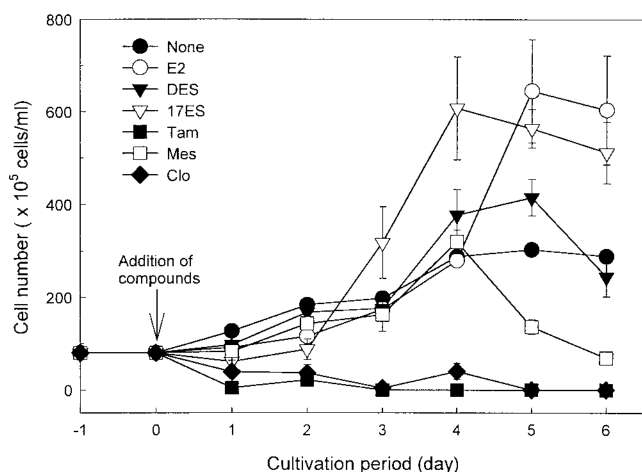


FIG. 5. Time-dependent effect of pharmaceutical chemicals on the proliferation of MCF-7 cells. Cells were cultured with 1 μ*M* pharmaceutical chemicals and 10 n*M* E2 in phenol red–free RPMI 1640 medium containing 1% cFBS for 6 d. Data are mean ± SD (n = 3). Abbreviations: none; hormone-free control; E2, 17β-estradiol; DES, diethylstilbestrol; 17ES, 17α-ethynylestradiol; Tam, tamoxifen; Mes, mestranol; Clo, clomiphene.

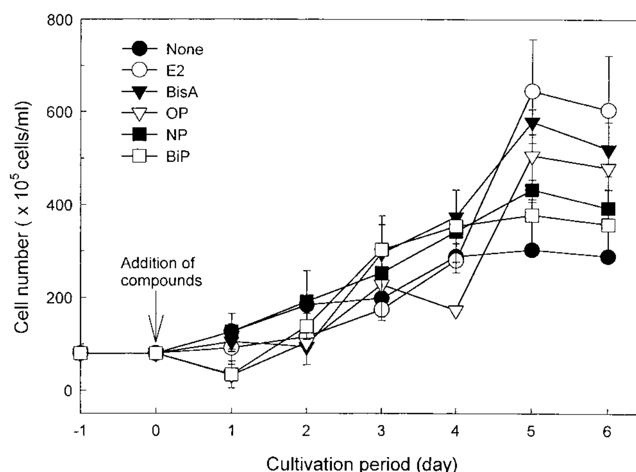


FIG. 6. Time-dependent effect of industrial chemicals on the proliferation of MCF-7 cells. Cells were cultured with 1 μ*M* industrial chemicals and 10 n*M* E2 in phenol red–free RPMI 1640 medium containing 1% cFBS for 6 d. Data are mean ± SD (n = 3). Abbreviations: none, hormone-free control; E2, 17β-estradiol; BisA, bisphenol A; OP, 4-octylphenol; NP, 4-nonylphenol; BiP, *p,p'*-biphenol.

have estrogenic agonists, were able to stimulate the proliferation of MCF-7 cells at 10 n*M*, while Q and L, which have estrogen antagonists, exerted a proliferation-inhibiting activity at all the concentrations tested.

Nonsteroidal estrogen antagonists, pharmaceutical chemicals such as Tam and Clo, have been reported to have a variety of effects in estrogen target tissues (Jordan, 1979; Clark and Maraverch, 1982). Although these chemicals are commonly referred to as antiestrogens, they have both estrogen agonist and antagonist properties in rats and humans (Luman and Klopper, 1975; Katzenellenbogan et al., 1979). In contrast, antiestrogens appear to act mainly as estrogen agonists in mice (Terenius, 1971) and as estrogen an-

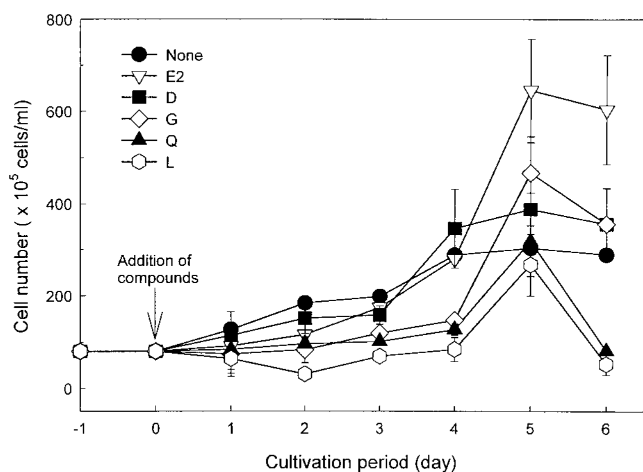
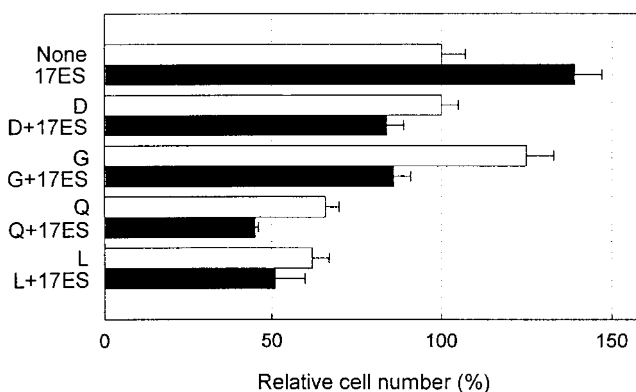


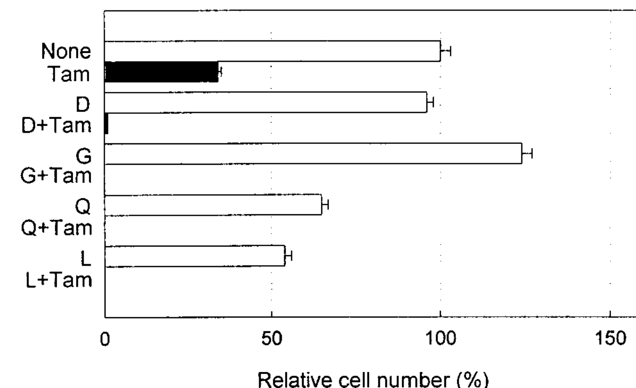
FIG. 7. Time-dependent effect of flavonoids on the proliferation of MCF-7 cells. Cells were cultured with 10 n*M* flavonoids and 10 n*M* E2 in phenol red–free RPMI 1640 medium containing 1% cFBS for 6 d. Data are mean ± SD (n = 3). Abbreviations: none, hormone-free control; E2, 17β-estradiol; D, daidzein; G, genistein; Q, quercetin; L, luteolin.

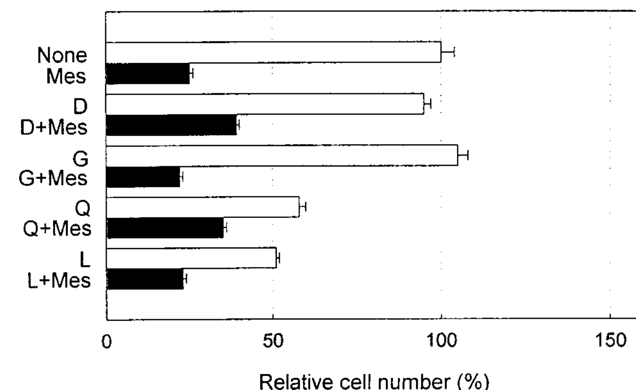
tagonists in chickens (Sutherland et al., 1977). Despite these various effects, Tam has proven to be effective in the management of human breast cancer patients (Sutherland, 1981). In the present study, Tam and Clo exhibited a strong proliferation-inhibiting activity at the concentrations ranging from 10 n*M* to 1 μ*M*. On the other hand, the pharmaceutical chemicals such as Mes exhibited proliferation-stimulating activity at 1 or 10 n*M;* and 17ES also exhibited proliferation-stimulating activity at concentrations ranging from 10 n*M* to 1 μ*M*.

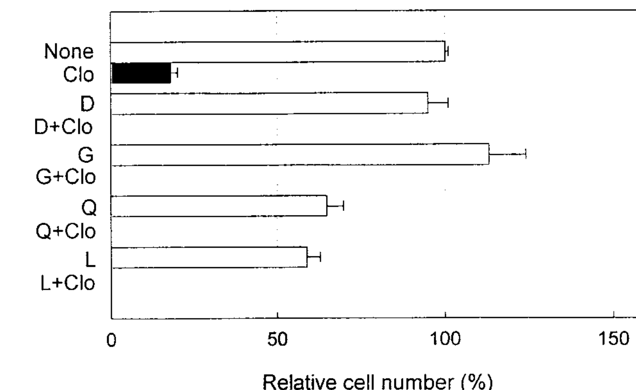
The first examination of the generational effects of hormone disruptors involved DES, a synthetic estrogen given to women in the early 1970s to prevent miscarriage. This drug caused increased rates of vaginal cancer, deformities of the uterus, abnormal pregnancies, and immune-system problems in daughters born to women who took the drug. In our study, 10 n*M* DES also exhibited a stronger proliferation-stimulating activity than 10 n*M* E2.

In addition, we found that the examples of suspected industrial chemicals included plastic compounds such as BisA, and the biodegradation products of detergents such as OP and NP, and nonchlorinated BiP. These chemicals induce cell proliferation in progesterone receptors in mammary tumor cells, and trigger mitotic activity in rat endometrium (Soto et al., 1991). However, maximal response in the proliferation assay was seen at concentrations of 10 μ*M*, and toxic effects occurred at higher concentrations. In the present study, we found that BisA, OP, NP, and BiP stimulated the proliferation of MCF-7 cells significantly in a dose-dependent man-









→

FIG. 8. Inhibition of pharmaceutical chemical–induced proliferation of MCF-7 cells by flavonoids. Cells were cultured with 1 μ*M* pharmaceutical chemicals and 10 n*M* flavonoids individually or in combination in phenol red–free RPMI 1640 medium containing 1% cFBS for 5 d. Results are expressed as the percentage for each compound tested over the hormone-free control. Data are mean ± SD (n = 4). Abbreviations: none, hormone-free control; 17ES, 17α-ethynylestradiol; Tam, tamoxifen; Mes, mestranol; Clo, clomiphene; D, daidzein; G, genistein; Q, quercetin; L, luteolin.

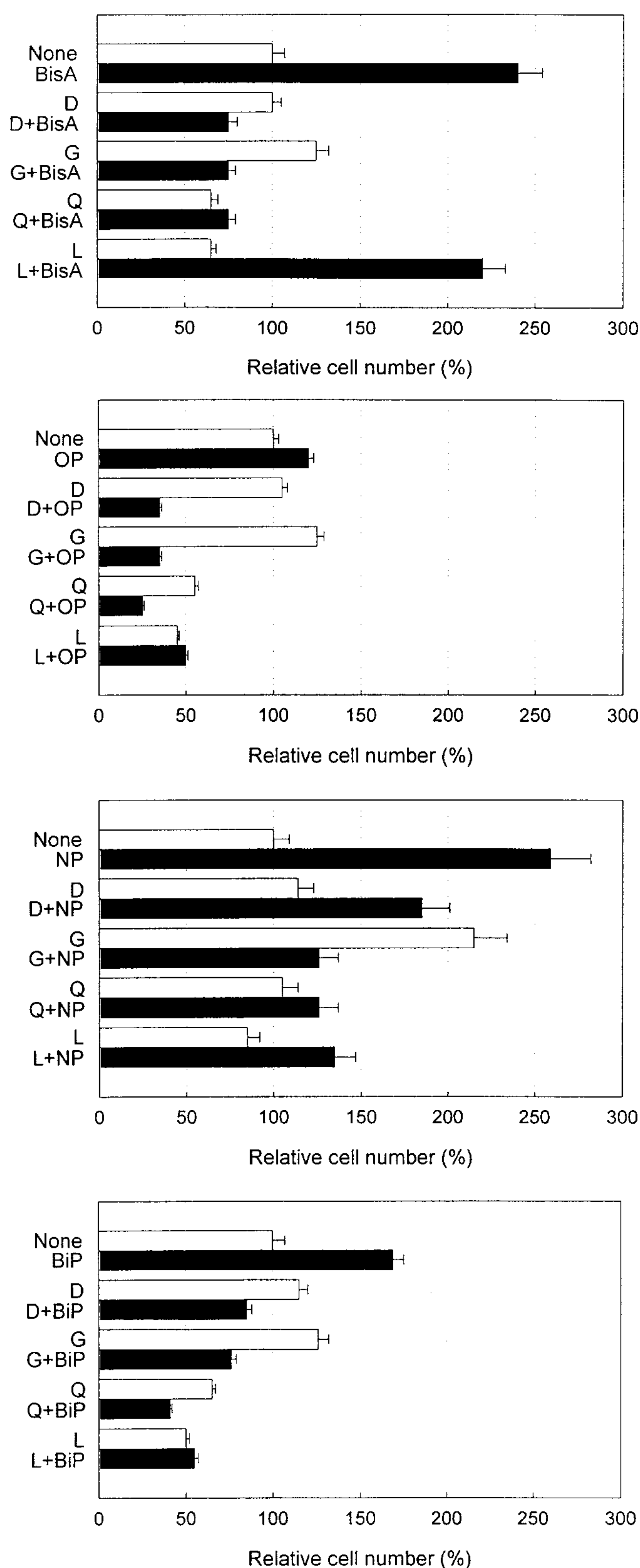


FIG. 9. Inhibition of industrial chemical–induced proliferation of MCF-7 cells by flavonoids. Cells were cultured with 1 μ*M* industrial chemicals and 10 n*M* flavonoids individually or in combination in phenol red–free RPMI 1640 medium containing 1% cFBS for 5 d. Results are expressed as the percentage of hormone-free control which was treated with EtOH/PBS. Data are mean ± SD (n = 4). Abbreviations: none, hormone-free control; BisA, bisphenol A; OP, 4-octylphenol; NP, 4-nonylphenol; BiP, *p,p′*-biphenol; D, daidzein; G, genistein; Q, quercetin; L, luteolin.

ner. Among them, 1 μ*M* BisA exhibited proliferation-stimulating activity as strong as 10 n*M* E2.

Also, our study revealed that 10 n*M* flavonoids were able to inhibit the proliferation of MCF-7 cells stimulated by environmental estrogens. In several epidemiological studies, an inverse relation has been suggested between the risk of breast or prostate cancer and the intake of soy foods or the urinary excretion of phytoestrogens (Markiewicz et al., 1993; Miksicek, 1993; Makela et al., 1994, 1995; Messina et al., 1994; Kurzer and Xu, 1997), although this could not be confirmed in other studies (Messina et al., 1994). The possibility still exists that the association between reduced breast and prostate cancer risk and phytoestrogens intake is not causal, and merely results from some other dietary characteristic. Despite the inconclusive epidemiological findings, several putative mechanisms that could account for the hypothesized chemopreventive effects of phytoestrogens have been proposed. Significantly, phytoestrogens have been suggested to have strong antiestrogenic effects, thereby inhibiting the development of hormone-related cancer (Makela et al., 1994; Messina et al., 1994). In this study, D and G exhibited some estrogenic activity in MCF-7 cells, while only Q and L exhibited antiestrogenic activity in the cells. All other phytoestrogens, including the flavonoids that are present in soy foods, showed only estrogenic activity. In previous in vitro studies, estrogenic or partial antiestrogenic activities were reported (Markiewicz et al., 1993; Ruh et al., 1995; Ingram et al., 1997; Messina et al., 1997). Several other mechanisms for the proposed chemopreventive effects of flavonoids have been suggested, including the induction of cancer cell differentiation, the inhibition of protein tyrosine kinases, the suppression of angiogenesis, and direct antioxidant effects (Markiewicz et al., 1993; Fotsis et al., 1997). These alternative mechanisms generally occur at flavonoid concentrations much higher (>5 μ*M*) than the concentrations at which estrogenic effects are detected (<100 n*M*). However, no unifying mechanism exists to explain the different effects of flavonoids within and between various species. Although D and G at 10 n*M* exhibited estrogenic activity in our study, we found that 10 n*M* flavonoids such as D, G, Q, and L were able to inhibit the estrogenic activity of 1 μ*M* environmental estrogens. In combination with NP, flavonoids had little effect on proliferation-inhibiting activity in MCF-7 cells, whereas when L was combined with BisA, it also had little effect on proliferation-inhibiting activity in MCF-7 cells.

Furthermore, in vitro and in vivo studies are necessary to elucidate the combine effect of flavonoids and environmental estrogens, and to clarify the mechanism by which flavonoids inhibits the expression of estrogenic activity.

ACKNOWLEDGMENT

This work was supported by CREST, Japan Science and Technology, Japan.

REFERENCES

Adlercruetz, H. Western diet and western disease: some hormonal and biochemical mechanisms and associations. Scand. J. Clin. Lab. Invest. 50:3–10; 1990.

Barkly, B. W.; William, H. K.; Nicoleno, G. Effects of serum and insulin on the sensitivity of the human breast cancer cell line MCF-7 to estrogens and antiestrogens. Cancer Res. 41:82–88; 1981.

Bradbury, R. B.; White, D. E. Oestrogens and related substances in plants. Vitam. Horm. 12:207–233; 1954.

Cheng, E.; Yoder, L.; Story, C. D.; Burrough, W. Estrogenic activity of some isoflavone derivatives. Science 120:545–546; 1954.

Clark, J. H.; Maraverch, B. M. The agonistic–antagonistic properties of clomiphene: a review. Pharmacol. Ther. 17:467–489; 1982.

Colborn, T.; Dumanoski, D.; Myers, J. P. Our stolen future. New York: Penguin Books; 1996:76.

Colborn, T.; Von Seal, F. S.; Soto, A. M. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ. Health Perspect. 101:378–384; 1993.

Cook, N. C.; Samman, S. Flavonoids-chemistry, metabolism, cardioprotective effects, and dietary sources. J. Nutr. Biochem. 7:66–76; 1996.

Farnsworth, N. R.; Bingel, A. S.; Cordell, G. A.; Crane, F. A.; Fong, H. H. S. Potential value of plants as sources of new antifertility agents II. J. Pharm. Sci. 64:717–754; 1975.

Fisher, S.; Cameron, G. S.; Baldwin, J. K.; Jasheway, D. W.; Patrick, K. E. Reactive oxygen in the tumor promotion stage of skin carcinogenesis. Lipids 23:592–597; 1988.

Fotsis, T.; Pepper, M. S.; Aktas, E., et al. Flavonoids, dietary-derived inhibitors of cell proliferation and in vitro angiogenesis. Cancer Res. 57:2916–2921; 1997.

Guillette, L. Endocrine disrupting environmental contaminants and developmental abnormalities in embryos. Hum. Ecol. Risk Assess. 1:25–36; 1995.

Ingram, D.; Sandres, K.; Kolybata, M.; Lopez, D. Case-control study of phytoestrogens and breast cancer. Lancet 350:990–994; 1997.

Katsuze, N.; Hogo, A.; Armelin, H. A.; Gorden, S. Control of ovarian cell growth in culture by serum and pituitary factors. Proc. Natl. Acad. Sci. 72(2):483–487; 1975.

Katzenellenbogan, B. S.; Bhakoo, H. S.; Ferguson, E. R.; Lan, N. C.; Tatee, T.; Tsai, T. S.; Katzenellenbogan, J. A. Estrogen and anti-estrogen action in reproductive tissues and tumors. Recent Prog. Horm. Res. 35:258–265; 1979.

Kurzer, M. S.; Xu, X. Dietary phytoestrogens. Annu. Rev. Nutr. 17:353–381; 1997.

Leclerg, G.; Heuson, J. C. Physiological and pharmacological effects of estrogens in breast cancer. Biochim. Biophys. Acta 560:427–455; 1979.

Lee, H. P.; Gourley, L.; Duffy, S. W.; Esteve, J.; Lee, J.; Day, N. E. Dietary effects on breast cancer risk in Singapore. Lancet 337:1197–1200; 1991.

Levenson, A. S.; Jordan, V. C. MCF-7: the first hormone responsive breast cancer cell line. Cancer Res. 57:3071–3078; 1997.

Luman, C. B.; Klopper, A. Antiestrogens: a review. Clin. Endocrinol. (Oxf.) 4:551–558; 1975.

Makela, S.; Davis, V. L.; Tally, W. C.; Korkman, J.; Salo, L.; Vihko, R.; Santti, R.; Korach, K. S. Dietary estrogens act through estrogen receptor-mediated processes and show no antiestrogenicity in cultured breast cancer cells. Environ. Health Perspect. 102:572–581; 1994.

Makela, S. I.; Pylkkanen, L. H.; Santti, R. S. S.; Adlercreutz, H. Dietary soybean may be antiestrogenic in male mice. J. Nutr. 125:437–445; 1995.

Markiewicz, L.; Garey, J.; Adlercreutz, H.; Gurpide, E. *In vitro* bioassays of non-steroidal phytoestrogens. J. Steroid Biochem. Mol. Biol. 45:339–405; 1993.

Messina, M.; Barnes, S.; Setchell, K. D. R. Phytoestrogens and breast cancer—commentary. Lancet 350:971–972; 1997.

Messina, M. J.; Persky, V.; Setchell, K. D. R.; Barnes, S. Soy intake and cancer risk: a review of the in vitro and in vivo data. Nutr. Cancer 21:113–131; 1994.

Miksicek, R. J. Commonly occurring plant flavonoids have estrogenic activity. Mol. Pharmacol. 44:37–43; 1993.

Mitchell, J. H.; Gardner, P. T.; McPhail, D. B.; Morrice, P. C.; Collins, A. R.; Duthie, G. G. Antioxidant efficacy of phytoestrogens in chemical and biological model systems. Arch. Biochem. Biophys. 360(1):142–148; 1998.

Murphy, P. A. Phytoestrogen content of processed soybean products. Food Technol. 36:62–64; 1982.

Price, K. R.; Fenwick, G. R. Naturally ocurring oestrogens in food. A review. Food Addit. Contam. 2:73–106; 1985.

Richard, L. E.; Benita, S. K. Effects of estrogens and antiestrogens on estrogen receptors dynamics and cancer cells. Cancer Res. 42:139–144; 1981.

Ruh, M. F.; Zacharewski, T.; Connor, K.; Howell, J.; Chen, I.; Safe, S. Naringenin, a weakly estrogenic bioflavonoid that exihibits antiestrogenic activity. Biochem. Pharmacol. 50:1485–1493; 1995.

Safe, S. Do environmental estrogens play a role in development of breast cancer in women and male reproductive problems? Hum. Ecol. Risk Assess. 1(2):17–23; 1995.

Setchell, K. D. R. Phytoestrogens: the biochemistry, physiology, and implications for human health of soy isoflavones. Am. J. Clin. Nutr. 68(S): 1333–1346; 1998.

Setchell, K. D. R.; Adlercreutz, H. Mammalian ligands and phytoestrogens. Recent studies on their formation, metabolism and biological role in health and disease. In: Rowlanol, I. R., ed. Role of the gut flora in toxicity and cancer. London: Academic Press; 1988.

Setchell, K. D. R.; Borriello, S. P.; Hulme, P.; Kirk, D. N.; Axelson, M. Non-steroidal estrogens of dietary origin: possible roles in hormone-dependent disease. Am. J. Clin. Nutr. 40:569–578; 1984.

Setchell, K. D. R.; Fenwick, G. R. Naturally occurring non-steroidal estrogens of dietary origin. In: Mclachlan, J., ed. Estrogens in the environment: influence on development. New York: Elsevier; 1985:69–85.

Sharpe, R.; Skakkenback, N. E. Are oestrogens involved in falling sperm counts and disorders of the male reproductive tract? Lancet 341: 1392–1395; 1993.

Soto, A. M.; Justicia, H.; Wray, J. W.; Sonnenchein, C. *p*-Nonyl-phenol: an estrogenic xenobiotic released from “modified” polytyrene. Environ. Health Perspect. 92:167–173; 1991.

Soto, A. M.; Sonnenschein, C.; Chung, K. L.; Fernadez, M. F.; Olea, N.; Serrano, F. O. The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants [Estrogens in the environment. Proceedings of the Estrogens in the Environmental III: Global Health Implications Conference]. Environ. Health Perspect. 103(S):113–122; 1995.

Stob, M. Naturally occurring food toxicants: oestrogens. In: Dechrigol Jr., M., ed. Handbook of naturally occurring food toxicants. Boca Raton, FL: CRC Press; 1983:81–100.

Sutherland, R. L. Estrogen antagonists in chick oviduct: antagonist activity of eight synthetic triphenlyethylene derivatives and their interactions with cytoplasmic and nuclear estrogen receptors. Endocrinol. 109:2061–2067; 1981.

Sutherland, R. L.; Mester, J.; Banliew, E. E. Tamoxifen is a potent “pure” antiestrogen in the chick oviduct. Nature 267:434–436; 1977.

Terenius, L. Structure activity relationships of antiestrogens with regard to interaction with 17β-estradiol in the mouse uterus and vagina. Acta Endocrinol. 66:402–431; 1971.

Wang, W.; Carl, M.; Higuchi. ; Rongging, Z. Individual and combinatory effects of soy isoflavones on the *in vitro* protenuation of lymphocyte activation. Nutr. Cancer 29(1):29–34; 1997.

Wang, X. U.; Murphy, P. A.; Cook, L.; Hendrich, S. Daidzein is a more bioavailable soymilk isoflavone than is genistein in adult women. J. Nutr. 124:825–832; 1994.