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Phytoestrogen Concentration Determines Effects on DNA Synthesis in Human Breast Cancer Cells

Chuanfeng Wang and Mindy S. Kurzer

Abstract: Thirteen isoflavonoids, flavonoids, and lignans, including some known phytoestrogens, were evaluated for their effects on DNA synthesis in estrogen-dependent (MCF-7) and -independent (MDA-MB-231) human breast cancer cells. Treatment for 24 hours with most of the compounds at 20–80 μM sharply inhibited DNA synthesis in MDA-MB-231 cells. In MCF-7 cells, on the other hand, biphasic effects were seen. At 0.1–10 μM, coumestrol, genistein, biochanin A, apigenin, luteolin, kaempferol, and enterolactone induced DNA synthesis 150–235% and, at 20–90 μM, inhibited DNA synthesis by 50%. Treatment of MCF-7 cells for 10 days with genistein or coumestrol showed continuous stimulation of DNA synthesis at low concentrations. Time-course experiments with genistein in MCF-7 cells showed effects to be reversed by 48-hour withdrawal of genistein at most concentrations. Induction of DNA synthesis in MCF-7 cells, but not in MDA-MB-231 cells, is consistent with an estrogenic effect of these compounds. Inhibition of estrogen-dependent and -independent breast cancer cells at high concentrations suggests additional mechanisms independent of the estrogen receptor. The current focus on the role of phytoestrogens in cancer prevention must take into account the biphasic effects observed in this study, showing inhibition of DNA synthesis at high concentrations but induction at concentrations close to probable levels in humans.

Introduction

Flavonoids, isoflavonoids, and lignans are naturally occurring diphenolic compounds widely present in vegetables, fruits, and whole grains. Some of these compounds are phytoestrogens, having been shown to exert estrogenic effects on the genital tract of female animals. Although not meeting this strict definition (1,2), a number of related compounds show effects suggestive of estrogenicity (2,3), such as binding of the estrogen receptor (ER), induction of specific estrogen-responsive gene products, and stimulation of breast cancer (BC) cell growth. For the purpose of this study, the term "phytoestrogen" will be used in the broader sense.

A growing body of literature suggests that phytoestrogen consumption may exert anticancer effects. *In vitro* experiments have shown specific phytoestrogens to inhibit growth of several tumor cell types, including mammary (4), leukemia (5), and gastric cancer cells (6). In animals, consumption of phytoestrogen-rich soy has been shown to protect rodents from experimentally induced cancer of the mammary gland (7,8) and colon (9,10). In humans the data are primarily epidemiologic. Studies have shown consumption of phytoestrogen-containing plant foods to be associated with decreased incidence of several cancers, including BC (11). In fact, it has been suggested that the low incidence of BC in Oriental women may be related to the consumption of an isoflavonoid-rich diet (12). Case-control studies showing low urinary lignan excretion in BC patients have led to the hypothesis that lignan consumption may also exert anticancer effects (13,14).

It is believed that phytoestrogens may contribute to a reduction in BC in part as a result of their antiestrogenic properties. These include competition with estradiol (E_2) for binding to the ER and type II binding sites (15–19) as well as inhibition of estrogen synthesis in human placental microsomes (20,21), preadipocytes (22,23), and choriocarcinoma cells (20). Phytoestrogens have also been found to induce sex hormone-binding globulin (17,24), potentially reducing free E_2 levels, and to decrease the estrogen-induced transcription of pS2 and ER in BC cells after long-term treatment (25).

Phytoestrogens also contain numerous nonhormonal properties that may result in anticancer effects. These include inhibition of viral reverse transcriptase (26), antioxidation and radical scavenging (27–29), antimutagenicity (30–32), and reduction of carcinogen-induced tumor growth (7–10). Other mechanisms may involve interactions with signal transduction factors such as tyrosine kinase (33–35) and protein kinase C (36) or DNA topoisomerase II (37–39).

Most studies of phytoestrogen effects on BC cell growth have been performed using genistein in estrogen-dependent MCF-7 cells. These reports suggest biphasic effects, with stimulation of growth at low concentrations and inhibition at high concentrations. Makela and co-workers (40) reported that, at 0.1–1.0 μM, genistein, biochanin A, and coumestrol stimulate growth of MCF-7 cells to 150–250%, and Mik-

The authors are affiliated with the Department of Food Science and Nutrition, University of Minnesota, St. Paul, MN 55108.

sicek (18) reported that 1 μM apigenin stimulates growth to 260%. Peterson and Barnes (4) showed high concentrations of genistein to inhibit growth of MCF-7 cells, with a 50% inhibitory concentration (IC_{50}) of 39 μM and >80% inhibition at 111 μM. In one report of biphasic effects, Wang and colleagues (25) showed stimulation of growth to 130–220% at 0.01–10 μM genistein and inhibition at >10 μM (25). Zava and Duwe (41) recently reported biphasic effects with genistein, showing stimulation to 150–400% at 0.001–10 μM and inhibition at >10 μM after 10 days of treatment.

There are few reports of phytoestrogen effects on estrogen-independent MDA breast cancer cells. Genistein has been shown to inhibit cell proliferation in MDA-MB-231 and MDA-468 cells, with IC_{50} values of 15 μM (42) and 24 μM (4), respectively.

Little is known about the actual exposure to phytoestrogens in humans consuming phytoestrogen-rich foods. The serum concentration of genistein in humans consuming soy products has been reported to be <1–10 μM (43–45). The results of *in vitro* studies suggest that, at these concentrations, stimulation, rather than inhibition, may be the primary effect of phytoestrogens on cell growth. Before recommendation of phytoestrogen consumption is considered as part of a cancer-preventive diet, it is essential to understand their potentially beneficial and harmful effects. It is therefore extremely important to broaden our understanding of the effects of phytoestrogens on BC cell growth at low and high concentrations, with particular attention to the effects at probable concentrations in humans.

The purpose of this study was to evaluate the direct effects of a number of phytoestrogens and related compounds on DNA synthesis (generally thought to reflect cell proliferation) in estrogen-dependent (MCF-7) and -independent (MDA-MB-231) human BC cells at low and high concentrations. The compounds studied were the isoflavonoids genistein, biochanin A, daidzein, and coumestrol; the flavonols kaempferol and quercetin; the flavones flavone, luteolin, apigenin, and chrysin; other flavonoids rutin and catechin; and the lignan enterolactone. The structures of the compounds tested are shown in Figure 1.

Methods

Materials

Genistein, kaempferol, chrysin, apigenin, quercetin, rutin, catechin, flavone, E_2, Dulbecco's modified Eagle's medium-nutrient mixture F-12 (DMEM-F-12) with and without phenol red, Dextran T-70, and Norit A activated charcoal were purchased from Sigma Chemical (St. Louis, MO), phosphate-buffered saline (PBS), fetal calf serum (FCS), trypsin, and antibiotic-antimycotic (10,000 U/ml penicillin G, 10,000 μg/ml streptomycin, 25 μg/ml amphotericin B) from GIBCO BRL (Grand Island, NY), coumestrol from Acros Organics (Fair Lawn, NJ), daidzein from ICN Biochemical (Aurora, OH), biochanin A from Aldrich Chemical (Milwaukee, WI), [^{3}H]thymidine from Amersham Life Science (Arlington Heights, IL), trichloroacetic acid (TCA) from EM Science (Gibbstown, NJ), and MCF-7 and MDA-MB-231 human breast cancer cell lines from American Type Culture Collection (Rockville, MD). Luteolin and enterolactone were kindly provided by Dr. Herman Adlercreutz (University of Helsinki, Finland).

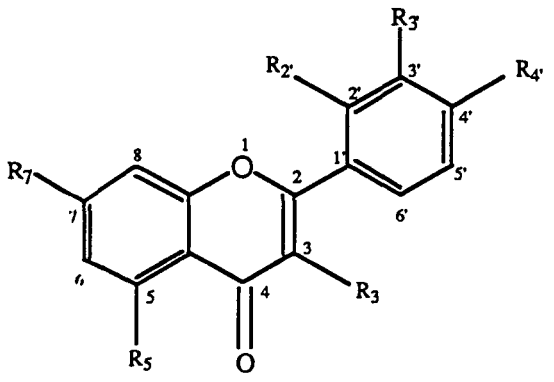
Cell Culture

Dextran-coated charcoal (DCC) was prepared as follows: 5 g of Norit A charcoal were washed, centrifuged four times for five minutes at full speed, and mixed with 0.5 g of Dextran T-70 in 200 ml of doubly distilled water for one hour with constant stirring at room temperature, then centrifuged at full speed for five minutes. The supernatant was then discarded. DCC-treated FCS (DCC-FCS) was prepared by mixing the DCC with 200 ml of FCS and incubating the mixture at 37°C for 90 minutes with constant stirring, then centrifuging it for 15 minutes at 3,200 *g*. After the FCS was reincubated with fresh DCC under the same conditions, the mixture was centrifuged for 30 minutes at 3,200 *g* and sterilized by filtration through a 0.2-μm filter (Nalge, Rochester, NY).

MCF-7 and MDA-MB-231 cells were maintained in T25 flasks with DMEM-F-12 containing 5% FCS and 1% antibiotic-antimycotic (5% FCS-DMEM-F-12) at 37°C in 5% CO_2. The medium was changed once every 48 hours, and the cells were passaged once each week. For the growth studies, the cells were grown to confluence in T175 flasks, then seeded in 24-well plates and incubated with 5% FCS-DMEM-F-12 overnight. The cells were then treated with serum-free and phenol red-free DMEM-F-12 for 24 hours. For the experimental treatments, the cells were then incubated with phenol red-free DMEM-F-12 containing 5% DCC-FCS and the compound of interest dissolved in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide in each well was 0.1%. DNA synthesis in the cells was evaluated by [^{3}H]thymidine incorporation (46).

Conditions varied slightly for the three types of experiments performed. For the dose-response experiments, 1–1.5 × 10^5 cells were seeded per well, and the cells were treated with each compound (except chrysin) for 24 hours at 0.01, 0.1, 1.0, 10, 50, and 100 μM. The tested concentrations of chrysin were 0.01, 0.1, 1.0, 10, 20, and 30 μM. For the genistein incubation and withdrawal experiment, 1.5 × 10^5 cells were seeded per well, and the cells were treated with genistein for 48 hours, then incubated with control medium for another 48 hours. The medium was changed and DNA synthesis was determined every 24 hours. For the experiments of long-term exposure to genistein and coumestrol, 5–7 × 10^4 cells were seeded per well, the experimental medium was changed once every 24 hours, and DNA synthesis was measured every other day for 10 days.

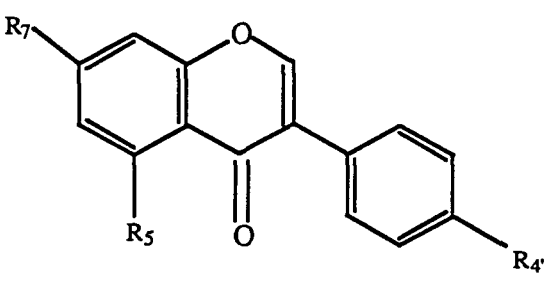
The sample size for each point was 8–10 for the dose-response studies, 5 for the genistein incubation and withdrawal studies, and 5–6 for the long-term genistein and coumestrol studies. Cell viability was tested with trypan blue



Flavonoid

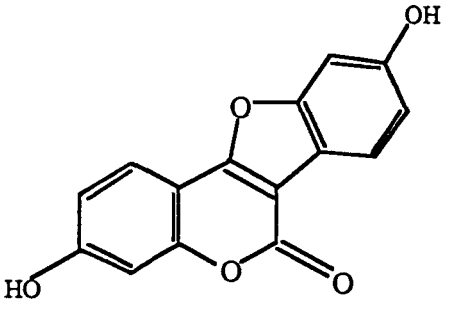
	$R_{2'}$	R_3	$R_{3'}$	$R_{4'}$	R_5	R_7
Flavone	H	H	H	H	H	H
Chrysin	H	H	H	H	OH	OH
Luteolin	H	H	OH	OH	OH	OH
Quercetin	H	OH	OH	OH	OH	OH
Rutin	H	OR*	OH	OH	OH	OH
Kaempferol	H	OH	H	OH	OH	OH
Apigenin	H	H	H	OH	OH	OH

R* = Rutinoside

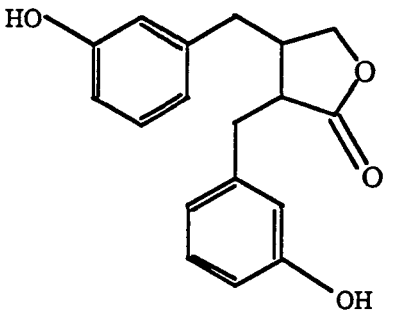


Isoflavone

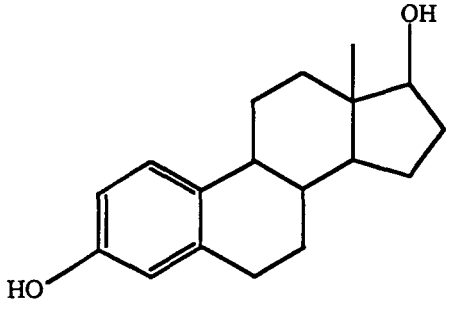
	R_5	R_7	$R_{4'}$
Genistein	OH	OH	OH
Biochanin A	OH	OH	OCH_3
Daidzein	H	OH	OH



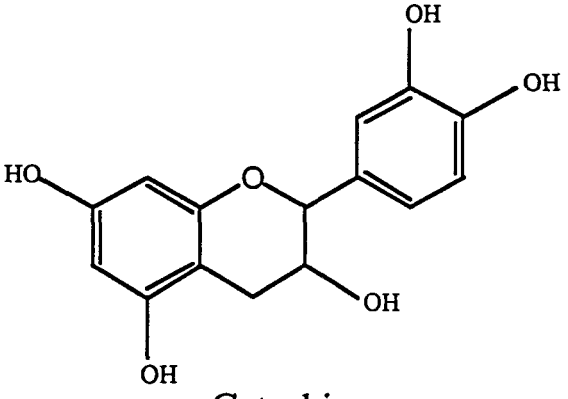
Coumestrol



Enterolactone



Estradiol



Catechin

Figure 1. Structures of genistein, biochanin A, daidzein, and coumestrol (isoflavonoids); kaempferol and quercetin (flavonols); flavone, luteolin, apigenin, and chrysin (flavones); rutin and catechin (flavonoids); enterolactone (lignan); and estradiol.

exclusion after 24 hours of treatment with the highest concentration of each compound.

Thymidine Incorporation

After incubation with experimental medium, the cells were washed with PBS three times and incubated with serum and phenol red-free DMEM-F-12 supplemented with [^{3}H]thymidine (2 μCi/ml, 0.4 ml/well) at 37°C for two hours. The medium was then removed, and the cells were washed with ice-cold PBS three times and fixed with methanol for 15 minutes. The cells were then treated with ice-cold 10% TCA for 10 minutes, washed twice with cold 5% TCA for 5 minutes, and extracted with 1 N NaOH overnight at room temperature (46). Aliquots of cell extracts were counted in a liquid scintillation counter (Beckman Instruments, Fullerton, CA).

Data Analysis

All experimental results were analyzed with one-way analysis of variance using Sigma Stat (Jandel Scientific, San Rafael, CA) and Microsoft Excel (Microsoft, Redmond, WA).

Results

Effects of E_2 on DNA Synthesis in MCF-7 and MDA-MB-231 Cells

Before the dose-response studies were performed, E_2 was used as a positive control to evaluate its ability to stimulate DNA synthesis in both cell lines (Figure 2). In MCF-7 cells, E_2 stimulated DNA synthesis significantly in the absence and presence of FCS. In the absence of FCS, induction was 150%, 250%, 250%, and 265% of control at 0.01, 0.1, 1.0, and 10 nM, respectively ($p < 0.01$). In the presence of 5% FCS, the induction increased to 320%, 490%, 520%, and 520% of control ($p < 0.01$). In MDA-MB-231 cells, E_2 significantly inhibited DNA synthesis in MDA-MB-231 cells to 90% of control at 1.0 and 10 nM ($p < 0.01$).

Dose-Response Studies of Isoflavonoid, Flavonoid, and Lignan Effects on DNA Synthesis in MCF-7 and MDA-MB-231 Cells

Cell viability studies showed no toxicity after 24 hours of treatment with the highest concentration of each compound. Cell viability was 94–98% in MCF-7 cells and 97–98% in MDA-MB-231 cells compared with 95–96% in control cells.

As shown in Figure 3, the isoflavonoids studied significantly stimulated DNA synthesis in MCF-7 cells at low concentrations, with maximal induction to 215% at 1.0 μM genistein, 185% at 0.1 μM coumestrol, 235% at 1.0 μM biochanin A, and 210% at 10 μM daidzein. The ranges in concentration causing stimulation were 0.01–10 μM for genistein and coumestrol, 0.1–10 μM for biochanin A, and 0.01–100 μM for daidzein. At high concentrations (50–100 μM), genistein, biochanin A, and coumestrol inhibited DNA synthesis, with IC_{50} values of 41.0, 37.7, and 42.5 μM, respectively (Figure 3, Table 1).

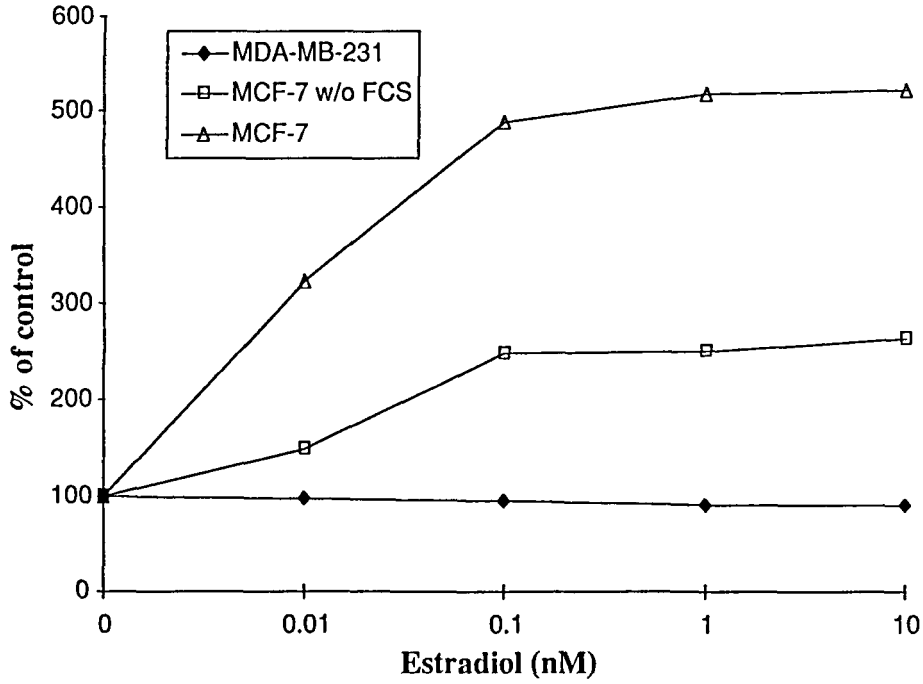


Figure 2. Effects of estradiol (E_2) on DNA synthesis in MCF-7 and MDA-MB-231 cells. Cells (1–1.5 × 10^5/well) were seeded overnight in 24-well plates with DMEM-F-12 containing 5% fetal calf serum (FCS), then incubated with serum-free and phenol red-free DMEM-F-12 for 24 hrs. Cells were then treated for 24 hrs with 0.01, 0.1, 1.0, or 10 nM E_2 with or without 5% dextran-coated charcoal-FCS, with 8–10 wells for each point. DNA synthesis was measured by [^{3}H]thymidine incorporation. For MCF-7 cells, all values are significantly different from control values ($p < 0.01$). For MDA cells, values at 1 and 10 nM E_2 are significantly different from control values ($p < 0.01$).

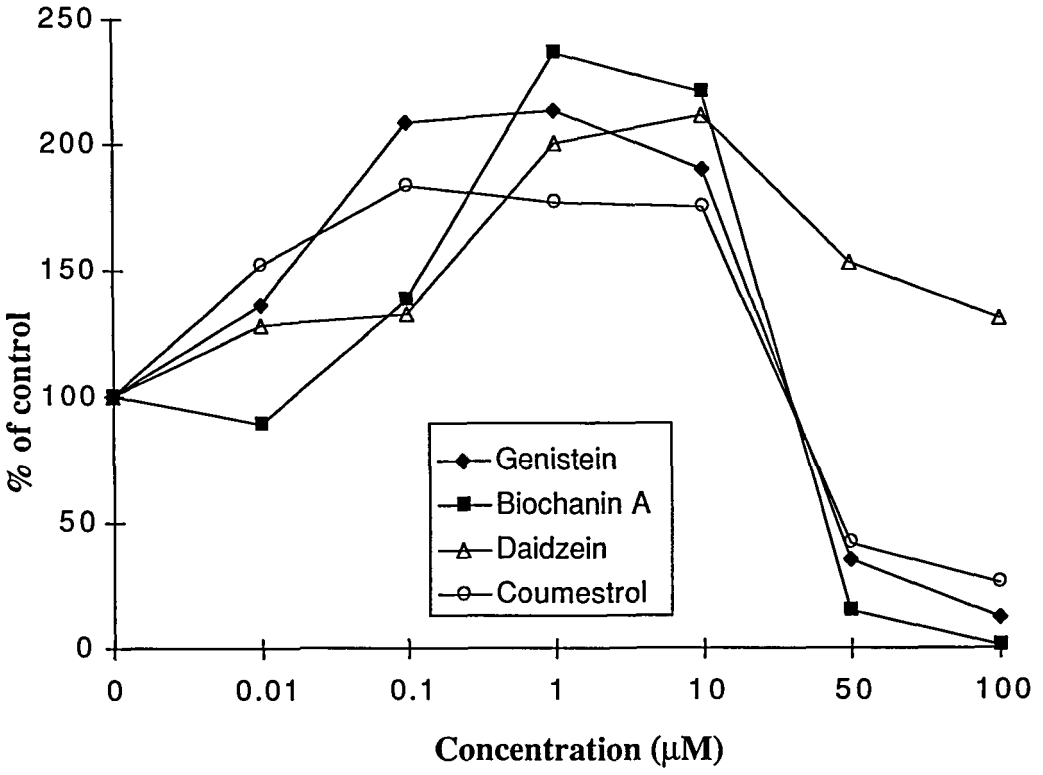


Figure 3. Effects of isoflavonoids on DNA synthesis in MCF-7 cells. MCF-7 cells (1–1.5 × 10^5/well) were seeded and incubated. Cells were then treated with each compound at 0.01, 0.1, 1.0, 10, 50, or 100 μM for 24 hrs in presence of 5% dextran-coated charcoal-FCS, with 8–10 wells for each point. DNA synthesis was measured by [^{3}H]thymidine incorporation. Values at all concentrations are significantly different from control ($p < 0.01$), except 0.01 μM biochanin A (not significant).

Table 1. Effects of Isoflavonoids, Flavonoids, and Enterolactone on DNA Synthesis in MCF-7 and MDA-MB-231 Cells[a]

	MCF-7			MDA-MB-231		
Compounds	Max stim,[b] % control	Concn, μM	IC_{50},[c] μM	Max stim,[d] *% control*	Concn, μM	IC_{50},[c] μM
Isoflavonoids						
Genistein	215	1.0	41.0			26.7
Daidzein	210	10				81.2
Biochanin A	235	1.0	37.7	110*	0.01	35.5
Coumestrol	185	0.1	42.5			24.4
Flavones						
Luteolin	150	10	29.8			17.2
Chrysin	120	10	23.0			>30.0
Apigenin	170	1.0	27.7			22.5
Flavone			61.0	110†	0.1	28.2
Flavonols						
Quercetin			88.0			37.0
Kaempferol	225	10	69.0			42.2
Other flavonoids						
Rutin	250	100				>100
Catechin			<0.01			>100
Lignans						
Enterolactone	210	10	82.0			>100

a: Cells (1–1.5 × 10^5/well) were seeded overnight in 24-well plates with DMEM-F-12 containing 5% fetal calf serum and incubated with serum-free and phenol red-free DMEM-F-12 for another 24 hrs. On the next morning, cells were treated with each compound (except chrysin) at 0.01, 0.1, 1.0, 10, 50, and 100 μM in the presence of 5% dextran-coated charcoal-fetal calf serum for 24 hrs, with 8–10 wells for each point. Tested concentrations of chrysin were 0.01, 0.1, 1.0, 10, 20, and 30 μM. DNA synthesis was measured by [^{3}H]thymidine incorporation.

b: Maximal stimulation of DNA synthesis. For MCF-7 cells, all values are significantly different from control, $p < 0.01$.

c: Concentration causing 50% inhibition (IC_{50}) was determined by plotting logarithm of compound concentration vs. DNA synthesis.

d: Statistical significance is as follows: *, significantly different from control, $p < 0.01$; †, significantly different from control, $p < 0.05$.

Several other flavonoids also showed biphasic effects on DNA synthesis in MCF-7 cells. The flavones luteolin, chrysin, and apigenin significantly stimulated DNA synthesis to a maximum of 150%, 120%, and 170% of control at 10, 10, and 1.0 μM, respectively (Table 1). These same compounds inhibited DNA synthesis at high concentrations, with IC_{50} values of 29.8 μM for luteolin, 23.0 μM for chrysin, and 27.7 μM for apigenin. The flavonol kaempferol stimulated DNA synthesis to 225% of control at 10 μM and inhibited DNA synthesis at high concentrations, with an IC_{50} of 69.0 μM. The lignan enterolactone was also found to have a biphasic effect on DNA synthesis, showing induction at 10–50 μM (with a peak value of 210% at 10 μM) and inhibition at high concentrations, with an IC_{50} of 82.0 μM.

Biphasic effects were also shown, although they were somewhat blunted, in the absence of FCS (Figure 4). With genistein treatment, induction was 125%, 145%, and 115% of control at 0.1, 1.0, and 10 μM, respectively; 50 and 100 μM genistein showed inhibition to 40% and 30% of control, respectively. With biochanin A treatment, 1.0 μM induced DNA synthesis to 120% of control and 50 and 100 μM inhibited DNA synthesis to 10% and <1% of control, respectively.

Flavone, quercetin, and catechin showed no stimulation of DNA synthesis at any concentrations but did show significant inhibition, with IC_{50} values of 61.0, 88.0, and <0.01 μM, respectively (Table 1). Rutin stimulated DNA synthesis to 250% of control at 100 μM but showed no inhibition at tested concentrations.

In MDA-MB-231 cells, most of the compounds significantly inhibited DNA synthesis at high concentrations, with no stimulation shown at any concentrations (Table 1). The isoflavonoids genistein, daidzein, biochanin A, and coumestrol inhibited DNA synthesis (Figure 5, Table 1), with IC_{50} values of 26.7, 81.2, 35.5, and 24.4 μM, respectively. The flavones luteolin, chrysin, apigenin, and flavone also inhibited DNA synthesis, with IC_{50} values of 17.2, >30, 22.5, and 28.2 μM, respectively (Table 1). The IC_{50} values for the flavonols quercetin and kaempferol were 37.0 and 42.2 μM, respectively. Rutin, catechin, and enterolactone were weak inhibitors, with IC_{50} values of >100 μM. In addition to inhibiting DNA synthesis at high concentrations, biochanin A and flavone showed slight stimulation to 110% of control at 0.01–1.0 and 0.1–1.0 μM, respectively.

Long-Term Effects of Genistein and Coumestrol on DNA Synthesis in MCF-7 Cells

When MCF-7 cells were exposed to genistein continuously for up to 10 days, low concentrations stimulated and high concentrations inhibited DNA synthesis persistently (Figure 6). Genistein at 0.1 and 1.0 μM showed sharp stimulation after six days to >300% of control. At 0.01 μM, DNA synthesis increased almost 200% by Day 10. At the same time, 50 and 100 μM genistein strongly inhibited DNA synthesis on Days 1–4, after which cytotoxicity occurred.

When cells were treated with coumestrol for 10 days, similar results were observed (Figure 7). DNA synthesis was significantly induced by 0.01–10 μM and inhibited by 50 and 100 μM coumestrol. The stimulation increased sharply after six days. The stimulation by 0.01 μM coumestrol was

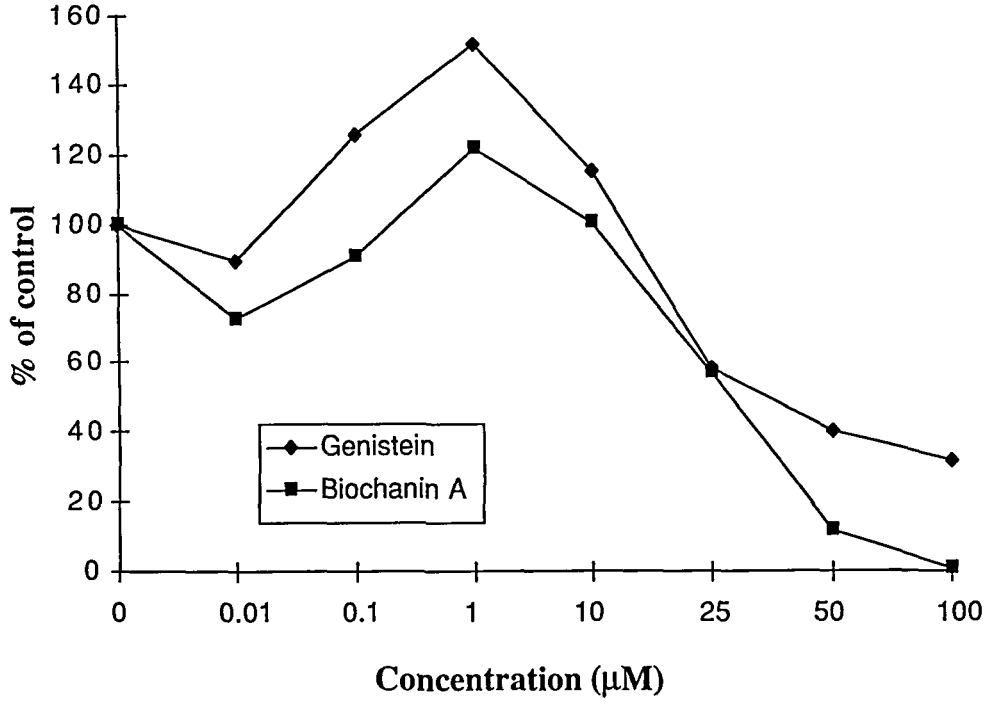


Figure 4. Effects of genistein and biochanin A on DNA synthesis in MCF-7 cells in absence of FCS. MCF-7 cells (1–1.5 × 10^5/well) were seeded and incubated. Cells were then treated with DMEM-F-12 containing 0.01, 0.1, 1.0, 10, 25, 50, or 100 μM genistein or biochanin A for 24 hrs in absence of serum, with 8–10 wells for each point. DNA synthesis was measured by [^{3}H]thymidine incorporation. Values at 0.01, 1, 25, 50, and 100 μM are significantly different from control ($p < 0.05$).

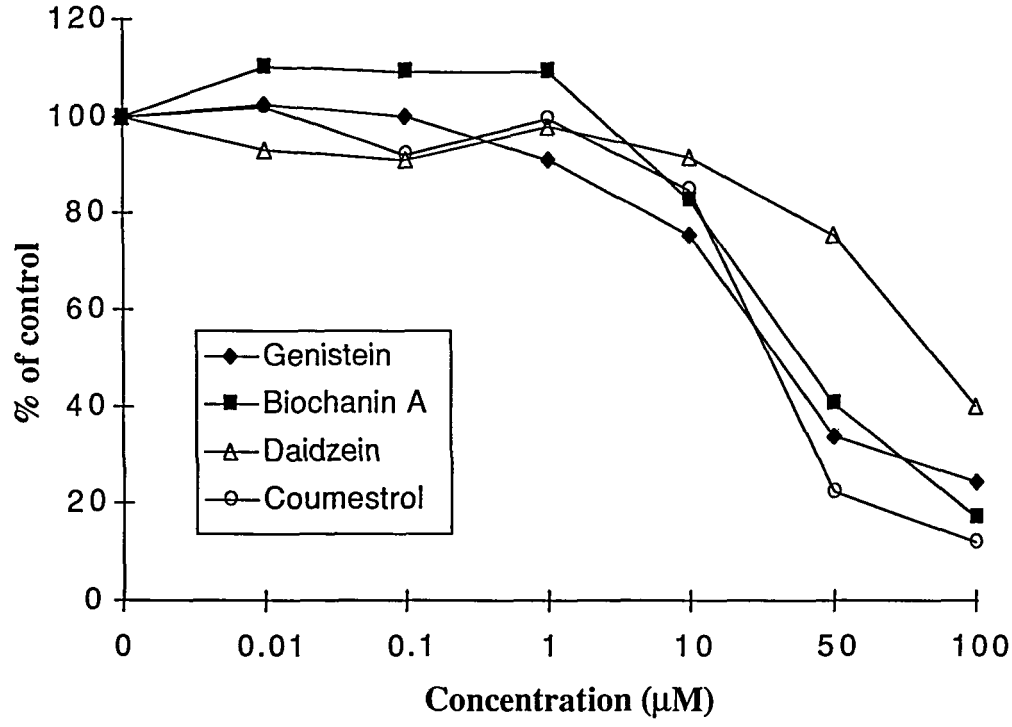


Figure 5. Effects of isoflavonoids on DNA synthesis in MDA-MB-231 cells. MDA-MB-231 cells (1–1.5 × 10^5/well) were seeded and incubated. Cells were then treated with each compound at 0.01, 0.1, 1.0, 10, 50, or 100 μM for 24 hrs in presence of 5% dextran-coated charcoal-FCS, with 8–10 wells for each point. DNA synthesis was measured by [^{3}H]thymidine incorporation. All values at 50 and 100 μM are significantly different from control ($p < 0.01$). Values at 0.01 and 0.1 μM are significantly different from control for daidzein and biochanin A only ($p < 0.01$). Values at 1.0 μM are significantly different from control for genistein and biochanin A only ($p < 0.01$). Values at 10 μM are significantly different from control for genistein, biochanin A, and coumestrol ($p < 0.05$).

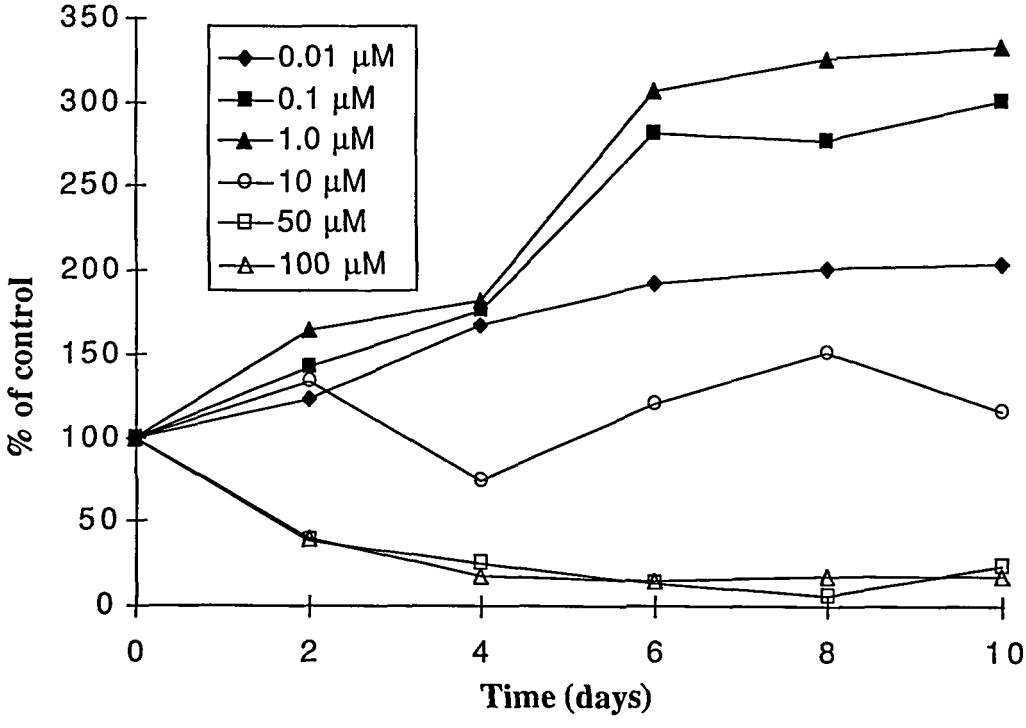


Figure 6. Long-term effects of genistein on DNA synthesis in MCF-7 cells. MCF-7 cells (5 × 10^4/well) were seeded overnight in 24-well plates and incubated. Cells were then treated with 0.01, 0.1, 1.0, 10, 50, or 100 μM genistein for 10 days, with 5–6 wells for each point. Medium was changed daily. DNA synthesis was measured every other day by [^{3}H]thymidine incorporation. All values for 0.01, 0.1, 1.0, 50, and 100 μM are significantly different from control ($p < 0.05$ for Day 2 and $p < 0.01$ for Days 4–10), except for 0.01 μM on Day 2 (not significant). For 10 μM, value at Day 4 is significantly different from control ($p < 0.05$).

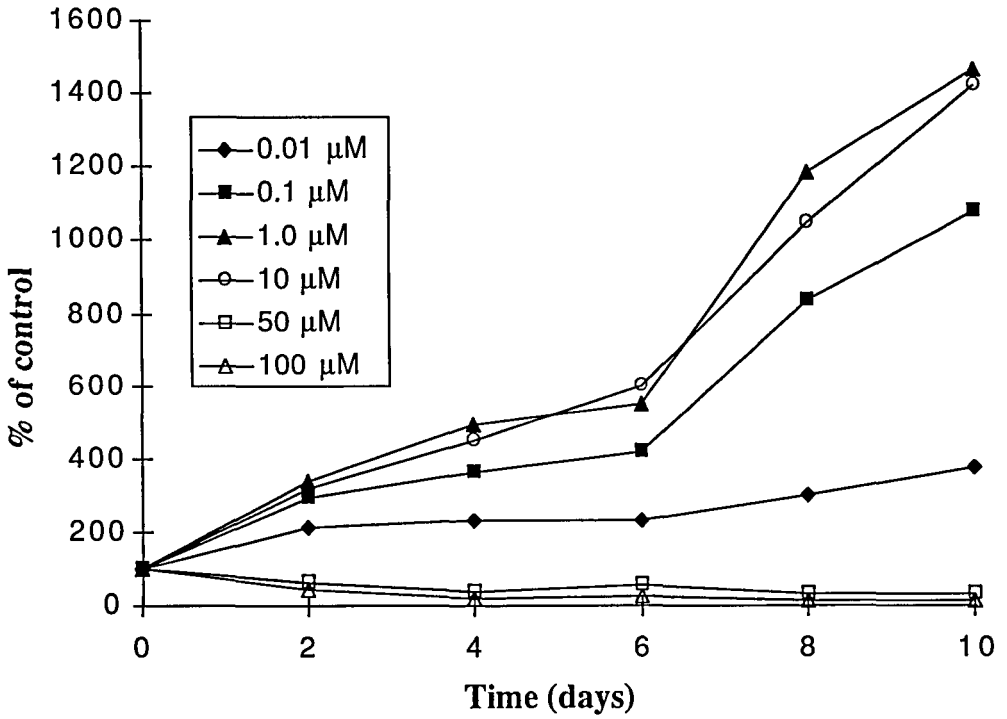


Figure 7. Long-term effects of coumestrol on DNA synthesis in MCF-7 cells. MCF-7 cells (8×10^4/well) were seeded overnight in 24-well plates and incubated. Cells were then treated with 0.01, 0.1, 1.0, 10, 50, or 100 μM coumestrol for 10 days, with 5–6 wells for each point. Medium was changed daily. DNA synthesis was measured every other day by [^{3}H]thymidine incorporation. All values are significantly different from control ($p < 0.01$).

210% of control after 2 days and 375% after 10 days. Stimulation by 0.1, 1.0, and 10 μM coumestrol was much greater, to 295%, 340%, and 320% of control after 2 days and 1,080%, 1,460%, and 1,420% of control after 10 days. Inhibition by 50 and 100 μM coumestrol persisted throughout the 10 days. After four days of treatment, cytotoxicity was observed in the cells treated with 100 μM coumestrol.

Effects of Genistein Incubation and Withdrawal on DNA Synthesis in MCF-7 Cells

Confirming previous studies, incubation with 0.01–10 μM genistein for 24 hours stimulated DNA synthesis significantly to 130–184% of control ($p < 0.01$), whereas incubation with 50 and 100 μM genistein showed inhibition to 39–55% of control ($p < 0.01$) (Table 2). After treatment with 0.01–10 μM genistein for an additional 24 hours (48 hrs total), stimulation of DNA synthesis was further increased to 144–263% of control. Incubation with 50 μM genistein for the additional 24 hours slightly inhibited DNA synthesis further, whereas incubation with 100 μM genistein sharply inhibited DNA synthesis to 13% of control.

After removal of genistein for the next 24 hours, DNA synthesis returned to the control level in those cells previously treated with 0.1–1.0 μM genistein. Although stimulation in the cells previously treated with 5 μM genistein was reduced, it was still significantly higher than the control level ($p < 0.01$). At the same time, DNA synthesis in cells previously treated with 0.01, 25, 50, and 100 μM genistein increased to 220%, 224%, 186%, and 66% of control, respectively. After removal of genistein for an additional 24 hours (48 hrs total), stimulation in most groups was decreased and close to the control level, although some groups still showed statistical differences ($p < 0.01$). For cells treated with 100 and 0.01 μM genistein, DNA synthesis increased to 368% and 282% of control, respectively.

Treatment with genistein for 24 hours followed by removal for 48 hours also showed reversible effects (data not shown).

Table 2. Time Course of Genistein Incubation and Removal on DNA Synthesis in MCF-7 Cells[a–c]

Genistein Concn, μM	Genistein Treatment		Genistein Removal	
	24 hrs	48 hrs	24 hrs	48 hrs
0.01	130 ± 12*	144 ± 12*	220 ± 31*	282 ± 20*
0.1	184 ± 13*	239 ± 17*	92 ± 9	84 ± 13†
1.0	183 ± 24*	263 ± 24*	110 ± 14	87 ± 12
5.0	154 ± 14*	242 ± 16*	192 ± 19*	138 ± 8*
10	141 ± 13*	252 ± 21*	257 ± 15*	127 ± 9*
25	89 ± 5	123 ± 11†	224 ± 22*	108 ± 4*
50	55 ± 12*	42 ± 12*	186 ± 31*	128 ± 20*
100	39 ± 13*	13 ± 17*	66 ± 9*	368 ± 13*

a: Values are means ± SD expressed as % control.

b: Cells (1.5×10^5/well) were seeded overnight in 24-well plates and incubated. Cells were then treated with 0.01, 0.1, 1, 5, 10, 25, 50, or 100 μM genistein for 48 hrs with 5 wells for each point, then with genistein-free medium for 48 hrs. Medium was changed daily. DNA synthesis was measured by [^{3}H]thymidine incorporation.

c: Statistical significance is as follows: *, significantly different from control, $p < 0.01$; †, significantly different from control, $p < 0.05$.

Discussion

As expected, E_2 significantly induced DNA synthesis in ER-positive MCF-7 cells but did not induce DNA synthesis in ER-negative MDA-MB-231 cells, confirming the significance of the ER in mediating the effects of E_2. Our serum-free experiments confirm previous reports showing blunted stimulation of estrogen-dependent cell growth by E_2 in the absence of serum or in the presence of heat-inactivated serum (47–49). These results are consistent with hypotheses concerning the effects on cell growth of interactions between serum growth factors and E_2. Binding of E_2 to the ER is thought to change the conformation of the ER, facilitating the binding between the E_2-ER complex and the estrogen response element on the DNA. Activation of the estrogen response element has been proposed to induce the expression of c-*fos* (49–52), forming a c-*fos*/c-*jun* heterodimer that activates the AP1 site, leading to cell proliferation. Growth factors in serum induce expression of c-*jun* and c-*fos*. The combination of E_2 and serum is thought to synergistically stimulate production of these transcription factors, causing much stronger growth induction than that induced by E_2 alone. Similar to our results with E_2, we found that genistein and biochanin A stimulated DNA synthesis in MCF-7 cells in the absence of serum, although more weakly than in the presence of serum. This is the first report to show such results with phytoestrogens. Because phytoestrogens are known to bind the ER, our results suggest that this stimulation of DNA synthesis may be mediated by the same mechanism as that of E_2.

Our dose-response results in MCF-7 cells confirm and expand on the results of previously reported studies. For most of the compounds tested, we found stimulation of DNA synthesis up to 250% at 0.1–10 μM and inhibition at 50–100 μM. Maximal stimulation was 215% for 1.0 μM genistein, 235% for 1.0 μM biochanin A, and 185% for 0.1 μM coumestrol. The genistein results in our experiment are similar to those of Wang and associates (25) and Zava and Duwe (41), who recently published studies showing biphasic effects of the phytoestrogen in MCF-7 cells. Wang and associates showed stimulation of cell growth (as reflected by protein content) at 0.01–10 μM genistein, with maximum stimulation to 220% at 0.1–1.0 μM and inhibition at >10 μM. Zava and Duwe showed stimulation of cell growth (as reflected by DNA content) to 150–400% at 0.001–10 μM and inhibition at >10 μM. The data from both groups reflect cumulative cell growth, whereas our data reflect growth rate.

Other studies, using various end points, have reported similar growth stimulation in MCF-7 cells treated with low concentrations of phytoestrogens. Makela and co-workers (40) reported stimulation of cell number to 150–250% of control in cells treated with genistein, biochanin A, and coumestrol at 0.1–1.0 μM. Welshons and others (53) showed half-maximal growth responses, as reflected by DNA content, of 0.0018, 0.0025, 0.04, and 0.3 μM for coumestrol, genistein, biochanin A, and daidzein, respectively. Other reports have shown stimulation of protein content to 190% and 150% of control by 1 μM daidzein and kaempferol (54), stimulation of cell number to 120% of control by 1 μM enterolactone (55), and stimulation of protein content to 200% of control by 1 μM enterolactone (54). Consistent with the study of Miksicek and co-workers (18), who measured cell number, we did not find stimulation of DNA synthesis by flavone.

Induction of DNA synthesis in ER-positive cells is consistent with the known estrogenic actions of phytoestrogens. Genistein, biochanin A, coumestrol, luteolin, kaempferol, and apigenin have been shown to bind the ER or type II estradiol-binding site in human BC and rat uterine cells (15–19). Daidzein, enterolactone, and kaempferol have been shown to induce expression of estrogen-dependent pS2 protein in MCF-7 cells (54). *In vivo* human studies have suggested that phytoestrogen consumption may induce hormonal effects in premenopausal (56–58) and postmenopausal women (59,60).

Our IC_{50} values are within the range of the few reported studies performed in MCF-7 and MDA cells. Compared with our IC_{50} of 41 μM for genistein in MCF-7 cells, others have shown IC_{50} values for genistein of 13 μM (61) for DNA synthesis and 36 μM (42), 39 μM (4), and 24–38 μM (6) for cell number. The IC_{50} for genistein in MDA-231 cells has been reported to be 15 μM for cell number (42), compared with our IC_{50} of 27 μM. The IC_{50} values for cell number in MDA-468 cells have been reported to be 24, 106, and 134 μM for genistein, biochanin A, and daidzein, respectively (4,6) compared with our values of 41, 38, and >100 μM. The IC_{50} for quercetin in MDA-435 cells has been reported to be 55 μM for cell number (62) compared with our value of 88 μM.

Many studies have shown inhibition of tumor cell growth by phytoestrogens, primarily genistein (63). Growth inhibition by high concentrations of phytoestrogens in estrogen-dependent and -independent BC cells may be mediated by mechanisms independent of the ER. Many mechanisms have been demonstrated, such as inhibition of tyrosine kinases (33–35), protein kinase C (36), and DNA topoisomerase II (37–39). In fact, certain anticancer drugs such as tyrphostin AG derivatives, a group of tyrosine kinase inhibitors, are similar in chemical structure to genistein (64).

As we have confirmed in our studies, the phytoestrogen concentrations found to inhibit BC cell growth have generally been >10 μM. Although there are few data on the levels of these compounds in human tissues, studies with genistein suggest that serum concentrations are normally <10 μM and most often <1 μM (43–45). Plasma genistein has been reported to be 0.1–0.6 μM in Japanese men and up to 0.01 μM in Finnish men (65). In another study, plasma genistein was reported to be 2–6 μM 6.5 hours after high soy consumption (45). Our data suggest the possibility that, at typical concentrations in humans, phytoestrogens and related flavonoids and lignans may stimulate, rather than inhibit, growth of estrogen-dependent tumors. This conclusion is in

contradiction to the conclusions of Zava and Duwe (41), who recently stated that "genistein has potent estrogen agonist and cell growth-inhibitory actions over a physiologically achievable concentration range (10 nM–20 μM)," despite results showing stimulation of MCF-7 cell growth at 1 nM–10 μM genistein. All these data must be interpreted with caution, because target tissue concentrations are unknown and may be higher or lower than serum concentrations.

Our genistein incubation and withdrawal time-course experiments showed stimulation and inhibition of DNA synthesis in MCF-7 cells to be reversible. In cells that had been treated with low levels of genistein for 48 hours, the stimulation of DNA synthesis gradually decreased to close to the control level over the next 48 hours after genistein withdrawal. In cells that had been treated with high levels of genistein, the inhibition was totally reversed over the next 48 hours. In fact, in cells that had been treated with 100 μM genistein, DNA synthesis increased to 368% of control. Parallel increases in cell number and protein (unpublished data) suggest that the rebound in DNA synthesis after genistein withdrawal reflects a rebound of normal DNA synthesis as opposed to unscheduled synthesis. The inhibitory effects of short-term treatment with 100 μM genistein are apparently reversible, suggesting the possibility of estrogenic effects of residual intracellular genistein. These results are similar to those of Peterson and Barnes (66), who showed that removal of genistein for four days reversed previous inhibition of cell growth induced by 10 μg/ml (37 μM) or 20 μg/ml (74 μM) genistein. We are unable to explain the rebound DNA synthesis in the group previously treated with 0.01 μM genistein.

In our long-term experiments, DNA synthesis in MCF-7 cells was stimulated for the entire 10-day period by 0.01–1.0 μM genistein or 0.01–10 μM coumestrol, reaching a maximum of 300% or 600% of control after 6 days, respectively. These results support those of Wang and co-workers (25), who showed that stimulation of cell protein by low concentrations of genistein in MCF-7 cells was maintained at 250% of control after five days of treatment. In an apparent contradiction, these authors also reported that MCF-7 cells treated with low concentrations (1 μM) of genistein for six days show downregulation of ER mRNA. They suggest that this indicates the antiestrogenic action of long-term treatment with genistein. If ER mRNA is downregulated over time, this downregulation clearly does not result in reduced cell growth. Taken together, these two apparently contradictory observations suggest that, in addition to the ER-dependent pathway, mechanisms independent of the ER may also be responsible for the induction of cell growth by low levels of genistein. It is also possible that ER expression is not regulated only at the level of transcription; posttranscriptional regulation may dominate ER expression (67).

With 10 days of coumestrol treatment, MCF-7 cells showed continued stimulation of DNA synthesis. Although actual control DNA synthesis decreased 30% by Day 10, synthesis in cells treated with 0.01–10 μM coumestrol increased continuously. This suggests that coumestrol stimulation overcomes the slowing of DNA synthesis induced by the contact inhibition caused by cell confluence.

Although short-term treatment of genistein was not cytotoxic, as shown by trypan blue exclusion, high concentrations of genistein and coumestrol caused cytotoxicity after four days. This suggests that the toxicity of high concentrations is time related.

In conclusion, most of the phytoestrogens and related compounds tested in this study showed stimulation of DNA synthesis in estrogen-dependent MCF-7 cells at low concentrations and inhibition of DNA synthesis in MCF-7 and estrogen-independent MDA-MB-231 cells at high concentrations. Although we observed inhibition at high levels, it is extremely important to consider that, at concentrations close to probable levels in humans, DNA synthesis was significantly induced in MCF-7 cells. At the same time, the situation is far more complex *in vivo*, and the observed effects reflect only a few of the multiple mechanisms by which phytoestrogens may influence tumor growth. In addition, one cannot assume that the effects will be identical in the presence of estrogen, as is true *in vivo*. Because of the great interest in possible cancer-preventive effects of dietary phytoestrogens, further studies are needed to clarify their effects at typical concentrations in the presence and absence of E_2, as well as possible synergistic effects.

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