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The Combination Effects of Phytoestrogens on Cell Proliferation, Cell Cycle Progression and Estrogen Receptor Alpha

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ABSTRACT

Phytoestrogens are plant chemicals that have been related to the occurrence of human breast cancers and other estrogen-dependent cancers. This study was designed to examine the potential combinational effects and estrogenic activity of phytoestrogens in MCF-7 human breast cancer cells. Results showed that the phytoestrogens biochanin A and daidzein inhibited the proliferation of MCF-7 cells in a dose-dependent manner. Both biochanin A and daidzein delayed the progression of the cell cycle from the G2/M phase to the G1 phase at growth inhibitory concentrations. Treatment with biochanin A down-regulated the level of estrogen receptor-alpha (ER-α) mRNA, but this down-regulation was not affected by the presence of the antiestrogen ICI182,780. When cells were treated with biochanin A at a growth inhibitory concentration of 40 μg/mL, co-administration of daidzein resulted in an enhanced inhibitory effect on the MCF-7 cell growth. In addition, neither the growth inhibition, nor the delayed cell cycle progression induced by biochanin A was affected by the addition of antiestrogen. These results suggest that the growth regulatory effects of phytoestrogens may be associated with cell cycle progression, and the underlying mechanism involves at least in part the modulation of a non-classical ER-α signal pathway.

Key words: phytoestrogen, fertility, estrogen receptor

INTRODUCTION

Phytoestrogens are natural plant substances that are present in certain plants and foliage including soybeans, alfalfa and citrus plants. They can occur in high concentrations (up to 5% dry mass) in clover, especially *Trifolium subterraneum* (subterranean clover), *T. pratense* (red clover) and closely related species. The presence of these chemicals has been proposed as having an important influence on ruminant fertility[(1)]. Phytoestrogens share structural similarity to natural animal estrogens, such as estradiol, and exhibit affinity for the estrogen receptor[(2,3,4)]. These compounds have been considered to be responsible for the depression of fertility observed in sheep grazing in clover pasture[(5)]. Phytoestrogens were also implicated in bovine infertility[(6)].

It has been shown that phytoestrogens in soybeans are metabolized in the digestive tract and can be detected in urine and plasma[(7,8,9)]. Recently, phytoestrogens have drawn much attention because of their possible cancer-preventing effects[(10,11)] and their ability to reduce the incidence of breast cancer in animals[(12,13)]. Epidemiological data suggest that a diet rich in phytoestrogens decreases the risk of breast cancer[(14)]. Women on traditional Asian low-fat, high-soy diet were found to have a lower risk of developing breast cancer and estrogen deficiency diseases such as heart disease and osteoporosis, than women on western diets. Such marked differences between women with Asian and Western diets are not due to genetic factors but are more likely dietary[(15)].

Daidzein, a hydroxylated isoflavone and biochanin A, the 4'-methyl ethers of genistein[(16)] are two important phytoestrogens in major pasture legumes *Trifolium subterraneum* (subterranean clover), and *T. pratense* (red clover) and in soybeans (up to 100-300 μg/100 g)[(17)]. *In vitro* studies showed that daidzein exhibits low but measurable binding affinity to both subtypes of estrogen receptor (ER) proteins ER-α and ER-β while biochanin A has very little affinity for either subtype[(3,18,19,20)]. Previous studies also showed that biochanin A or daidzein alone inhibited cell proliferation and DNA synthesis in several cancer cell lines[(21,22,23)]. Analysis of serum or urine in human revealed the presence of multiple phytoestrogens suggesting that phytoestrogens may act cumulatively[(24)]. However, studies on the combinational effects of phytoestrogens on cell growth and their estrogenic activity on ER have been rather limited or controversial[(25,26,27)]. In this study, we investigated not only the individual but also the combined growth

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regulatory effects of daidzein and biochanin A using an estrogen model system, the human breast cancer cells. We also analyzed the effects of phytoestrogens on cell cycle progression in this model system. Lastly we showed that biochanin A regulated the level of ER-α mRNA and continued to exert its regulatory effects even in the presence of an antiestrogen ICI182,780.

MATERIALS AND METHODS

I. *Chemicals*

Biochanin A and daidzein were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Research Biochemicals International (Natick, MA, USA), respectively. Solutions of biochanin A and daidzein were prepared in dimethylsulphoxide (DMSO) and stored at -20˚C in the dark.

II. *Cell Culture Conditions*

MCF-7 human breast cancer cells were grown in phenol red-free DMEM supplemented with either 10% fetal bovine serum (FBS) or 3X dextran/charcoal stripped FBS (DC-FBS). Cultures were maintained at 37˚C in a humidified atmosphere of air/CO_2 (95/5) and fed every other day.

III. *Cell Proliferation Assays*

Cells were first cultured in DMEM medium supplemented with 10% FBS until they reached confluence. These cells were cultured for another 24 hr in DMEM medium. Cells were then collected by trypsinization, counted and seeded in culture flasks or 96-well culture plates in medium containing 10% DC-FBS. After culturing for 24 hr to ensure attachment of cells, medium was removed and fresh medium supplemented with 10% DC-FBS alone or with test concentrations of phytoestrogen was then added. DMSO was added to the same dilution (0.1%, v/v) in all samples. The cells were then incubated at 37˚C. For cell grown in culture flasks, the number of viable cells in each sample was determined by trypan blue dye exclusion assay in which viable cells remain transparent, whereas non-viable cells appear blue under the microscope. Cell proliferation for cells grown in 96-well plates was determined by using mitochondrial activity assays as described previously[28,29,30]. In particular, the WST-1 colorimetric assay kits (Boehringer Mannheim UK Ltd, Bell Lane, East Sussex, UK) was used in accordance with the manufacturer's instructions. This kind of assay, which considers metabolic activity to be an index for cell proliferation is similar to those assays using other tetrazolium salt, 3-(4,5-dimethylthiszol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), to determine cell proliferation[29,30]. The absorbance at 450 nm (formazan dye) and 650 nm (cellular background) was measured individually for each well after the addition of WST-1 reagent. Subtracting the absorbance at 650 nm from the absorbance at 450 nm then gives an index that is directly correlated to the cell number in each well[31].

IV. *Cell Cycle Analysis*

A previously established procedure[32] was used to analyze the cell cycle distribution of MCF-7 cells treated with biochanin A or daidzein. In summary, cells were grown to confluence in a medium containing 10% FBS. The medium was then removed and cells were cultured in medium containing no serum for another 24 hr. Cells were collected and reseeded in medium containing DC-FBS for 24 hr prior the addition of tested chemicals. After treatment, the cells were washed twice with PBS and suspended in phosphate-buffered NaCl solution (PBS) containing 50 mg/mL propidium iodide, 0.1% sodium citrate and 0.1% Triton X-100 at the density of 1×10^6/mL. Cells were then incubated overnight at 4˚C in the dark and analyzed with flow cytometry (Epics Elite ESP XL, Coulter). Each analysis was repeated at least twice.

V. *Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR)*

Total RNA from MCF-7 cells treated with biochanin A alone or with ICI182,780 for 2 days were prepared as described by Hsu *et al.*[31]. The RT reaction was performed using 0.1-0.2 μL of total RNA in a 20-μL reaction mixture. A portion of the RT product containing an equal amount of cDNA from each sample was then subjected to PCR. The PCR reaction mixture contained 2 μL of 10X PCR buffer, 1.6 μL of 50 μM $MgCl_2$, 11.7 μL of DEPC-treated water, 1.5 μL of RT-reaction mixture, 1 μL of 10 mM dNTPs, l unit of Taq DNA polymerase, and 2 μL of ER-alpha primers (5'-GGAGATTCTGATGATTGGTCT-3' and 5'-CATCTCCAGGAGCAGGTCAT-3'; 250 μg/mL) or ribosomal protein L19 (RPL19) primers (5'-CTGAAGGTCAAAGGGAATGTG-3' and 5'-GGACAGAGTCTTGATGATCTC-3'; 125 μg/mL). The RPL19 is constitutively expressed and used as a control for the RT-PCR reaction[33].

The ER-alpha and RPL19 cDNA fragments were amplified by standard PCR procedure (35 cycles; 94˚C/1 min, 60˚C/1 min, 72˚C/1 min). The 477 base pairs (bp) and 194 bp amplified product for ER-alpha and RPL19, respectively, was fractionated on 2% agarose gel and stained with ethidium bromide.

IV. *Statistical Analysis*

The method of least squares ANOVA was used for data analysis[34,35]. The means of treatment were compared by the orthogonal contrast with the comparisons of each individual treatment vs. control blank.

RESULTS

I. *The Effects of Daidzein and Biochanin A on the Cell Proliferation of MCF-7 Cells*

Both biochanin A and daidzein showed biphasic regulation effects on the cell proliferation of MCF-7 cells (Figure 1). Low concentrations (<10 μg/mL) of daidzein or biochanin A stimulated cell proliferation while cell proliferation was inhibited at higher concentrations. This observation agrees well with studies previously reported in T-47D and other human breast cancer cells[21,27]. Limited cell death was observed at 40 μg/mL biochanin A or 50 μg/mL daidzein as determined by trypan blue dye exclusion assay. The number of viable cells after the treatment of 40 μg/mL biochanin A was comparable with the starting cell number. This observation suggested that biochanin A at this concentration range did not cause significant cell death and the observed decline in the number of viable cells was probably due to cell growth inhibition. Previous study also showed that altered cell permeability of MCF-7 cells determined by the release of lactate dehydrogenase was observed primaring at higher concentrations (>80 μg/mL) of biochanin A[21]. Therefore, although one cannot completely rule out the possibility that the effects seen at higher concentrations (50 μg/mL for daidzein and 40 μg/mL for biochanin A) are due to cytotoxicity of the tested phytoestrogen since detailed cytotoxic assays were not performed, the cytotoxic effects were expected to be minimal or limited. At higher concentrations of phytoestrogens (>75 μg/mL for daidzein and >60 μg/mL for biochanin A), however, cells became detached and floated in the culture and the majority of the floating cells appeared non-viable (data not shown).

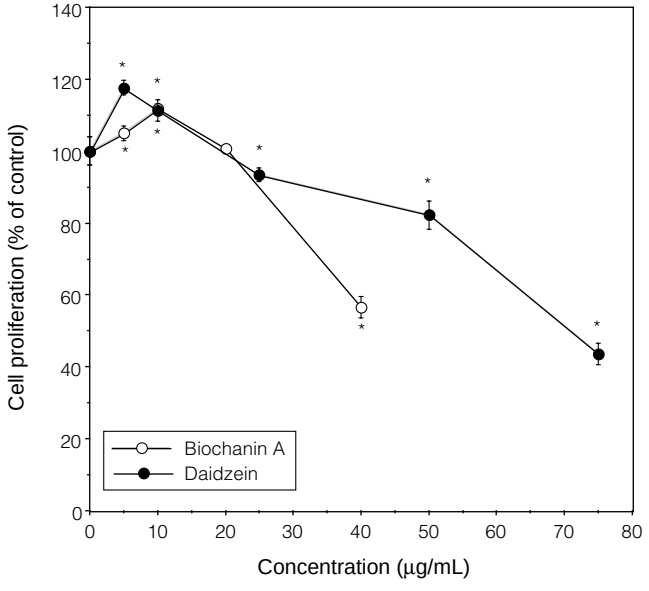

Figure 1. The growth regulation of MCF-7 human breast cancer cells by biochanin A and daidzein. (A) MCF-7 cells seeded in 96 well plates were cultured in DMEM containing 10% DC-FBS for the indicated period of time and the number of viable cells was determined. (B) Cells were incubated with biochanin A or daidzein for 2 days and the number of viable cells was determined by WST-1 assay as described in the Materials and methods. The number of viable cells after grown for 2 days in control vehicle was designated 100%. The * indicates a significant difference ($p < 0.05$) with respect to the control value. Data are the mean of five replications and similar experiment was performed at least twice.

In the presence of a growth-inhibitory concentration of biochanin A (40 μg/mL), co-administration of various concentrations of daidzein (5-75 μg/mL) produced an enhanced growth regulatory effect (Figure 2A, Figure 3A). For example, cell proliferation was inhibited by 72.4% when both biochanin A (40 μg/mL) and daidzein (75 μg/mL) were present as compared to a 43.5% and a 56.5% inhibition by biochanin A or daidzein alone at the same concentrations, respectively (Figure 2A). At lower concentrations of biochanin A (5-20 μg/mL), the effects of co-administrated daidzein appeared to mask the effect of biochanin A and no synergistic effects was detected (Figure 2B, C and D, Figure 3B).

II. *Cell Cycle Progression of Daidzein- and Biochanin A-treated MCF-7 Cells*

Figure 4 shows the distribution of the MCF-7 cell cycle phases after treatment with daidzein or biochanin A for different times. In the controls (Figure 4), the percentage of MCF-7 cells in the S and G2/M phases peaked at 6 and 12 hr, respectively. At 24 hr, more than half of the control cells previously in the G2/M phase reentered the G1 phase. In contrast, for cells treated with daidzein, the S and G2/M populations were the highest at 12 and 24 hr, respectively. Most of the daidzein-treated cells did not re-enter the G1 phase until 48 hr. Thus treatment with daidzein evidently prolonged the cell cycle by delaying progression from one cell cycle phase to the next. There was no significant build-up in the G1 phase, that indicates blockage in the G1 to S transition, was observed in any of the treatment (Figure 4). Biochanin A also delayed the cell cycle progression in MCF-7 cells (Figure 4). Cells treated with biochanin A accumulated at the G2/M phase and reentered the G1 phase very slowly. Biochanin A also caused cells at the S phase to progress into the G2/M phase more slowly than the control cells (Figure 4).

III. *The Effects of Antiestrogen on the Biochanin A Induced Cell Growth Inhibition, Cell Cycle Progression and the Level of ER-α mRNA*

The addition of antiestrogen ICI182,780 did not block the growth inhibition induced by biochanin A in MCF-7 cells (Figure 5A). Cell cycle analysis revealed that the delay in cell cycle progression caused by biochanin A was likewise not affected by the presence of the antiestrogen (Table 1). The level of ER-α mRNA was down-regulated by the growth-inhibitory concentration of biochanin A (Figure 5B). This down-regulation was not significantly

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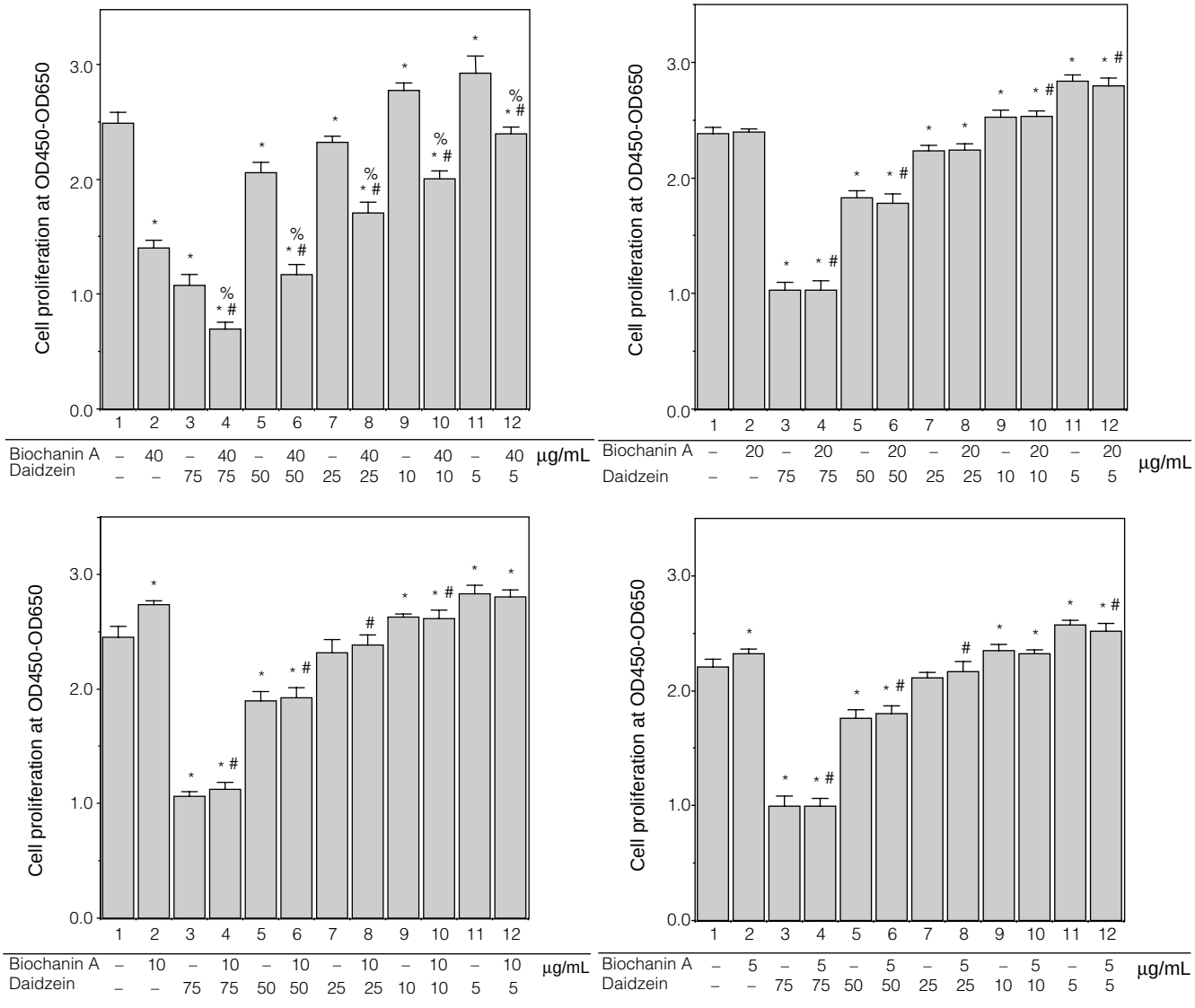

Figure 2. Combined growth regulation effect on MCF-7 cells. (A)-(D). MCF-7 cells were incubated with biochanin A at 40, 20, 10 or 5 µg/mL, respectively, for 4 days in the presence of indicated concentrations of daidzein. Cell proliferation was measured using the WST-1 assay. Values are mean ± SD (n=5). Significant differences ($p < 0.05$) compared to the 0.1% DMSO vehicle-treated samples control, to biochanin A treatment alone, to daidzein treatment alone at the same concentrations are indicated by an asterisk (*), a pound sign (#) and a percentage sign (%), respectively.

Table 1. Cell cycle progression of MCF-7 cells treated with biochanin A and antiestrogen ICI182,780

Time	Treatment[a]	Percentage of cells (%)		
		G1/G0	S	G2/M
0 hr		79.6 ± 2.0	17.7 ± 0.4	2.6 ± 2.6
24 hr	Control[b]	73.2 ± 9.9	12.9 ± 7.7	9.0 ± 7.1
	BioA	53.9 ± 5.5	41.4 ± 3.2	4.8 ± 2.4
	BioA + ICI	63.7 ± 0.9	35.6 ± 1.6	0.7 ± 0.7
48 hr	Control	68.1 ± 7.9	27.4 ± 6.1	3.9 ± 2.3
	BioA	69.4 ± 2.0	13.6 ± 3.6	17.1 ± 1.5
	BioA + ICI	57.9 ± 12.4	33.0 ± 12.6	9.1 ± 0.2

[a]MCF-7 cells were treated with 40 µg/mL biochanin A alone (bioA) or simultaneously with 40 µg/mL ICI182,780 (bioA+ICI) for 0-48 hr and the percentages of cells at each phase of the cell cycle were analyzed (mean ± SEM, n = 2).
[b]vehicle.

blocked by the co-administrated ICI182,780 although the mRNA level of ER-α did appear to decline somewhat in cells treated with ICI182,780 alone. These results thus suggest that the growth inhibition induced by biochanin A is not mediated through the classical ER-α dependent pathway(s) in MCF-7 cells at the tested concentration. Although previous reports[18,20,36] indicated that the effects of phytoestrogens could be mediated through another subtype of ER protein, ER-β, it was difficult to determine the potential effects of biochanin A on ER-β in this cell line since the levels of ER-β mRNA in MCF-7 cells were below our RT-PCR detection limit. Other investigators also reported the lack of ER-β expression in MCF-7 cells[37,38].

DISCUSSION

In this study, we examined the growth regulatory effects of two phytoestrogens, daidzein and biochanin A in the model system for estrogen response, the MCF-7 cells. In agreement with previous studies, the inhibition of cell growth was dose-dependent, while growth stimulation was observed at low concentrations of both phytoestrogens. At growth inhibitory concentrations, however, the cell cycle progression of daidzein-treated MCF-7 cells was altered such there was an extended period of time before cells entered the G2/M phase from the S phase. Biochanin A showed similar effects on the cell cycle progression in MCF-7 cells. These results suggested that cell cycle regulation might be involved in the phytoestrogen-induced regulatory mechanism. Down-regulation induced by biochanin A on the levels of ER-α was also observed in MCF-7 cells. This implies that a response to estradiol in human breast cancer cells can be decreased upon administration of phytoestrogens.

In response to the estrogen treatment, the level of ER mRNA was found to decrease by 50% in MCF-7 cells, which was considered to be an important step on estrogen action[39]. In this study, we investigated whether the effect of biochanin A was mediated through ER-pathway. Previous studies have suggested that the phytoestrogens act *via* the classical ER dependent pathways. For instance, Sathyamoorthy *et al*.[40] showed that phytoestrogen regulate the expression of the estrogen responsive gene pS2 suggesting an ER-dependent pathway was activated by phytoestrogens. However, there is also other evidence that suggests that the phytoestrogens might not regulate cell growth exclusively through the classical ER pathways. Previous study showed that genistein at the growth-stimulatory concentration (1 μM) also caused a down-regulation of ER levels[23]. Thus, it indicated that down regulation of ER expression might not be associated with reduced cell growth. Likewise, although compared to biochanin A, daidzein has a higher affinity for either ER protein subtype α or β *in vitro*[3,20], it is biochanin A that shows the higher potency in human breast cancer cells and other cancer cells[21,25,27]. Similar observation on other phytoestrogens was reported. For instance, although genistin binds more weakly to the ER proteins than does genistein, it regulates the growth of MCF-7 cells more strongly than genistein[18]. Our present results also found that biochanin A had higher potency in MCF-7 cells, with an IC_{50} that was approximately half that of daidzein (Figure 1). Further, we found that none of the effects induced by biochanin A (i.e. a reduction in the number of viable cells, a delay in the cell cycle progression, and a down-regulation of ER-α mRNA) were blocked by the presence of the "pure" antiestrogen ICI182,780. Although some of the above discrepancies might be due in part of different experimental designs and conditions, our present results strongly suggest that biochanin A might mediate its growth inhibition through non-classical ER pathways and that instead of the ligand binding site on ER-α proteins, a novel target site might be involved.

The biological efficacy of daidzein, biochanin A and genistein in combination was studied in human bladder cancer cells. Combined regimen was found to have a greater inhibitory effect than any single compound[25]. The growth of tumor cells in mice also found that a mixture of

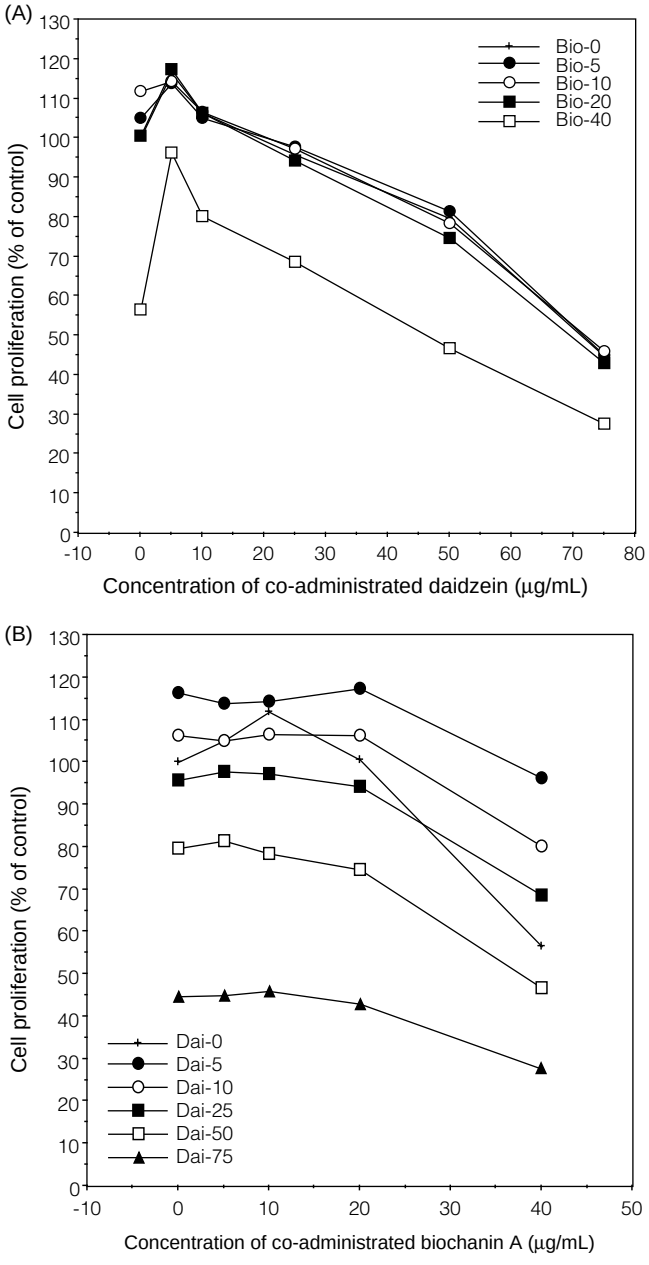

Figure 3. The effects of co-administration of two phytoestrogens on the regulation of MCF-7 cell proliferation. (A) MCF-7 cells were incubated with daidzein at increasing concentration (0-75 μg/mL) alone or with biochanin A at 5, 10, 20 or 40 μg/mL for 4 days and the cell viability was determined by WST-1 assay. (B) MCF-7 cells were incubated with biochanin A at increasing concentration (0-40 μg/mL) alone or with daidzein at 5, 10, 25, 50 or 75 μg/mL for 4 days and the number of viable cells was determined by WST-1 assay. The cell viability obtained from samples treated with 0.1% DMSO vehicle only in parallel was designated 100%.

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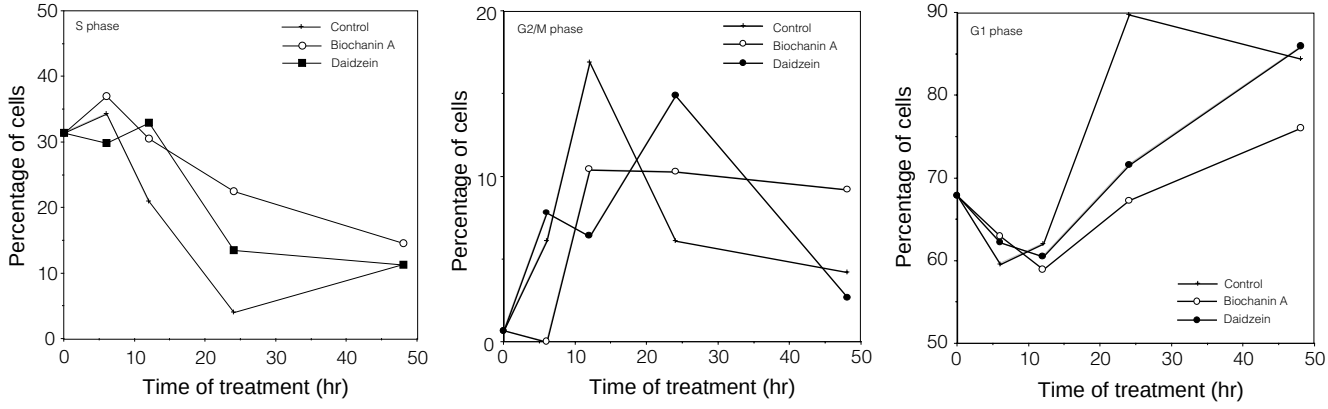

Figure 4. The effects of daidzein and biochanin A on the cell cycle progression of MCF-7 cells as determined by flow cytometry. The MCF-7 cells were incubated with daidzein or biochanin A at the concentration of 50 or 40 μg/mL, respectively, for 0-48 hr. The percentage of cells in each cell cycle phase was determined by flow cytometry (n = 2).

daidzein, biochanin A and genistein was more potent than genistein alone[25]. Verma *et al.*[26] reported that two plant products, curcumin and genistein, were able to synergistically inhibit cell growth induced by estrogenic pesticides. In the present study, the growth inhibitory effect of biochanin A at 40 μg/mL enhanced by the presence of daidzein was also observed (Figure 2A). However, at growth-stimulatory (5 and 20 μg/mL) and growth-neutral (20 μg/mL) concentrations of biochanin A, we did not detect combinatory synergistic effect between daidzein and biochanin A. The growth stimulatory effects of biochanin A appeared to be blocked by daidzein that was present simultaneously (Figure 2, Figure 3A). The reason for these observations is not yet clear, but these results suggest that different mechanisms may be involved in the growth stimulation and inhibition induced by these phytoestrogens. Further investigation will be required to identify the precise mechanisms involved.

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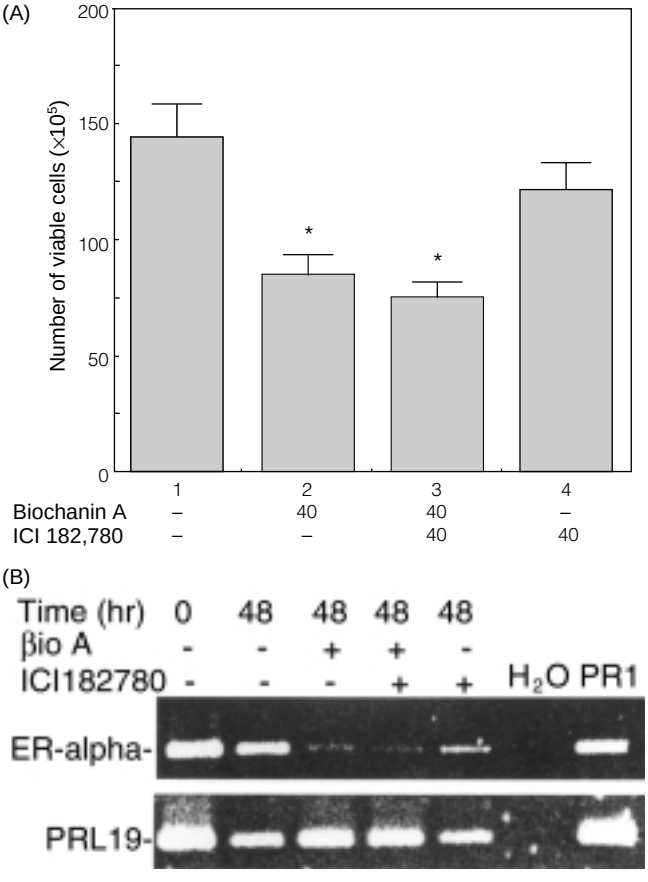

Figure 5. Effects of co-administered antiestrogen ICI182,780 on the biochanin A induced cell regulation. (A) MCF-7 cells were incubated with 40 μg/mL biochanin A alone or with 40 μg/mL ICI182,780 for 2 days. The number of viable cells was determined by trypan blue exclusion assay. Data are presented as mean ± SD. * indicates significant difference ($p < 0.01$) compared to the DMSO vehicle control. (B) The levels of ER-α mRNA in MCF-7 cells treated with biochanin A and ICI182,780. MCF-7 cells were incubated with 40 μg/mL biochanin A alone or with 40 μg/mL ICI182,780 for 2 days. Total RNA was prepared from each sample and RT-PCR was performed to detect the level of ER-α mRNA. PR1: RNA from ER expression PR1 cells was used in the RT-PCR reaction. H_2O: water was used in substitution for RNA in the PCR reaction.

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