AD_____

AWARD NUMBER DAMD17-97-1-7035

TITLE: Molecular Mechanism of Action of Genistein and Related Phytoestrogens in Estrogen Receptor Dependent & Independent Growth of Breast Cancer Cells

PRINCIPAL INVESTIGATOR: Srivani Balabhadrapathruni

CONTRACTING ORGANIZATION: University of Medicine and Dentistry
Of New Jersey
Piscataway, New Jersey 08854

REPORT DATE: July 1998

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE			Form Approved OMB No. 0704-0188
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.			
1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE July 1998	3. REPORT TYPE AND DATES COVERED Annual (1 Jul 97 - 30 Jun 98)	
4. TITLE AND SUBTITLE Molecular Mechanism of Action of Genistein and Related Phytoestrogens in Estrogen Receptor Dependent & Independent Growth of Breast Cancer Cells			5. FUNDING NUMBERS DAMD17-97-1-7035
6. AUTHOR(S) Srivani Balabhadrapathruni			
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Medicine and Dentistry of New Jersey Piscataway, New Jersey 08854			8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER
11. SUPPLEMENTARY NOTES			
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited			12b. DISTRIBUTION CODE

ABSTRACT.

During the first year of my pre-doctoral fellowship, I have demonstrated that genistein binds weakly to the estrogen receptor (ER) with an IC_{50} value of 900 nM (task 1). Quercetin and genistein also bind to the type-II estrogen binding sites, and exert cell growth inhibition in MCF-7 and MDA-MB-468 cells (task 2). In circular dichroism studies, decreased intensity of the spectra leading to a random recombinant ER structure was observed with genistein (task 3). Genistein (10 μM) stimulated the growth of MCF-7 cells at 24 h, however, by 72 h, it was growth inhibitory at all doses (task 5). A major cell cycle block at G_2/M phase was observed with genistein treatment and was accompanied by alterations in cyclin B1 levels (tasks 7 and 8). In addition, we show that cell growth inhibition by genistein and quercetin is associated with decreased polyamine levels. Our results provide insights into the ER-dependent and -independent mechanisms of action of genistein in breast cancer cell growth.

14. SUBJECT TERMS Breast Cancer			15. NUMBER OF PAGES 39
			16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

NA Where copyrighted material is quoted, permission has been obtained to use such material.

NA Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

SB Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

NA In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

NA In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

NA In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

NA In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Sridevi B 6-30-98

PI - Signature Date

TABLE OF CONTENTS

A. INTRODUCTION

The primary focus of my research in the past year had been to understand the complex behavior of the phytoestrogen genistein in promoting or inhibiting the growth of human breast cancer cells.

Epidemiological studies suggest that Oriental women consuming high vegetarian diets are at a lower risk for breast cancer (1,2,3). The anticancer effects of the vegetarian diets are attributed to the phytoestrogens (flavones, isoflavones, coumestans and lignans), present abundantly in soy products, fruits, vegetables, and whole-grains (4,5,6). Hormone-dependent cancers such as breast cancer is promoted by endogenous estradiol (7). Estradiol (E_2) binds the estrogen receptor (ER), the complex then binds estrogen response element (ERE) and increases the transcription of growth-related genes (8). Owing to the structural similarity to estradiol, and potential antiestrogenic effects (9,10), phytoestrogens are being studied for their potential use in chemoprevention.

Genistein is an isoflavone that has been shown to have both stimulatory (11,12) as well as inhibitory roles (13,14,15) in cancers in humans and animals. In breast cancer, the growth enhancing effects of genistein are attributed to its estrogen-like activity (16,17), while its antiproliferative actions were explained either as antiestrogenic (18) or as estrogen-independent (19,20). This study is a systematic evaluation of the dose-dependent estrogenic, antiestrogenic or ER-independent pathways through which genistein exerts its diverse effects on breast cancer cells.

We explored the mechanism of ER-based estrogenic actions of genistein by determining the binding affinities of genistein to ER using cellular and recombinant ER (rER). To understand how the molecular structure of genistein is associated with estrogenic or antiestrogenic properties, we compared it with other structurally related compounds such as quercetin, daidzein, biochanin A and kaempferol in ER binding. To further delineate the ER-mediated actions of genistein, we conducted circular dichroism (CD) studies. These data are expected to provide evidence for any secondary structural changes in ER protein after its binding to genistein. The changes in spectral intensity caused by estradiol are compared to that obtained with different concentrations of genistein.

Estradiol also binds to another class of protein besides ER, called the type II binding site (EBS) with low affinity (21). Reported evidence suggests that the function of EBS may not be to bind physiological concentrations of estradiol (22). However, there is some evidence indicating that plant flavonoids may be the ligands for EBS and mediate cell growth inhibition by these compounds (21). To explore this possibility for phytoestrogens' action independent of ER, we quantified EBS and determined their affinity for phytoestrogens relative to estradiol in ER-positive MCF-7 and ER-negative MDA-MB-468 breast cancer cells.

Cell proliferation is strictly controlled in normal cells whereas in cancer cells, a strict restriction point control is lacking, so they continue to grow under conditions in which normal cells are dormant (23). Recently, considerable interest has been paid to the role of cyclins in regulating cell cycle progression, and these proteins may be a target for

chemopreventive agents (24). To comprehend the mechanism of action of genistein and its structural basis in mediating growth inhibition, we studied the cell cycle distribution after treatment with genistein and other structurally similar phytoestrogens in breast cancer cells. To further identify the targets of this molecule in cancer cells, we determined the levels of cyclin B1 protein. Cyclin B1 is needed for the successful progression of cells from S to M phase and for the completion of mitosis (25).

Polyamines are cellular cations, present abundantly in all cells (26). They are involved in cell growth and differentiation (26). Previous studies reported that estradiol based growth in breast cancer cells is mediated through an increase in intracellular polyamine levels (26,27,28). Ornithine decarboxylase (ODC) is the first and a rate limiting enzyme in the synthesis of cellular polyamines (26). To gain insight into the estrogenic or antiestrogenic actions of genistein, we determined ODC activity and polyamine levels after treatment of breast cancer cells with this compound.

Our results in the first year provide some important clues to the mechanism of action of genistein in breast cancer cells. In addition to genistein, we studied other structurally related compounds. The chemical structures of the phytoestrogens used in this study are shown in Figure 1.

A summary of my accomplishments during this report period is given below.

Tasks for this report	Status
1. Months 1-3	Binding affinities of phytoestrogens with cellular and recombinant ER- **completed**
2. Months 4-6	Binding affinities of phytoestrogens with type-II EBS - **completed**
3. Months 7-12	Molecular conformation of phytoestrogen-bound ER- **Partially completed, work in progress.**
Tasks proposed for the second and third years	
5. Months 17-19	Cell growth studies - **completed**
7. Months 27-28	Cell cycle progression studies- **completed**
9 Months 29-36	Cyclin levels determination - partially **completed**
* Additional tasks	Measurement of polyamine levels - **completed**
	Determination of ODC activity - partially **completed**

*These experiments were not proposed, but provide additional information on the estrogenic or antiestrogenic actions of genistein.

Presentations

1. Submitted an abstract and presented a poster at the Annual Meeting of the American Association of Cancer Research, 1998 in New Orleans (COPY ATTACHED).
2. Gave an oral presentation titled "Genistein treatment causes G_2/M cell cycle arrest and cyclin B1 accumulation in breast cancer cells: a possible mechanism for the preventive action of genistein", in the focus session during the Annual Retreat on Cancer

Research, organized by The Cancer Institute of New Jersey and the New Jersey Commission on Cancer Research, May 1998.

Award. Received the "Gallo Award", presented by the Cancer Institute of New Jersey for "Outstanding Cancer Research", May 29, 1998 (COPY ATTACHED).

A brief description of methods used, results and discussion follows.

B. METHODS

1. Cell culture. MCF-7 cells are maintained in Dulbecco's modified Eagle's medium (DMEM) with 100 μg/ml penicillin, 100 μg/ml streptomycin, 2 μg/ml insulin, and 10% fetal bovine serum (FBS). For two weeks before each experiment, cells were grown in phenol red free DMEM as phenol red has estrogenic effects (29). FBS was treated with dextran coated charcoal (DCC) to remove endogenous estrogens and added to the medium as described previously (30). MDA-MB-468 cells were grown in Improved minimum essential medium with 5% FBS, 2 mM glutamine, and 100 μg/ml each of penicillin and streptomycin.

Genistein, quercetin, daidzein, kaempferol were purchased from Sigma Chemical Co. (St Louis, MO). Stock solutions were made in dimethylsulfoxide (DMSO), aliquoted and frozen until use. Recombinant ER was purchased from Panvera Corp (Madison, WI).

2. Competitive binding assay of phytoestrogens with labeled estradiol. To prepare cellular ER, MCF-7 cells from 10 T-150 flasks were harvested into PBS and sonicated in TEDG buffer (10 mM Tris, 1 mM EDTA, (pH 7.5), 1 mM dithiothreitol (DTT) and 10% glycerol). The cellular homogenate was centrifuged at 100,000 x g to obtain the cytosol. The cytosol (250 μl) was incubated for 3 h at 4 °C with 5 nM [^{3}H]-E_2 and unlabeled genistein or quercetin (10^{-5} - 10^{-8} M). After incubation, DCC suspension was added to the samples to remove free E_2. The samples were then centrifuged at 750 x g for 5 min at 4 °C. Supernatant was sedimented through a 10-30% sucrose-TEDG buffer. [^{14}C]-labeled bovine serum albumin and γ- globulin were used as internal markers. Gradients were centrifuged in a Beckman SW60 rotor at 53,000 rpm for 16 h. Fractions were collected into 5 ml scintillation fluid and the bound estradiol was counted using a scintillation counter. Nonspecific binding was determined in parallel samples incubated with a 200-fold molar excess of unlabeled estradiol. Concentration dependent, competitive binding curves were determined for each compound. Results are expressed as percent [^{3}H]-E_2 bound in the absence (100%) or presence of the competitor.

Human recombinant ER (500 fmols/ml) was incubated with 5 nM [^{3}H]-E_2 and different concentrations of the phytoestrogens, and treated as described above for cellular ER. In parallel experiments, receptor was incubated with 5 nM [^{3}H]-E_2 and increasing concentrations of unlabeled E_2.

3. **Type-II EBS analysis in human breast cancer cells.** Type-II EBS were measured by a whole cell assay as described by Ranelletti et al (31). MCF-7 and MDA-MB-468 cells (5×10^4/well) were plated into Multiwell plates with the appropriate medium. After 24 h, the cells were refed with fresh media without serum. The cells were then dosed with increasing concentrations (4-50 nM) of [^{3}H]-estradiol alone or with 200-fold molar excess of diethyl stilbestrol (DES) and incubated for 3 h at 37 °C. After 3h, the cells were kept on ice for 30 min and washed 4 times in 1 ml volumes of ice cold HBSS. To each well, 1 ml of 100% ethanol was added and incubated at 37 °C. Bound estradiol was measured by a liquid scintillation counter. Competition for type-II EBS was determined by incubating cells with 40 nM [^{3}H]-E_2 plus increasing concentrations of one of the phytoestrogens. Radioactivity bound to the cells was determined and IC_{50} values were calculated for each of the compounds, from the concentration dependence of the displacement of [^{3}H]-E_2 from type-II EBS.

4. Circular dichroism measurements. The secondary structural changes in the human recombinant ER (1.5 μM protein) was studied in the presence of genistein, using CD spectroscopy. Genistein (0.5-10 μM) or same concentrations of estradiol were incubated with the ER at room temperature overnight in storage buffer (50 mM Tris, 1 mM sodium vanadate, 1 mM EDTA, 500 mM KCl, 10% glycerol and 2 mM DTT). All CD spectra were recorded using an AVIV 62D spectropolarimeter with a water-jacketed cell of 1 mm path length. The spectral wavelength range measured was between 200-260 nm. The cell temperature was kept constant with a circulating water bath at 25 °C. The α-helicity was calculated from the reported value of molar elipticity of a 100% helical peptide, (θ) (222 nm) = 33,500 deg cm^2 $dmol^{-1}$.

5. [^{3}H]-thymidine incorporation assay. Cells (0.5×10^6) were plated in 60 mm culture dishes and allowed to adhere for 24 h. The cells were dosed with different concentrations of phytoestrogens for 24, 48 and 72 h. One hour before the treatment time ended, one μCi/ml of [^{3}H]-thymidine was added to the cells. The radioactivity incorporated into the cellular DNA was measured by liquid scintillation counting.

6. **Flow cytometry.** To determine the effect of phytoestrogens on cell cycle progression, flow cytometric analysis was conducted. We plated 2×10^6 cells in 100 mm dishes. At 24, 48 and 72 h after the treatment of cells with different concentrations of phytoestrogens, the media was removed and 2 ml of the buffer containing 40 mM sodium citrate, 250 mM sucrose, and 5% DMSO was added. The samples were then frozen at -70 °C until further analysis. For DNA analysis, the cells were thawed, citrate buffer removed, and trypsinized for 10 min on ice. Then a solution of trypsin inhibitor and RNase were added for 10 min and stained with propidium iodide in citrate buffer (130 μg/ml). Cells were analyzed by a Coulter Flow Cytometer. Distribution of cells in different phases of cell cycle was calculated using cytological software.

7. Western blot analysis of cyclin B1. Cells were plated as described above for Flow cytometry. Twenty four hours after treatment with genistein and quercetin, cells were harvested in PBS and pellets stored at -70 °C until further analysis. Cell pellets were solubilized in 300 µl of a buffer containing 150 mM Tris, 150 mM NaCl, 50 mM NaF, 0.2% SDS, 1% NP40, 2 mM EDTA, 100 µM Na_3PO_4. Thirty µg protein was electrophoretically separated on a 10% polyacrylamide gel. The proteins were transferred to PVDF immobilon membrane. After blocking overnight with 2% non-fat dry milk, blots were incubated for 3 h with purified monoclonal mouse anticyclin B1 (NeoMarkers, CA) antibody, followed by horseradish-peroxidase labeled anti-mouse secondary antibody. Protein was visualized with a chemiluminiscence based detection system and the intensity of the bands were read by a scanning densitometer.

8. Determination of ODC activity. MCF-7 cells were harvested after treatment with genistein. The cell pellet was resuspended in 300 µl cold assay buffer and stored at -70 °C. Thawed samples were sonicated and centrifuged at 12,000 x g for 10 min at 4 °C. To 100 µl of the supernatant, 200 µl of assay buffer and 0.08 mM pyridoxal phosphate (final) was added. The reaction was initiated by adding 100 µl L-ornithine and 0.25 µCi of L-[1-^{14}C]-ornithine to yield a final concentration of 0.5 mM ornithine. The released $^{14}CO_2$ was absorbed on a GF/C filter paper during incubation and was measured using the liquid scintillation counter. The amount of protein in cell lysates was determined and ODC activity expressed as pmole/mg protein/min.

9. Determination of intracellular polyamine levels. Cells (2 x 10^6) were plated and after 24, 48 and 72 h treatment with genistein and quercetin, were harvested in 1 x PBS. The cell pellet was treated with 300 µl of 8% sulfosalicylic acid, and sonicated for 15 sec on ice. The lysate was incubated on ice for 1 h and centrifuged at 10,000 x g for 5 min in a microfuge tube to remove the precipitated protein. Polyamine levels were determined by high performance liquid chromatography (HPLC) after derivatization to their dansyl derivatives. 1,6-diaminohexane was used as an internal standard.

C. RESULTS

I. Results of Task I: Binding affinities of phytoestrogens with ER, months 1-3.

To determine if phytoestrogens interact with the ER, we studied their binding with ER, relative to unlabeled estradiol using sucrose gradient analysis. Recombinant as well as cellular ER prepared from MCF-7 cells were used in the experiments. The inhibition of [^{3}H]-E_2 binding to cellular and recombinant ER by genistein is shown in Figures 2 and 3, respectively. The IC_{50} value, the concentration of phytoestrogens needed to displace 50% of the [^{3}H]-E_2 bound to cellular ER or recombinant ER is given in Table 1. The order of relative affinities of the phytoestrogens for ER as compared to estradiol are as follows; genistein >> quercetin > daidzein ≥ biochanin A ≥ kaempferol. The IC_{50} values of different compounds showed comparable rank order with cellular and recombinant ER.

However, [^{3}H]-E_2 from recombinant ER is more easily displaced by phytoestrogens than cellular ER.

II. Results of Task 2: Interaction of phytoestrogens with type-II EBS, months 4-6.

In order to investigate the ER-independent actions of phytoestrogens, we measured relative binding affinities of phytoestrogens for type-II EBS. First, we determined type-II EBS in MCF-7 and MDA-MB-468 cells according to the whole-cell assay (31). In the ER-positive MCF-7 cells, the specific binding of [^{3}H]-E_2 was determined by competing out with DES. Two classes of specific binding sites were observed, one between 5 and 10 nM and another at 40 nM concentration of radiolabeled E_2 (data not shown). These sites correspond to the ER (saturating at 5-10 nM concentrations) and type-II EBS (showing saturation at 40 nM concentration), as described in a previous study (32). Further experiments to determine competition of genistein for type-II EBS were conducted using 40 nM [^{3}H]-E_2 in MCF-7 cells. In MDA-MB-468 cells, 30 nM [^{3}H]-E_2 saturated the type-II EBS, and this concentration was used to determine competition by phytoestrogens.

Among the phytoestrogens studied, quercetin bound to the type-II EBS with high affinity, with an IC_{50} of 35 nM and 590 nM in MDA-MB-468 and MCF-7 cells, respectively (Figure 4, Table 2). Genistein, however was less potent requiring >2 μM concentrations to inhibit [^{3}H]-E_2 binding in both the cell lines (Table 2). Kaempferol and daidzein did not compete for type-II EBS with [^{3}H]-E_2, up to 10 μM concentration.

III. Results of Task 3: Changes in molecular conformation of phytoestrogen-bound ER using CD studies, months 7-12.

To gain insight into the conformational alterations caused by genistein on ER, we conducted CD studies using recombinant human ER. The recombinant ER was incubated overnight at room temperature in storage buffer, to eliminate any time dependent changes in the protein during analysis with the compounds. Increasing concentrations of genistein were added to the rER and CD spectra recorded at 25 °C. The results are presented in Figure 5. The CD spectrum of ER alone showed a minima spectra at 225 nm and a shoulder band at 212 nm. Genistein and estradiol decreased the intensity of the CD spectra. Calculation of the percentage of α-helix content was made from the multiple linear regression program developed by Yang et al (33), which gave an estimated α-helix contents of 26.9 % for untreated ER, 17.5% and 13.5% with 10 μM estradiol and genistein, respectively. These results are however, in a preliminary stage and more experiments will be performed to understand the ligand dependent changes in the ER.

In addition to the three tasks required to be completed in the first year, I also worked on other tasks proposed for second and third years of my fellowship. Results from these experiments are presented below.

IV. Results of Task 5. Cell proliferation studies, months 17-19.

In order to relate binding affinity data to biological effects of phytoestrogens, we determined the effects of these compounds on cell growth. MCF-7 and MDA-MB-468 cells were treated with 0, 10, 25, 50 and 100 μM phytoestrogens for 24, 48 and 72h. Cell growth was determined by [^{3}H]-thymidine incorporation assay. In MCF-7 cells, 10 and 25 μM genistein treatment for 24 h caused a 27% and 39% increase in cell growth, respectively. However, the higher concentrations (50 and 100 μM) were growth inhibitory. Furthermore, by 72 h of treatment, all doses of genistein were growth inhibitory (Figure 6). Genistein significantly reduced the growth of MDA-MB-468 cells at all concentrations used and the concentration needed to decrease the growth of ER-negative cells was 4-fold lower than that needed for the ER-positive cells. The IC_{50} values for the phytoestrogens in inhibiting the growth of MCF-7 and MDA-MB-468 cells are presented in Table 3. Quercetin, although less effective than genistein, was equally potent in inhibiting cell growth of ER-positive and -negative cells. Biochanin A and kaempferol were less potent cell growth inhibitors where as daidzein did not have a significant effect in decreasing the growth of these breast cancer cells at the concentrations used.

VI. Results of Task 7. Cell cycle progression after treatment with phytoestrogens, months 27-28.

Flow cytometric analysis of the untreated breast cancer group showed a majority of the cells in the G_0/G_1 (65-70%) phase, and only a small percentage of cells in the G_2/M phase (10-13%) of the cell cycle in both MCF-7 and MDA-MB-468 cells. We observed a time and dose-dependent accumulation of the G_2/M phase cells after treatment with genistein in MDA-MB-468 cells (Figure 7). The increase in the population of G_2/M cells was associated with a corresponding decrease in the percentage of cells in S and G0/G1 phases of the cell cycle. Genistein treatment (100 μM) for 24 h caused 70% of the MDA-MB-468 cells and 42.4% of the MCF-7 cells to arrest at the G_2/M phase (Table 4 and 5 respectively). Quercetin treatment also caused G_2/M block, although to a lesser extent compared to genistein. The effects of G_2/M block by genistein and quercetin were much more prominent in MDA-MB-468 cells. Kaempferol was half as effective as quercetin, while biochanin A and daidzein did not have significant effects on cell cycle distribution in MDA-MB-468 cells.

VII. Results of Task 8. Western Immunoblot for cyclin protein determination, months 29-36.

Genistein and quercetin caused a significant G_2/M block in the breast cancer cells. Therefore, further studies were conducted to study the effects of phytoestrogens on the levels of cyclin B1 as it is involved in G_2/M progression. Treatment of the breast cancer cells with genistein resulted in a biphasic response in cyclin B1 levels. After 24 h of treatment, 50 μM genistein caused an accumulation of cyclin B1 protein compared to control in MCF-7 cells (Figure 8A) and 25 μM genistein increased cyclin B1 levels in

MDA-MB-468 cells (Figure 9). However, at 100 μM concentration, genistein caused a drastic decrease in the levels of this protein in both the cell lines.

In contrast to genistein, quercetin-treated MDA-MB-468 cells showed a rise in cyclin B1 levels (Figure 9) amounting to a 37% increase with 100 μM treatment compared to controls as determined by densitometric scanning. Similarly, cells treated with the positive control nocodazole (known microtubule inhibitor) (34) resulted in a progressive accumulation of cyclin B1 levels after 24 h (Figure 8B).

VIII. Results of additional experiments. In addition to the studies proposed, we conducted other experiments which enhanced our understanding of estrogen-dependent and independent actions of genistein in breast cancer cell growth.

Effect of genistein treatment on ODC activity. The polyamine biosynthestic enzyme ODC activity increased significantly within 4 h of genistein treatment (Figure 10A). However, at later time points, the enzyme activity was greatly decreased in MCF-7 cells (Figure 10 B).

Effect of genistein on intracellular polyamine levels. Genistein treatment for 24 h did not have a significant effect on the levels of putrescine, spermidine and spermine in MCF-7 cells. However, at 48 h and 72 h, there was a decrease in all the polyamine levels (Figure 11). Similar results were obtained with quercetin treatment (data not shown).

D. DISCUSSION

In this report, we present data on the effects of phytoestrogens on a number of parameters likely to be important in the biological action of these compounds. Competitive binding of phytoestrogens to cellular and recombinant ER shows genistein to have higher binding affinity to ER compared to other structurally related compounds, quercetin, daidzein, biochanin A and kaempferol. Assessment of type-II EBS in MCF-7 and MDA-MB-468 cells revealed a relatively higher binding affinity of quercetin to these sites compared to other compounds. Studies on cell growth inhibition showed quercetin to be the most active compound in MCF-7 cells with IC_{50} of 17.3 ± 2.7 μM, where as genistein is the most active compound in MDA-MB-468 cells with an IC_{50} of 8.8 ± 1.6 μM. Thus, even though genistein has relatively higher binding affinity for ER, compared to other compounds, this affinity does not seem to be important in its antiproliferative effects. Our studies on cell cycle kinetics showed genistein-induced arrest of MDA-MB-468 cells in the G2/M phase, and a biphasic response in cyclin B1 levels. These results will be potentially important in the identification of cellular targets of genistein, and phytoestrogens in general.

The concentration of genistein effective in displacing 50% of estradiol from ER was reported previously to be growth stimulatory in breast cancer cells (35). Therefore, it has been a great concern that at physiologically relevant concentrations (1 nM - 10 μM), genistein stimulates ER-positive breast cancer cells via an estrogenic response (35). In

our study, although genistein, daidzein, biochanin A and kaempferol at 10 μM stimulated MCF-7 cell growth in 24 h experiments, growth inhibition was evident by 3 days with genistein treatment. Also, we found the growth stimulation of 10 μM genistein only in the absence of estradiol and not when it is present (data not shown). These results are comparable to those reported by others (36,37). Similarly, Wang et al (17) reported that when MCF-7 cells were treated with 1 μM genistein for 6 days, ER mRNA was downregulated. Taken together, these results suggest that: (i) physiologically achievable concentrations of genistein may not be growth stimulatory in the presence of circulating E_2 levels, and (ii) with chronic exposure, genistein may be working through other ER-independent mechanisms to inhibit the growth of breast cancer cells, even at low concentrations. In this study, quercetin bound weakly to the ER, but was growth inhibitory at all concentrations studied. These results are comparable to other studies, in which the quercetin concentration needed to bind the ER, was growth inhibitory (36).

Previous studies reported that flavonoids bind the type-II EBS and may cause a reduction in breast cancer cell growth. Quercetin was shown to induce type-II EBS in MDA-MB-231 cells (38) and this was associated with decreased cell growth. In our studies, quercetin bound to type-II EBS with higher affinity than other compounds, both in MCF-7 and MDA-MB-468 cells, as estimated from competition binding curves. The concentrations needed to half-saturate type-II EBS were 590 and 35 nM in MCF-7 and 468 cells respectively. However, binding affinity for genistein was lower than that of quercetin. Furthermore, biochanin A and kaempferol, which have very little affinity for ER had virtually no affinity for type-II EBS. Therefore, it is unlikely that type-II EBS play a significant role in the antiproliferative effects of most phytoestrogens.

We report for the first time, the concentrations of phytoestrogens needed to displace 50% of radiolabeled E_2 bound to the recombinant ER. To understand the structural basis for ER-binding by genistein, we compared the effects of other structurally similar compounds in their ability to bind ER. Daidzein is similar in structure to genistein, except that it does not have the 5-OH group on its A ring (Figure 1). Biochanin A has the 5-OH group, but its 4'-OH group on B ring is methylated. Kaempferol differs from genistein in that its phenolic B ring is attached to the 2 position (instead of 3 position in genistein) of the pyran C ring and quercetin differs from kaempferol with an extra OH group on 5' position of the B ring (36). In a previous study, it was hypothesized that that the 4'-OH group of phenolic B ring and its spatial orientation with the 7-OH group on A ring are the reasons for genistein's estrogenicity (36,39). Therefore, when the 4'-OH group is methylated (biochanin A) or its orientation is changed (kaempferol), the binding affinity to ER is decreased (36,40), which is similar to what we observed. However, in our studies, daidzein, which is similar to genistein in this respect, has 10-fold lower binding with ER compared to genistein, suggesting that other factors beside the structure are important in ER binding.

Our results demonstrate that genistein decreased breast cancer cell growth, by arresting the proliferating cells in the G_2/M phase of the cell cycle. The effects of genistein increased in a dose- and time- dependent manner in MDA-MB-468 cells. The G_2/M block was associated with a decrease in the percentage of cells in the S and G0/G1

phases of the cell cycle. Similar to growth inhibition, the effects of genistein in increasing the population of G2/M cells were much more pronounced in the MDA-MB-468 cells compared to MCF-7 cells, suggesting that the estrogenic effects of genistein are not completely abated in MCF-7 cells. Our results confirm the previous reports of G_2/M phase arrest by genistein in MCF-7 cells (41), and by quercetin in 468 cells (42). The inability of other phytoestrogens in this study to inhibit cell cycle progression at G_2/M phase suggests that disruption of cell cycle is specific for quercetin and genistein.

In eukaryotic cells, cyclin B1 accumulates during the late S and G_2 phases and allows entry of cells into the M phase and is rapidly degraded at the end of mitosis, allowing the cells to divide. In this study, genistein treatment resulted in the accumulation of cyclin B1 compared to untreated cells at 50 μM concentration in MCF-7 cells and at 25 and 50 μM concentration in MDA-MB-468 cells, suggesting that G2/M phase arrest is associated with the inhibition of cyclin B1 degradation. However, at 100 μM concentration, in both the cell lines, genistein treatment drastically decreased cyclin B1 levels after 24 h. Microscopic examination of the cells treated with highest dose of genistein showed morphological features of apoptosis, suggesting apoptosis as a reason for the observed decrease in cyclin B1 levels. In contrast to genistein, cyclin B1 levels increased after quercetin treatment for 24 h, similar to the microtubule inhibitor nocodazole. These results indicate that the mechanism of G_2/M arrest by genistein and quercetin may be mediated via different pathways. It is unclear whether the observed alterations in cyclin B1 levels after treatment with genistein and quercetin are a result of a change in synthesis or degradation of this protein. Further studies will be directed towards answering this question.

Polyamine levels are increased several-fold in breast tumors compared to normal tissues (43), and have been reported to be involved in the estrogenic effects of cell growth (44). ODC, a key enzyme in the polyamine biosynthetic pathway, is transcriptionally regulated by estradiol and links the estrogenic effects and polyamine synthesis cascade. All the three polyamines, putrescine, spermidine and spermine levels decreased only after 48 and 72 h of genistein treatment in MCF-7 cells.

In conclusion, our results demonstrate that the antiproliferative effects of genistein are subdued in ER-positive breast cancer cells, because of its weak estrogenic potential. However, its role in cell growth inhibition involves several pathways, including disruption of cell cycle progression, alterations in cyclin B1 levels and disruption of polyamine pathway. Genistein induced alterations in different pathways followed similar trend in both ER-positive and negative cells leading to reduced cell growth, thus its antiproliferative effects are unlikely to be mediated by ER.

E. RECOMMENDATIONS IN RELATION TO THE STATEMENT OF WORK

I completed the first two tasks proposed for the first year of my grant period. Circular dichroism spectroscopy studies are in progress. In addition to the tasks in the first year, I completed most of the work from tasks 5, 7 and 9 proposed for subsequent years. I will be writing a manuscript soon with the data. I started some preliminary work in the polyamine pathway with the phytoestrogens, which will help broaden my understanding of the effects of phytoestrogens in breast cancer, and to elucidate the mechanism(s) of action of genistein in breast cancer cells.

F. CONCLUSIONS.

This study provides new information on the effects and mechanism of action of five phytoestrogens, genistein, quercetin, biochanin A, daidzein and kaempferol on breast cancer cell growth. All the phytoestrogens showed weak binding to the ER in MCF-7 cells compared to E_2. Genistein is a potent breast cancer cell growth inhibitor in MCF-7 and MDA-MB-468 cells, and these effects appear to be independent of ER. In MDA-MB-468 cells, type-II EBS may have contributed to the antiproliferative effects of quercetin, because of relatively high affinity of quercetin for type-II EBS. However, since other compounds have low or no affinity for these sites, type-II EBS are unlikely to contribute to the effects of most phytoestrogens. We identified G_2/M cell cycle arrest, disruption of cyclin B1 levels, and decrease in intracellular polyamine levels as potential pathways through which genistein and quercetin mediate their growth inhibition. Genistein and quercetin may have potential chemotherapeutic effects, and our results are valuable in understanding the targets of these drugs.

G. REFERENCES

1. Dunn, J.E., Jr. Cancer epidemiology in populations of the United States-emphasis in Hawaii and California- and Japan. Cancer Res., 35:3240-3245, 1975.

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

3. Adlercreutz, H., Honjo, H., Higashi, A., Fotsis, T., Hamaten, E., Hasegawa, T. and Okada, H. Urinary excretion of lignans and isoflavonoid phytoestrogens in Japanese men and women consuming a traditional Japanese diet. Am J Clin Nutr., 54:1093-1100, 1991.

4. Adlercreutz, H. Does fiber-rich food containing animal lignan precursors protect against both colon and breast cancer? An extension of the fiber hypothesis. Gastroenterology, 86:761-764, 1984.

5. Adlercreutz, H., Heikkinen, R., Woods, M., Fotsis, T., Dwyer, J.T. and Goldin, B.R. Excretion of the lignans enterolactone and enterodiol and of equol in omnivorous and vegetarian postmenopausal women and in women with breast cancer. Lancet, 2:1295-1299, 1982.

6. Murkies, A.L , Wilcox, G , and Davis, S.R. Phytoestrogens. Clinical Review. J Clin Endocrinology and Metabolism, 83:297-303, 1998.

7. Vignon, F. and Rochefort, H. Mechanism of action of tamoxifen and metabolites in MCF-7 human breast cancer cells. In: V.P. Hollander (ed.), Hormone responsive tumors, pp. 135-153. New York: Academic Press, 1985.

8. Kiang, D.T., Frenning, D.H., Goldman, A.I., Aslensa, V.F. and Kenneedy, B.J. Estrogen receptors and responses to chemotherapy and hormonal therapy in advanced breast cancer. N Engl J Med., 229:1330 1334, 1984.

9. Miksicek, R.J. Commonly occurring plant have estrogenic activity. Mol Pharmacol., 44:37-43, 1993.

10. Jordan, V.C., Mittal, S., Gosden, B., Koch, R. and Lieberman, M.E. Structure-activity relationships of estrogens. Environ Health Persp., 61:97-110, 1985.

11. Rao, C.V., Wang, C-X ,Simi, B , Lubet, R , Kelloff, G , Steele, V and Reddy, B. Enhancement of experimental colon cancer by genistein. Cancer Res., 57:3717-3722, 1997.

12. Petrakis, N , L , Barnes, S , King, E B, Lowenstein, J , Wiencke, J , Lee, M , M , Miike, R , Kirk, M and Coward, L. Stimulatory influence of soy protein isolate on breast secretion in pre- and postmenopausal women. Cancer Epidemiol Biomarkers Prev, 5:785-794, 1996.

13. Ingram, D , Sanders, K , Kolybaba, M and Lopez, D. Case-control study of phyto-estrogens and breast cancer. Lancet, 350:990-994, 1997.

14. Lamartiniere, C.A., Moore, J., Holland, M. and Barnes, S. Neonatal genistein chemoprevents mammary carcinogenesis. Proc Soc Exptl Biol Med., 208:120-123, 1995.

15. Sharma, O.P., Adlercreutz, H., Strandberg, J.D., Zerkin, B.R. and Coffey, D.S. Soy of dietary source plays a preventive role against the pathogenesis of prostitis in rats. J Steroid Biochem Mol Biol., 43:557 564, 1992.

16. Sathyamoorthy, N., Thomas, T. and Phang, J.M. Stimulation of pS2 expression by diet-derived compounds. Cancer Res., 54:957-61, 1994.

17. Wang, T.T.Y., Sathyamoorthy, N., and Phang, J.M. Molecular effects of genistein on estrogen receptor mediated pathways. Carcinogenesis., 17:271-275, 1996.

18. So, F , V , Guthrie, N , Chambers, A , F and Carroll, K , K. Inhibition of proliferation of estrogen receptor-positive MCF-7 human breast cancer cells by flavonoids in the presence and absence of excess estrogen. Cancer Letters, 112:127-133, 1997.

19. Peterson, G. and Barnes, S. Genistein inhibition of the growth of human breast cancer cells: independence from estrogen receptors and the multi-drug resistance gene. Biochem Biophy Res Commun., 179:1:661 667, 1991.

20. Monti, E. and Sinha, B.K. Antiproliferative effect of genistein and adriamycin against estrogen-dependent and independent human breast carcinoma cell lines. Anticancer Res., 14:1221-1226, 1994.

21. Scambia, G., Ranelletti, F.O. and Panici, P. Type-II estrogen binding in a lymphoblastoid cell line and growth-inhibitory effect of estrogen, anti-estrogen and bioflavonoids. Int J Cancer., 46:1112-1116, 1990.

22. Markaverich, B.M., Roberts, R.R., Alejandro, M.A., Johnson, G.A., Middleditch, B.S., and Clark, J.H. Bioflavonoid interaction with rat uterine type II binding sites and cell growth inhibition. J Steroid Biochem, 30:71-78, 1988.

23. Sherr, C , J. Cancer cell cycles. Science, 274:1672-1677, 1996.

24. Sherr, C.J. G1 phase progression: cycling on cue. Cell, 79:551-555, 1994.

25. King, R.W , Deshaies, R.J , Peters, J.M and Kirschner, M.W. How proteolysis drives the cell cycle. Science, 274:1652-1659, 1996.

26. Tabor, C , V Tabor, H. Polyamines. Annu Rev Biochem., 53:749-790, 1984.

27. Thomas, T. and Thomas, T.J. Estradiol control of ODC mRNA, enzyme activity and polyamine levels in MCF-7 breast cancer cells: therapeutic implications. Breast Cancer Res Treatment., 29:189-201, 1993.

28. Kendra, K , L and Katzenellenbogen, B , S. An evaluation of the involvement of polyamines in modulating MCF-7 human breast cancer cell proliferation and and progesterone receptor levels by estrogen and antiestrogen. J Steroid Biochem. ,28:123-128, 1987.

29. Berthois, Y., Katzenellenbogan, J.A., and Katzenellenbogan, B.S. Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. Proc Natl Acad Sci USA., 83:2496-2500, 1986.

30. Thomas, T., Trend, B., Butterfield, J.R., Janne, O.A., and Kiang, D.T. Regulation of ornithine decarboxylase gene expression in MCF-7 breast cancer cells by antiestrogens. Cancer Research, 49:5852-5857, 1989.

31. Ranelletti, F.O , Piantelli, M , Carbone, A , Rinelli, A , Scambia, G , Benedetti Panici, P and Mancuso, S. Type-II estrogen binding sites and 17 beta-hydroxysteroid dehydrogenase activity in human peripheral-blood mononuclear cells. J Clin Endocr Metab., 67:888-892, 1988.

32. Markaverich, B.M , Gregory, R.R , Alejandro, M , Kittrell, F.S , Medina, D , Clark, J.H , Varma, M and Varma, R.S. Methyl p-hydroxyphenyllactate and nuclear type II binding sites in malignant cells: metabolic fate and mammary tumor growth. Cancer Research, 50:1470-1478, 1990.

33. Yang, J.T , Wu, C.S and Martinez, H.M. Calculation of protein conformation from circular dichroism. Methods Enzymol., 130:208-269, 1974.

34. Tishler, R.B , Lamppu, D.M , Park, S and Price, B.D. Microtubule-active drugs taxol, vinblastine, and nocodazole increase the levels of transcriptionally active p53. Cancer Research, 55:6021-6025, 1995.

35. Wang, C and Kurzer, M.S. Phytoestrogen concentration determines effects on DNA synthesis in human breast cancer cells. Nutrition and Cancer, 28:236-247, 1997.

36. Zava, D.T and Duwe, G. Estrogenic and antiproliferative properties of genistein and other flavonoids in human breast cancer cells in vitro. Nutrition and Cancer, 27:31-40, 1997.

37. Peterson, G and Barnes, S. genistein inhibits both estrogen and growth factor-stimulated proliferation of human breast cancer cells. Cell Growth & Differentiation, 7:1345-1351, 1996.

38. Scambia, G., Ranelletti, F.O., Panici, P.B. Quercetin induces Type-II estrogen-binding sites in estrogen-receptor-negative (MDA-MB231) and estrogen-receptor-positive (MCF-7) human breast-cancer cell lines. Int J Cancer., 54:462-66, 1993.

39. Barnes, S., and Peterson, T.G. Biochemical targets of the isoflavone genistein in tumor cell lines. Proc.Soc.Exptl.Biol.Med., 208:103-107, 1995.

40. Kuo, S-M. Antiproliferative potency of structurally distinct dietary flavonoids on human colon cancer cells. Cancer Letters., 110:41-48, 1996.

41. Pagliacci, M.C , Smacchia, M , Migliorati, G , Grignani, F , Riccardi, C and Nicoletti, I. Growth-inhibitory effects of the natural phytoestrogen genistein in MCF-7 human breast cancer cells. Eur J of Cancer, 30A:1675-1682, 1994.

42. Avila, M.A., Velasco, J.A., Cansado, J. and Notario, V. Quercetin mediates the down-regulation of mutant p53 in the human breast cancer cell line MDA-MB468. Cancer Research, 54:2424-2428, 1994.

43. Manni, A , Grove, R , Kunselman, S and Aldaz, M. Involvement of the polyamine pathway in breast cancer progression. Cancer Letters, 92:49-57, 1995.

44. Pegg, A. Polyamine metabolism and its importance in neoplastic cell growth and as target for chemotherapy. Cancer Research, 48:759-774, 1988.

TABLE 1

Table 1. Concentrations of estradiol and phytoestrogens needed to displace 50% [^{3}H]-E_2 bound to cellular or human recombinant ER

Phytoestrogen	IC_{50} concentration, nM	
	MCF-7 ER	Recombinant ER
Estradiol	5	2
Genistein	900	60
Quercetin	4000	880
Daidzein	8100	600
Biochanin A	8600	920
Kaempferol	950	> 1000

IC_{50} values for the compounds were determined from the competition binding curves, as the concentration required to give 50% inhibition of specific binding of [^{3}H]- E_2 to the ER, (calculated on the basis of total receptor peak as 100%).

TABLE 2

Table 2. Concentrations of phytoestrogens needed to displace 50% [^{3}H]-E_2 bound to type-II EBS in MCF-7 and MDA-MB-468 cells

Phytoestrogen	IC_{50} concentration, nM	
	MCF-7	MDA-MB-468
Genistein	2100	2200
Quercetin	590	35
Daidzein	>10000	>10000
Biochanin A	>10000	4000
Kaempferol	>10000	> 10000

IC_{50} values for the compounds were determined from the competition binding curves (as described in methods for type-II EBS) and was calculated as the concentration required to give 50% inhibition of specific binding of [^{3}H]- E_2 to the type-II EBS.

TABLE 3

Table 3. IC_{50} values for phytoestrogens needed for cell growth inhibition

PHYTOESTROGEN	CELL LINE	
	MCF-7	MDA-MB-468
	IC_{50}, μM	
GENISTEIN	37 ± 3.8	8.8 ± 1.6
QUERCETIN	17.3 ± 2.7	18.1 ± 1.6
BIOCHANIN A	40 ± 4.1	44 ± 10.1
KAEMPFEROL	50 ± 3.9	47 ± 2.9
DAIDZEIN	>100	>100

Effective concentration of phytoestrogens needed to inhibit the growth of breast cancer cells by 50% was calculated from growth curves obtained from thymidine incorporation assay, after 72 h of treatment. The values are the average of 2 separate experiments conducted in triplicate.

TABLE 4

Table 4. Effect of phytoestrogens on cell cycle distribution in MDA-MB-468 cells after 24 h.

PHYTOESTROGEN, 100 μM	% CELLS AT 24 H:		
	G0/G1	S	G2/M
CONTROL	70 ± 6.8	20 ± 10.2	10 ± 3.9
GENISTEIN	27 ± 4.3	3 ± 0.9	70 ± 4.3
QUERCETIN	35 ± 5.1	5 ± 2.1	60 ± 5.2
KAEMPFEROL	50 ± 4.2	20 ± 4.9	30 ± 6.5
BIOCHANIN A	70 ± 6.1	15 ± 3.32	15 ± 2.2
DAIDZEIN	60 ± 6.6	20 ± 6.2	20 ± 2.6

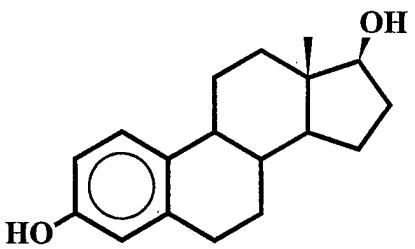
Cell cycle distribution was determined using Flow cytometry in MDA-MB-468 control and cells treated with 100 μM phytoestrogens at 24 h. Percentage of cells in each phase of the cell cycle was determined using cytological software.

TABLE 5

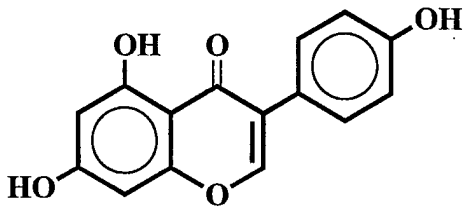
Table 5. Phytoestrogens and nocodazole treatment on cell cycle distribution in MCF-7 cells

% CELLS AT 24 H AFTER TREATMENT			
TREATMENT	G0/G1	G2/M	S
GENISTEIN			
CONTROL	68.1 ± 0.7	10 ± 2.1	21.9 ± 1.4
10 μM	62.7 ± 8.2	17.2 ± 0.07	25.9 ± 0.01
25 μM	61.1 ± 0.2	13.6 ± 1.6	25.21 ± 1.6
50 μM	55.4 ± 0.2	17.6 ± 1.6	27 ± 1.4
100 μM	40.4 ± 1.2	42.4 ± 0.9	15.5 ± 1.8
QUERCETIN			
CONTROL	61.8 ± 0.8	6.03 ± 0.3	32.1 ± 0.8
10 μM	56.64 ± 1.0	9.46 ± 1.1	33.9 ± 4
25 μM	53.49 ± 2.0	22.6 ± 1.7	23.91 ± 3.6
50 μM	50.65 ± 1.8	34.55 ± 1.6	14.8 ± 0.2
100 μM	50.5 ± 1.6	37.2 ± 2.3	12.3 ± 1.5
NOCODAZOLE			
CONTROL	56.83 ± 2.6	13.6 ± 2.3	29.6 ± 3.6
0.006 μM	49.5 ± 1.9	19.4 ± 2.1	31.7 ± 0.6
0.06 μM	53.9 ± 0.7	26.9 ± 0.5	19.1 ± 0.5
0.6 μM	13.5 ± 0.4	67.4 ± 0.4	19.05 ± 0.5
6 μM	12.6 ± 1.5	72.9 ± 0.6	14.5 ± 1.6

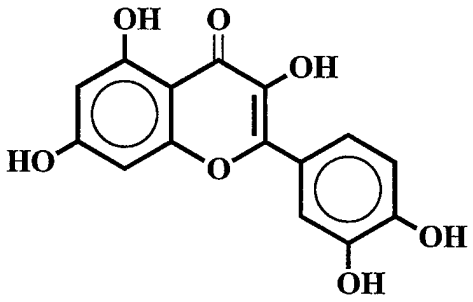
Cell cycle distribution was determined in MCF-7 cells after treatment with genistein, quercetin and nocodazole for 24 h using Flow cytometric analysis.



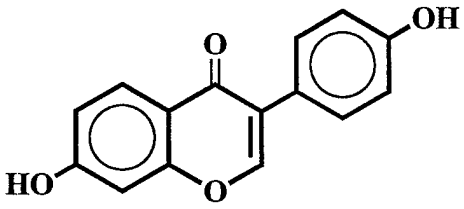
17- β-ESTRADIOL



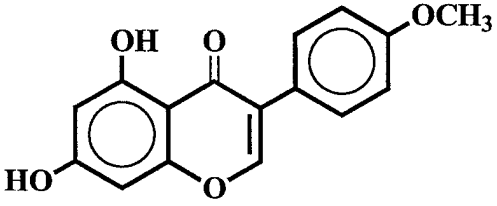
GENISTEIN



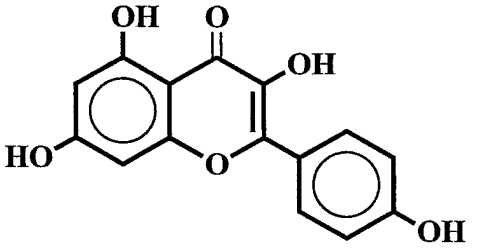
QUERCETIN



DAIDZEIN



BIOCHANIN A



KAEMPFEROL

FIG. 1. CHEMICAL STRUCTURES OF ESTRADIOL AND PHYTOESTROGENS

FIGURE 1

FIGURE 2

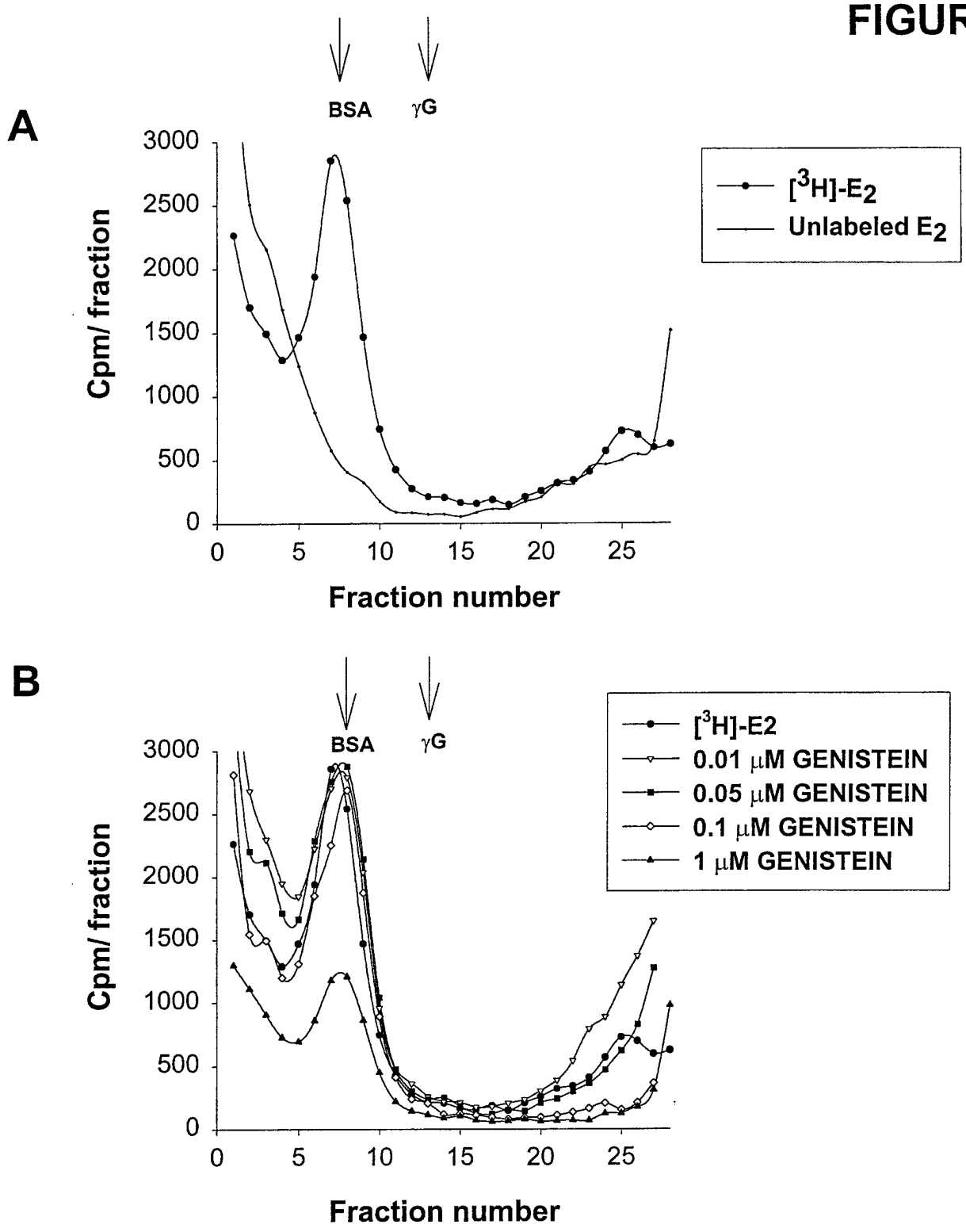
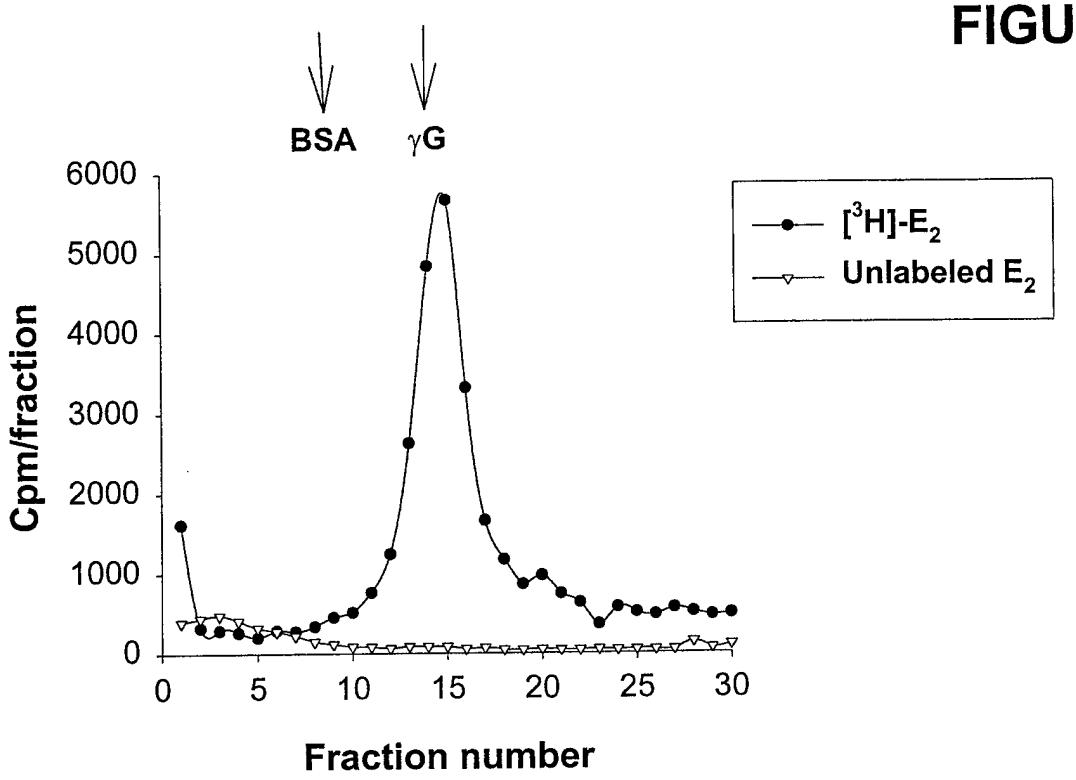


FIG. 2. Inhibition of [^{3}H]-E_2 binding to ER by genistein. Cytosol from MCF-7 cells was incubated with 5 nM [^{3}H]-E_2 alone or together with (A) 200-fold excess E_2 or (B) increasing concentrations of genistein at 4 °C for 3 h. Free hormones were removed with charcoal and hormone bound receptor was sedimented through a 10-30% linear sucrose gradient. Fractions were collected and counted. [^{14}C]-labeled bovine serum albumin (BSA, 4.5S) and [^{14}C]globulin (γG, 7S) were included as internal markers.

FIGURE 3

A



B

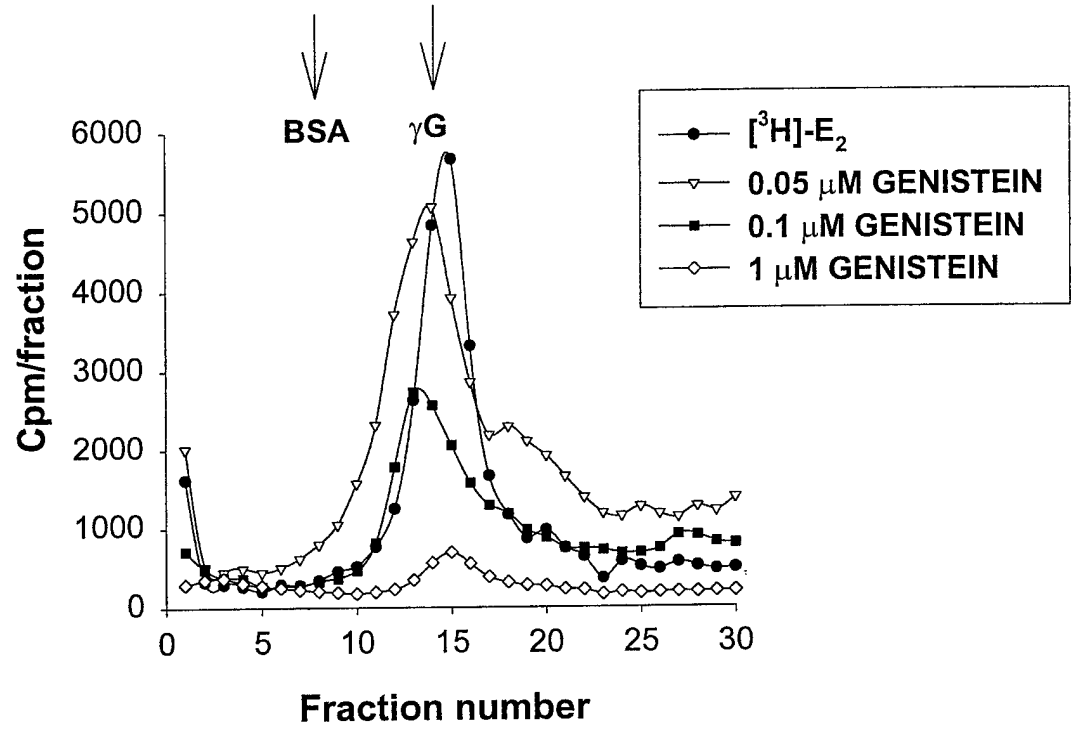


FIG. 3. Inhibition of [^{3}H]-E_2 binding to human recombinant ER. ER was incubated with radiolabeled E_2 alone or with (A) 200-fold excess unlabeled E_2 or (B) increasing concentrations of genistein and treated as described in Fig. 2. Arrows indicate sedimentation of [^{14}C]-labeled bovine serum albumin (BSA) and [^{14}C]globulin (γG).

FIGURE 4

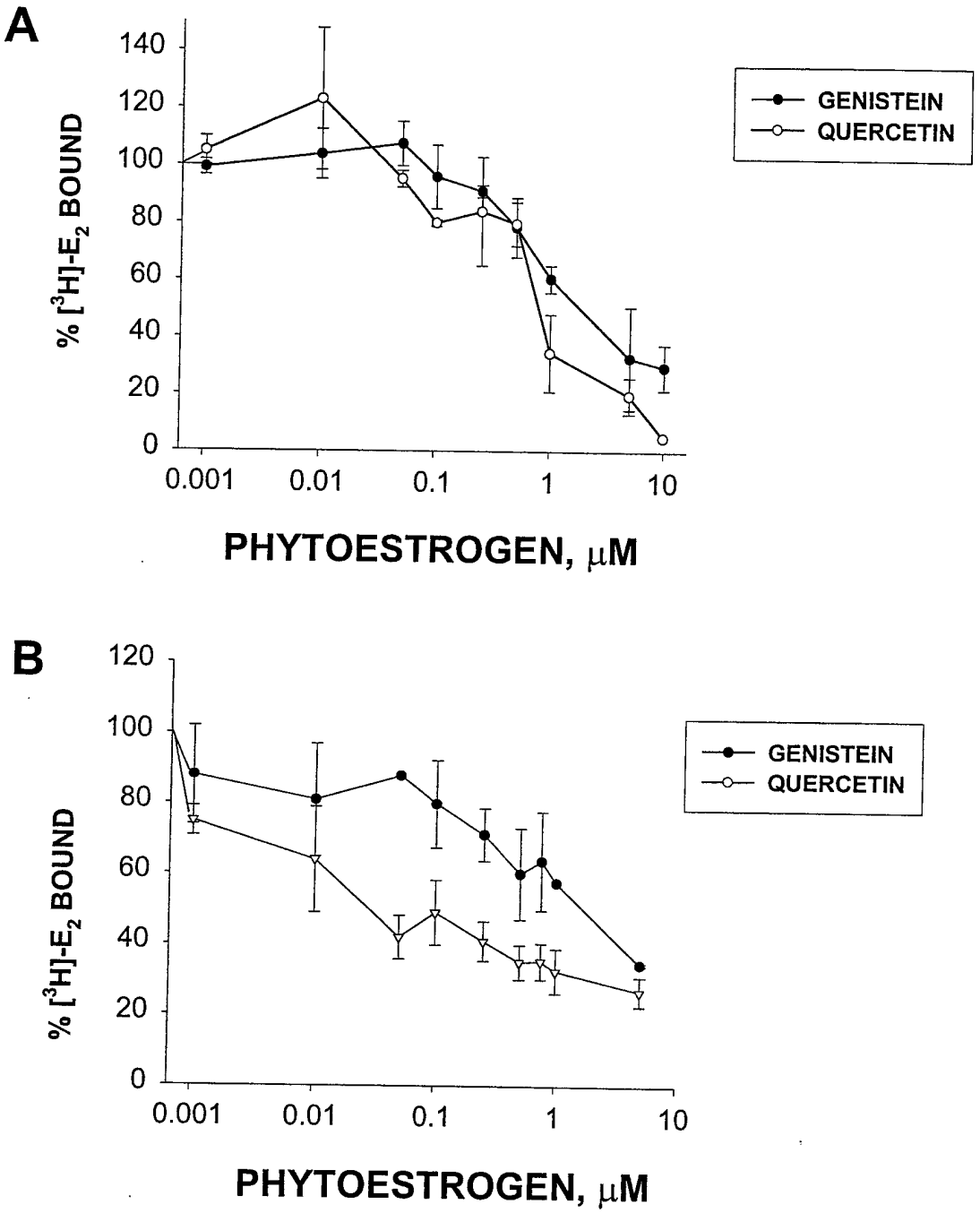
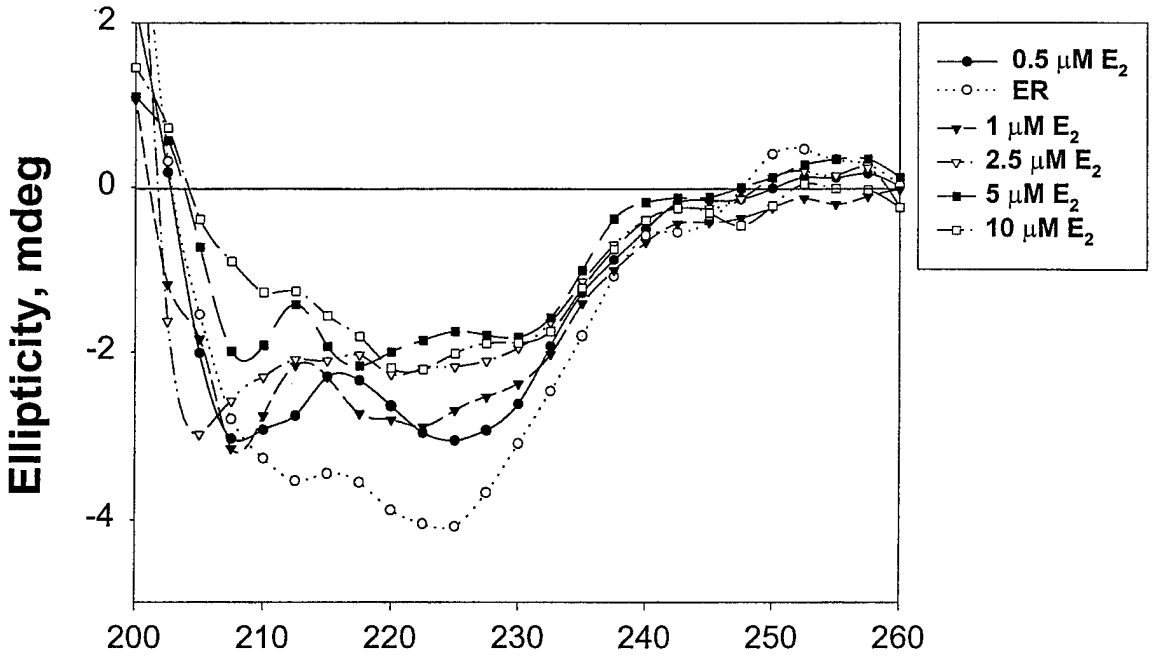


FIG 4. Competition for type-II EBS by genistein and quercetin in (A) MCF-7 and (B) MDA-MB-468 cells. Cells were incubated with increasing concentrations of phytoestrogens with or without 40 nM or 30 nM [^{3}H]-E_2, respectively for 3 h. Type-II EBS were estimated as described in Methods. Non-specific binding was determined using 200-fold excess DES. Results are expressed as per cent [^{3}H]-E_2 bound in the absence (100%) or presence of the phytoestrogen.

FIGURE 5

A



B

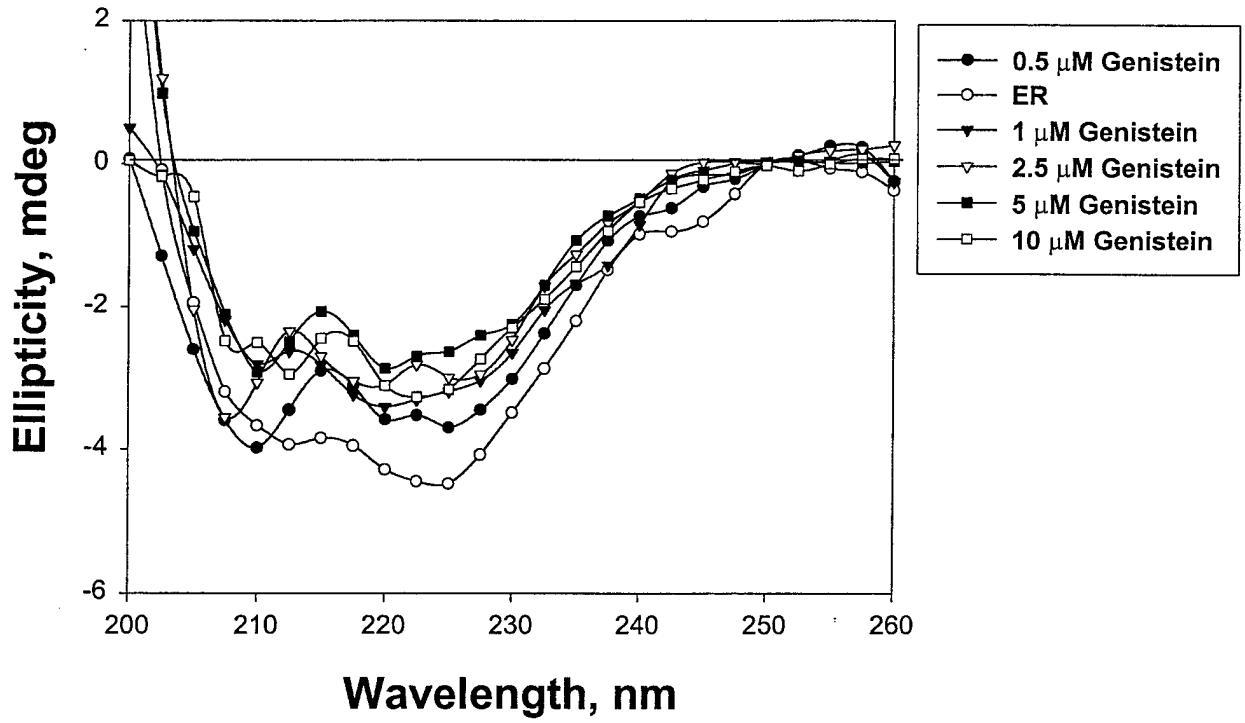


FIG. 5. Circular dichroism spectra of human recombinant ER in the presence of increasing concentrations of (A) E_2 and (B) genistein at, 25 °C. The ER was incubated in the storage buffer overnight at room temperature. The spectra were corrected for the contribution from the buffer.

FIGURE 6

EFFECT OF GENISTEIN ON THE GROWTH OF MCF-7 AND MDA-MB-468 BREAST CANCER CELLS

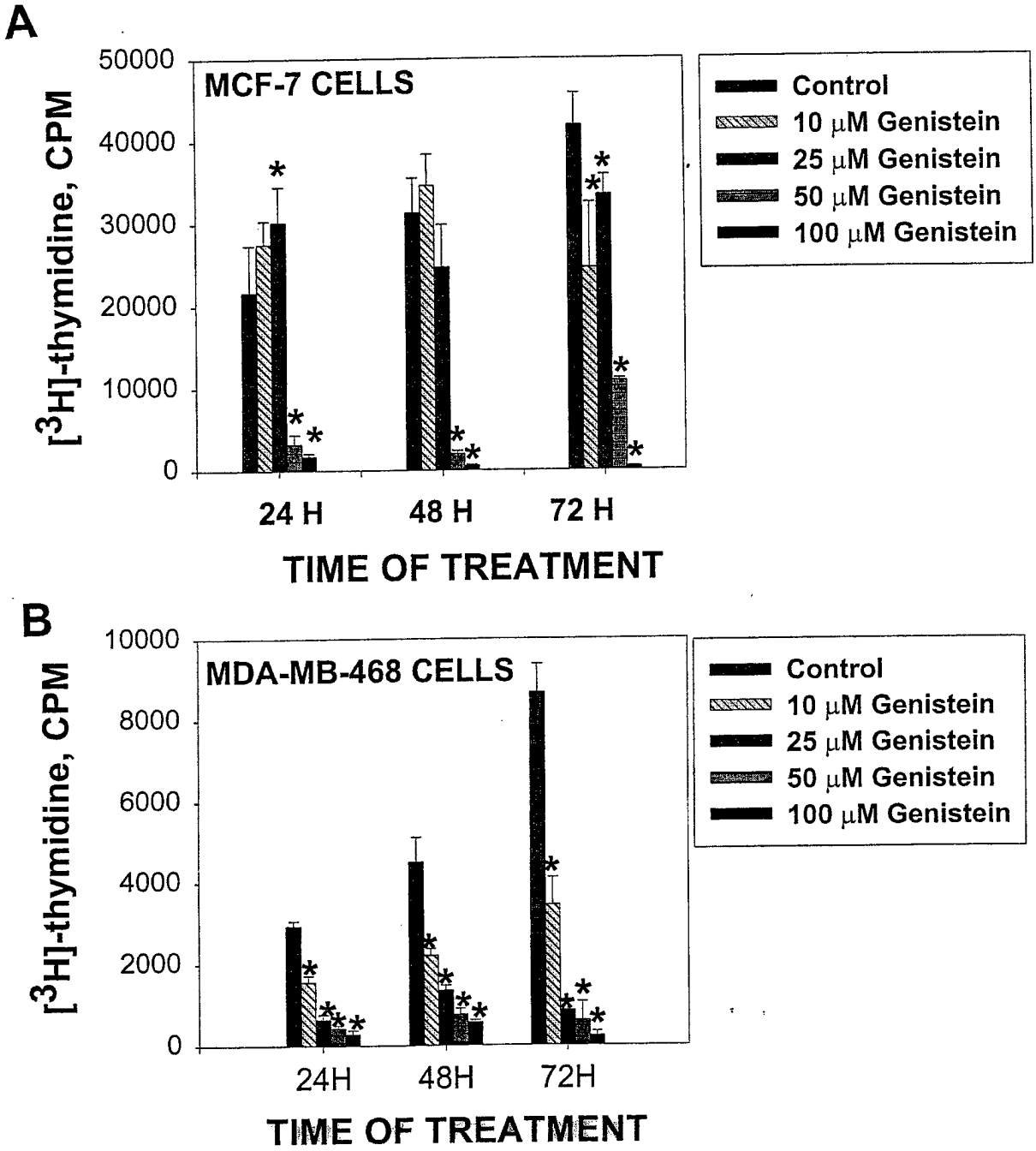


FIG. 6. Breast cancer cell growth was determined based on the amount of DNA synthesized, by thymidine incorporation assay. Cells were treated with genistein for 24, 48 and 72 h respectively in triplicates. One hour before the end of the treatment, cells were pulsed with 1 μCi/ml radioactive thymidine. Radioactivity incorporated into the cellular DNA was measured by scintillation counting. Points are average of two separate experiments conducted in triplicate. Bars are SD. * Significantly different from control, $p<0.05$.

EFFECT OF GENISTEIN ON CELL CYCLE DISTRIBUTION IN MDA-MB-468 HUMAN BREAST CANCER CELLS

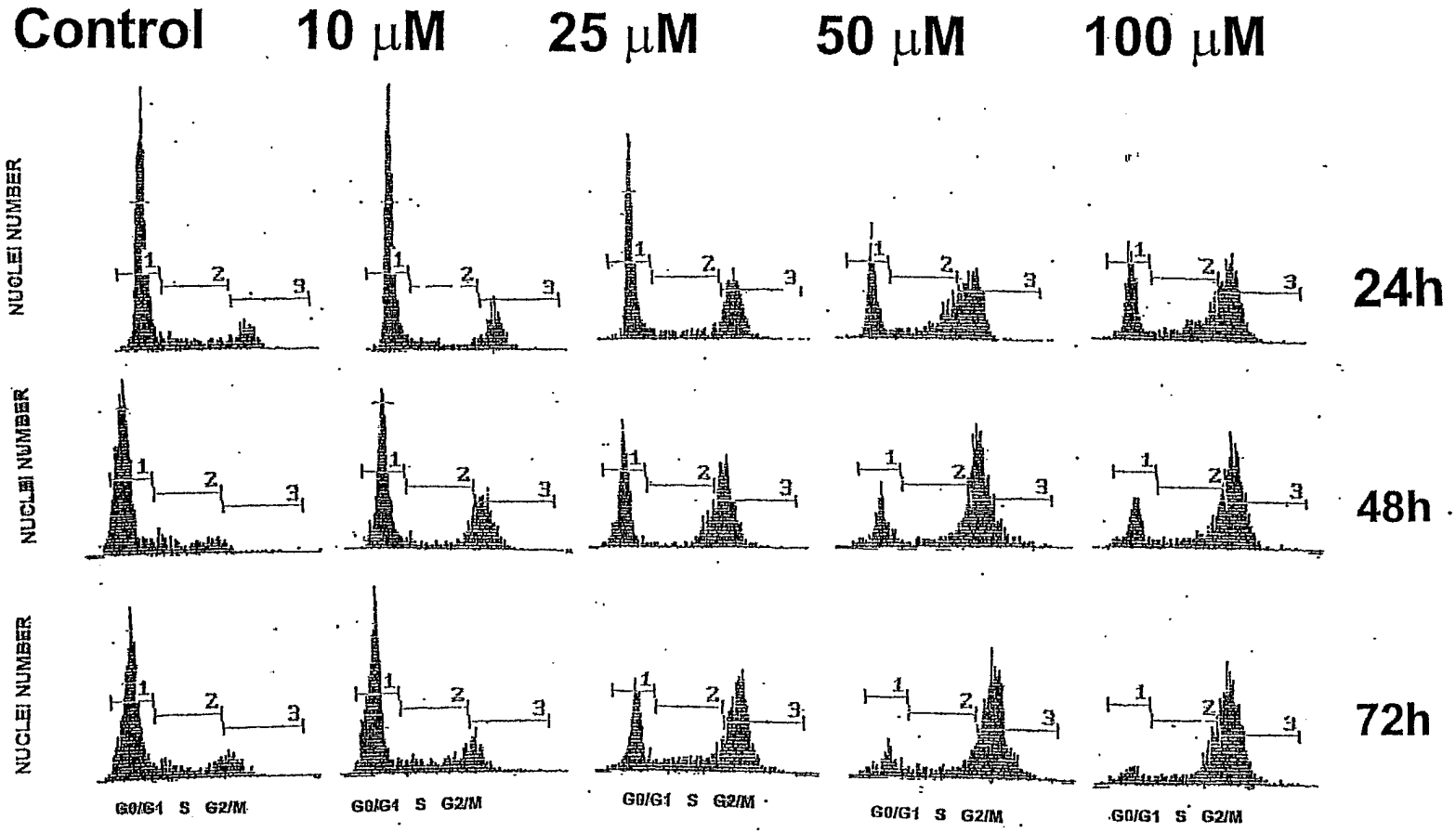


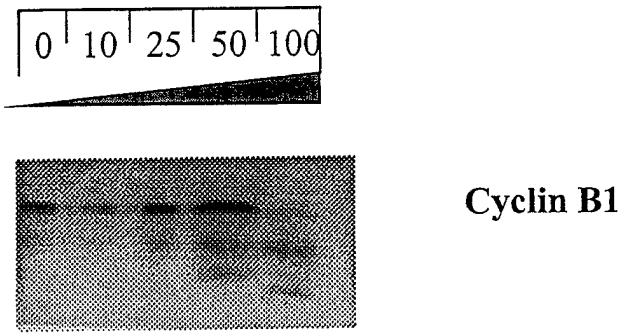
FIG 7. Flow cytometric analysis showing cell cycle distribution (%) of MDA-MB-468 cells after treatment with 0, 10, 25, 50 and 100 μM concentrations of genistein for 24, 48 and 72 h respectively. Percentage of DNA in G0/G1, S and G2/M phases are depicted.

FIGURE 7

FIGURE 8

EFFECT OF GENISTEIN AND NOCODAZOLE ON CYCLIN B1 LEVELS IN MCF-7 CELLS

A



B

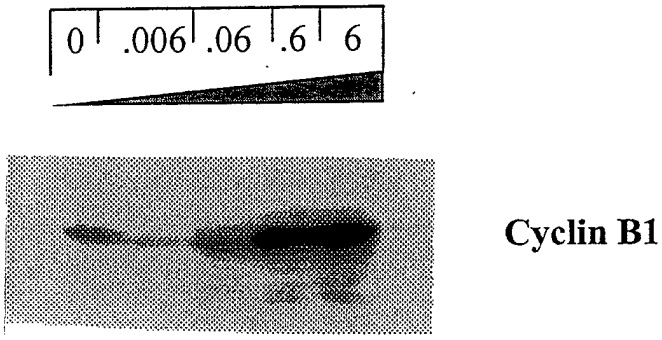


FIG 8. Western blots showing cyclin B1 levels in MCF-7 cells dosed with 0, 10, 25, 50, 100 μM genistein (Panel A) and 0, 0.006, 0.06, 0.6 and 6 μM nocodazole (Panel B) for 24 h.

FIGURE 9

EFFECT OF GENISTEIN AND QUERCETIN ON CYCLIN B1 LEVELS IN MDA-MB-468 CELLS

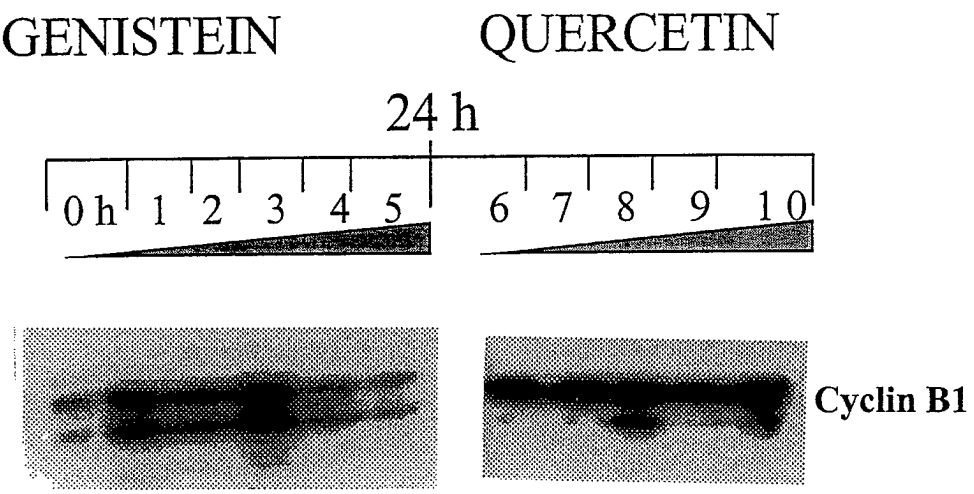


FIG 9. Western blots showing cyclin B1 levels after treatment with genistein at 0, 10, 25, 50,-100 μM concentrations (lanes 1 though 5, respectively) or quercetin at 0, 10, 25, 50 and 100 μM concentrations (lanes 6-10, respectively), in MDA-MB-468 cells. 0 h represents control cells at time zero.

FIGURE 10

EFFECT OF GENISTEIN ON ODC ACTIVITY IN MCF-7 CELLS

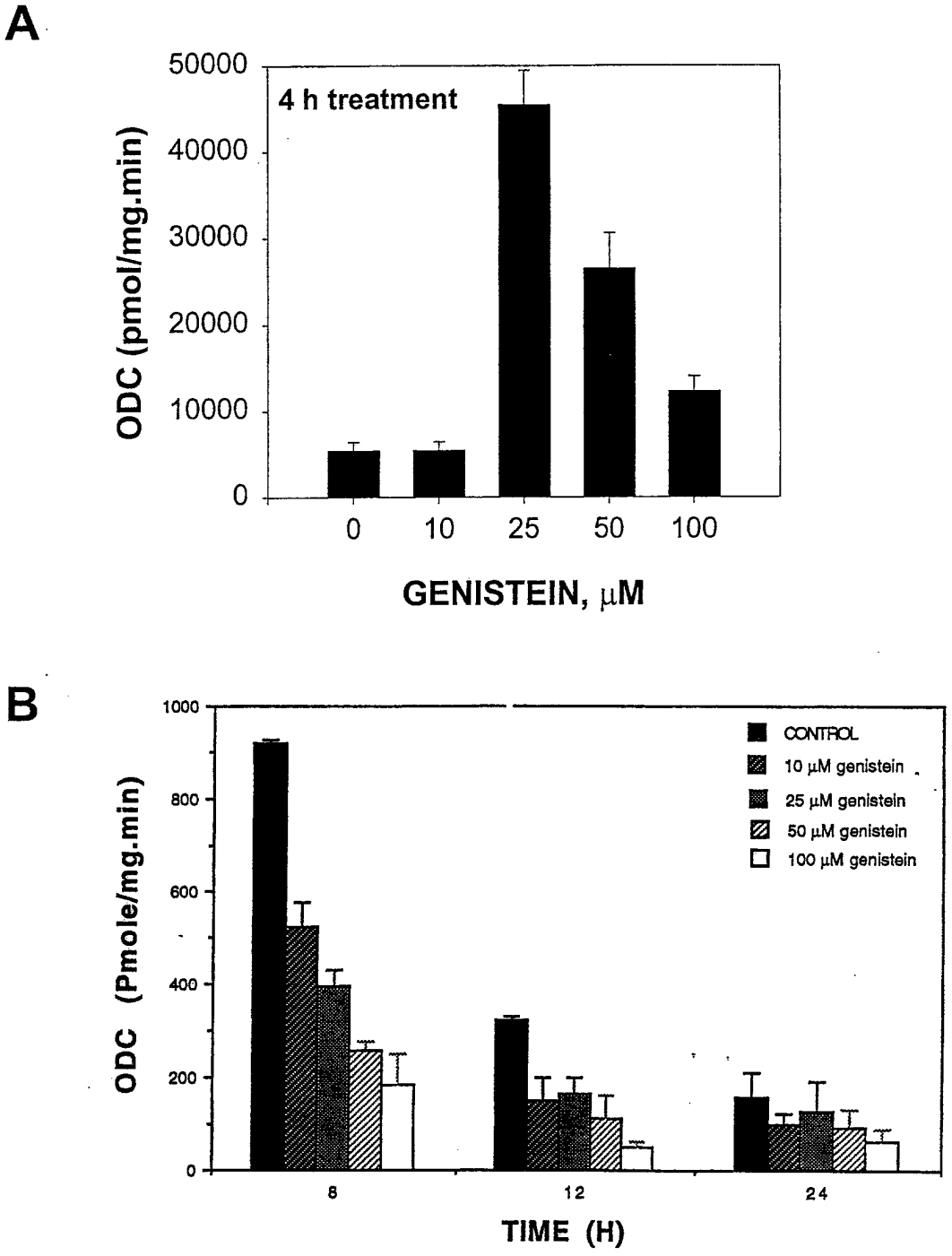


FIG 10. MCF-7 cells were treated with genistein for 4 h (Panel A) and for 8, 12 and 24 h (Panel B). The cells were then harvested and ODC activity determined as described in Methods. Results are an average of triplicate experiment. Bars represent SD.

EFFECT OF GENISTEIN TREATMENT ON INTRACELLULAR POLYAMINE LEVELS IN MCF-7 CELLS

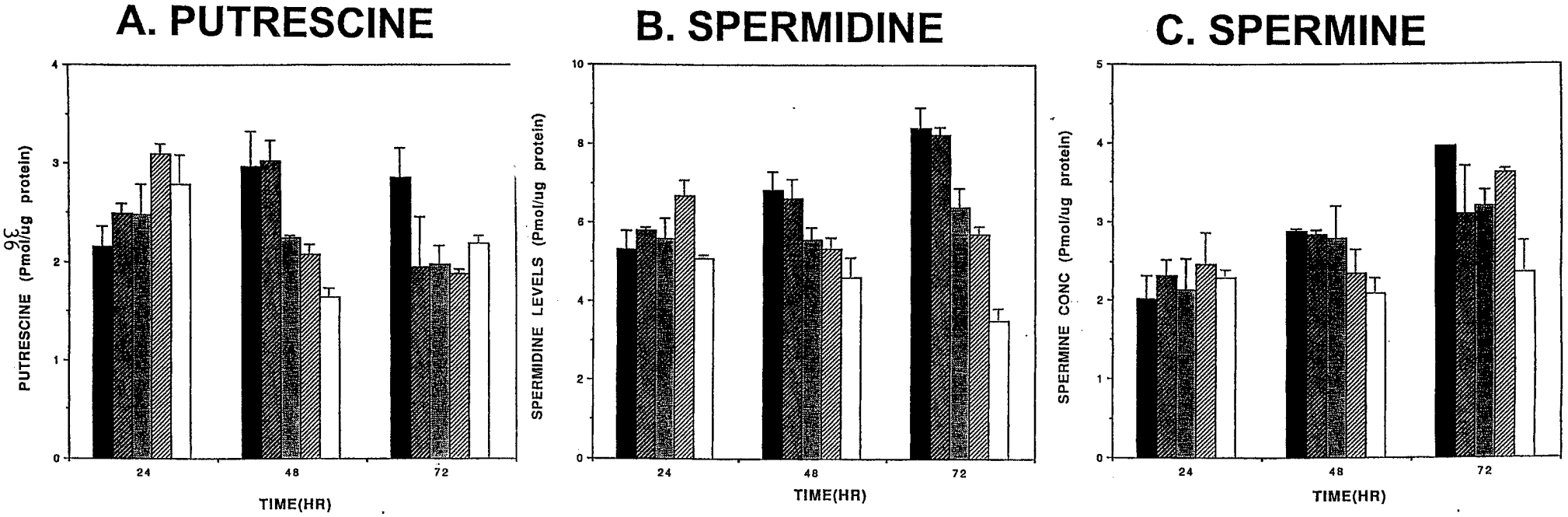


FIG 11. MCF-7 cells were treated with 0, 10, 25, 50 and 100 μM genistein for 24, 48 and 72 h. The cells were harvested after the appropriate time and Putrescine (Panel A), Spermidine (Panel B) and Spermine (Panel C) levels were determined as described in Methods. The results are an average of 2 separate experiments conducted in triplicate. Bars represent SD.

FIGURE 11

APPENDIX 1

Acronym and symbol definitions in alphabetical order

CD	Circular dichroism
DCC	Dextran coated charcoal
DES	Diethyl stilbestrol
DMEM	Dulbecco's minimum essential medium
DMSO	Dimethyl sufoxide
EBS	Estrogen binding sites
ER	Estrogen receptor
E2	Estradiol
FBS	Fetal bovine serum
HPLC	High performance liquid chromatography
MEM	Minimum essential medium
ODC	Ornithine decarboxylase
$[^3H]$-E_2	Tritiated estradiol
rER	Recombinant estrogen receptor

#2644 The effect of dietary oltipraz and genistein on DNA adducts induced by the potent mammary carcinogen dibenzo[*a,l*]pyrene in female Sprague-Dawley rats. Smith, W.A., Arif, J.M. and Gupta, R.C. *University of Kentucky, Lexington, KY 40536.*

Oltipraz a synthetic dithiolethione and genistein an isoflavone and component of soy products have both been shown to exhibit potential chemopreventive properties *in vitro* and *in vivo*. In this study we have examined the effect of these two suspected anticarcinogens *in vivo* on DNA adducts induced by the environmental carcinogen, dibenzo[*a, l*]pyrene (DBP). Female S/D rats were provided dietary oltipraz (500 mg/kg diet), genistein (100 mg/kg diet) or control diet for 7 days prior to a single oral dose of DBP (2.2 mg/kg, b.w.). The animals remained on this diet for 5 days post-DBP dosing and were euthanatized at this time. Treatment with DBP resulted in the formation of 1 major and at least 6 minor DNA adducts in the mammary gland and lung while several additional adduct spots were detected in the liver. Adducts were the highest in the mammary gland with a level of 121 ± 46 adducts/10^9 nucleotides while lung and liver adducts were 2.5–3 fold lower with levels of 44 ± 13 and 48 ± 16 adducts/10^9 nucleotides, respectively. Intervention with oltipraz or genistein produced no qualitative differences in the DNA adduct pattern and had similar effects on individual adduct levels therefore, we report only on total binding. Oltipraz inhibited DBP-DNA adducts in all three tissues by 35–48% but was most effective in the liver. Genistein, however, did not inhibit DBP-DNA adducts in the mammary gland and in fact moderately enhanced adduct levels in both the lung (27%) and liver (35%). This study indicates that oltipraz may be an effective anticarcinogen during the pre-initiation stages of carcinogenesis while genistein most likely exerts its chemoprotective effects at later stages of cancer development.

#2645 Inductions of p53, p21, and MDM2 are associated with G2/M arrests in metastatic melanoma cells treated with genistein in vitro. Rauth, S., Boddie, E., Pezzuto, J., Pisha, E., Green, A. *Dept. of Surgical Oncology, University of Illinois at Chicago, IL 60612 USA.*

Genistein,a major isoflavone of soybeans, inhibits growth and induces terminal cell differentiation in many tumor cell types. Our recent studies show that genistein significantly inhibits growth and induces differentiation in metastatic melanoma cells. We also show that sensitivity of the cells to genistein's action depended on cellular p53. Based on these observations and the report that genistein treatment causes single- and double-strand breaks in DNA, we hypothesized that p53 plays a role in genistein's action. In the present study, we sought to determine whether p53, p53-mediated p21, and MDM2 induction occurs in the DNA damage response pathways leading to cell cycle arrest following genistein treatment. Metastatic melanoma cell lines UISO-MEL-2 and UISO-MEL-4, which contain p53 with no mutations in exons 5 thru 8 of the gene, were used in our study. The results showed that genistein induced p53, p21, and MDM2 in a dose- and time-dependent manner. Inductions of p53 and its downstream effector genes were associated with cell cycle arrest at G2/M checkpoint.
(Supported by American Cancer Society National Grant)

#2646 The natural tyrosine kinase inhibitor genistein inhibits growth and adhesion of sarcoma cell lines in vitro. Rauth, S., Green, A., Das Gupta, T.K., and Bornstein, A. *Dept. of Surgical Oncology, University of Illinois at Chicago, IL 60612 USA.*

Genistein (4',5,7 trihydroxyisoflavone) demonstrated strong growth inhibitory effects against several cancers both *in vitro* and *in vivo*. Our recent studies showed that it inhibited growth and induced differentiation in metastatic melanoma cell lines. We also showed that the chemosensitivity of the cells depended on the status of cellular p53. In the present study, we investigated genistein's effects on sarcoma cell lines in culture. The fibrosarcoma cell line HT1080 (p53-negative), the osteosarcoma cell line SAOS-2 (p53-negative), and the Ewing's sarcoma cell line UISO-ES3 (p53-positive) were used in our study. Those cells were treated with different concentrations of genistein for different time periods. The results showed that genistein significantly inhibited cell adhesion and growth in a dose- and time-dependent fashion. Treatment with 100 μM of genistein resulted in complete inhibition of cell adhesion and growth in 24 hrs. Exposure to 200 μM concentrations resulted in loss of adhesion of 50% of the cells at 2 hrs., and, by 6 hrs. after treatment, more than 95% of the cells were detached. The molecular mechanisms by which genistein mediates its effects in sarcoma cell types remain to be elucidated.
(Supported by American Cancer Society National Grant)

#2647 Protection against DMBA-induced breast cancer in female rats by lifetime soy consumption. Hakkak, R., Korourian, S., Ronis, M.J., Irby, D., Kodlaya, S., Shelnutt, S.R., and Badger, T.M. *Arkansas Children's Nutrition Center, Departments of Pediatrics and Pathology, University of Arkansas for Medical Sciences, and Arkansas Children's Hospital Research Institute, Little Rock, AR 72202.*

Breast cancer is one of the leading causes of cancer death among women. Epidemiologic studies have suggested a relationship between diets high in soy-products and a low incidence of breast cancer. It has been demonstrated that neonatal injection of genistein, the major isoflavinoid phytoestrogen found in soy products, results in chemoprotection against DMBA-induced mammary carcinogenesis in the rat model. Since most of the soy consumption is through diet, it is important to know if soy diets have similar effects as injections of purified isoflavone. In the present study, female Sprague-Dawley were maintained on AIN-93 diet which sources of the protein were casein (control) or Isolate Soy Protein four weeks prior to breeding and continued throughout the study. At day 50 of age, all female pups from both diets received via gavage 80 mg/kg dimethylbenz[a]anthracene (DMBA). All rats were palpated twice weekly for mammary tumors and they were killed when 100% rats on the casein diet developed tumors. Rats on the soy diet took a longer period of time for mammary tumors to develop compared to rats on the casein diet (2 weeks). At end of the experiment rats on soy diet had an 84% incidence of tumors compared to 100% of rats on the casein diet. The number of tumors per rat on soy diets were less than (5.74 Vs 3.81) rats on casein diet. These results suggest that dietary soy consumption can protect against the development of DMBA-induced mammary tumors. Supported by USDA A256251-5100-001-025.

#2648 Genistein treatment causes G2/M cell cycle arrest and cyclin B1 accumulation in MCF-7 breast cancer cells: A possible mechanism for the preventive action of genistein. Balabhadrapathruni, S., Thomas, T., Yurkow, E., and Thomas, T.J. *UMDNJ-Robert Wood Johnson Medical School, and Rutgers Univ., New Brunswick, NJ 08903.*

Genistein (4',5,7-trihydroxyisoflavone) is a constituent of soy foods, the consumption of which is believed to contribute to the low incidence of breast cancer in oriental women. In order to understand the mechanism of action of genistein, we studied the effects of genistein on long-term cell growth, cell cycle phase kinetics, and cyclin B1 accumulation using MCF-7 breast cancer cells. Genistein inhibited cell growth in a dose-dependent manner, with an IC_{50} value of 50 μM as determined by total DNA content over a period of 6 days. At 100 μM concentration, genistein inhibited cell proliferation by ~90%. Flow cytometric analysis of cells treated with 100 μM genistein for 24 h showed a 3-fold increase in the percentage of cells in G_2/M phase (47%) of cell cycle. Western immunoblot analysis showed a 2- to 3-fold increase in the level of cyclin B1 protein in cells treated with genistein compared to controls at the 16 h time point. These data suggest a possible mechanism for the action of genistein involving mitotic arrest due to an accumulation of cyclin B1 in breast cancer cells. Future studies will be directed to understand the nature of genistein interaction(s) with cyclins and cyclin/CDK complexes.

This work was supported by a pre-doctoral Fellowship from the U.S. Army's Breast Cancer Research Program (DAMD17–97-1–7035).

#2649 Role of BTG2 and possibly p53 in growth inhibition by genistein in LNCaP, PC3, and DU145 human prostate cancer cells. Davies, J.A., Walden, P., and Bosland, M.C. *Departments of Environmental Medicine and Urology, NYU Medical Center, New York, NY 10016.*

The low prostate cancer rates in Asian countries may be attributable to high dietary intakes of soy. The major soy isoflavone genistein inhibits growth of human prostate cancer cell lines LNCaP, PC3, and DU145. We examined the mechanism of these growth inhibiting effects of genistein at non-cytotoxic (dye-exclusion) concentrations of 1.85–93 μM; genistein serum levels in Japanese consuming traditional diets are approx. 5 μM. Tritiated thymidine uptake was reduced dose-relatedly at all concentrations in all 3 lines. Genistein induced apoptosis in LNCaP cells (TUNEL assay). This was confirmed by flow cytometry, with 35% of cells being hypodiploid after 72 h exposure to a high dose of genistein. In contrast, genistein caused a partial G2/M block in PC3 and DU145 cells. The PC3 and DU145 lines have inactivating p53 mutations, but LNCaP cells express wild-type p53 which, in response to cellular stresses, transcriptionally activates genes promoting G1 arrest and apoptosis. Genistein, similar to etoposide as positive control, increased expression (Northern blot) in LNCaP cells of the antiproliferative p53 target gene BTG2 in a dose-related fashion with a maximum after 2 h exposure. PC3 and DU145 cells did not express BFTG2. These data suggest involvement of p53 in growth inhibition of LNCaP but not PC3 or DU145 cells by genistein. (Supported by CA72290 and CaP CURE.)

#2650 Potential chemopreventive properties of glutathione conjugate of benzylselenocyanate against colon carcinogenesis in rats. Reddy, B.S., Kawamori, T., Rosa, J., and El-Bayoumy, K. *American Health Foundation, Valhalla, NY 10595.*

The major goal of this study was to develop organoselenium compounds with maximal chemopreventive efficacy yet lowest possible toxicity. The chemopreventive efficacy of selenium compounds depend on the form in which they are administered, suggesting that their metabolism is important in exerting their biological effects. Our preliminary results indicate that glutathione conjugates of chemopreventive organoselenium compounds, benzylselenocyanate (BSC) and 1,4-phenylenebis (methylene)selenocyanate (*p*-XSC) are putative metabolites that may be responsible for their biological effects. The present study was designed to evaluate the potential chemopreventive activity of glutathione conjugate of BSC (BSe-SG) against colonic aberrant crypt foci (ACF) which are premalignant lesions. BSe-SG and BSC were synthesized in our laboratory. Male F344 rats were fed the control and experimental diets containing 10 ppm BSC (4.1 ppm Se) and 10 and 20 ppm BSe-SG (1.8 and 3.6 ppm Se) starting one week prior to the first of two weekly s.c. injections of azoxymethane (15 mg/kg body wt.) and until the animals were killed 8 weeks later for ACF evaluation. Administration of BSC and BSe-SG significantly suppressed the colonic ACF. Additionally, the degree of inhibition of ACF is more pronounced with BSe-SG in a dose-dependent manner

The 1998 Annual Retreat on Cancer Research in New Jersey

May 29, 1998

GALLO AWARD

Presented by

The Cancer Institute of New Jersey

For Outstanding Cancer Research

Srivani Balabhadrapathruni

Research Titled

Genistein Treatment Causes G_2/M Cell Cycle Arrest and Cyclin B1 Accumulation in Breast Cancer Cells: A Possible Mechanism for the Preventive Action of Genistein

T. S. Ravikumar, MD
Associate Director for Education and Training
The Cancer Institute of New Jersey
Co-Chair, The 1997 Annual Retreat on Cancer Research in New Jersey

William N. Hait, MD, Ph.D.
Director, The Cancer Institute of New Jersey
Professor of Medicine & Pharmacology
UMDNJ - Robert Wood Johnson Medical School