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

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Investigations during the past year focused on growth inhibitory effects of phytoestrogens and their role as weak estrogens in breast cancer cell growth. Genistein, biochanin A, kaempferol and daidzein had growth stimulatory effects in ER-positive MCF-7 breast cancer cells at 10 μ M. To understand the estrogenic effects of genistein, we measured its interaction with human recombinant ER α and β . ER α or β bound to [³ H]-E ₂ showed a 6S dimeric form in sucrose gradients, whereas both receptors labeled with [¹⁴ C]-genistein sedimented as the 4S monomeric form. In EMSA experiments, we found that genistein facilitated ER β binding to ERE. In contrast, genistein did not have a significant effect on ER α -ERE binding. Northern blot analysis showed a slight decrease in the level of ornithine decarboxylase mRNA at 8 h with 100 μ M genistein treatment. Genistein and quercetin inhibited ER-negative MDA-MB-468 breast cancer cell growth with IC ₅₀ values of 8.8 and 18.1 μ M, respectively. The other compounds were less effective. The mechanism of growth inhibition by genistein and quercetin involved G ₂ /M cell cycle arrest, changes in cyclin B1 levels and apoptosis. Our results indicate that genistein and quercetin may be useful in the treatment of ER-negative tumors. Results of our studies on MDA-MB-468 cells have been documented and submitted for publication.						
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FOREWORD

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(5) INTRODUCTION

Phytoestrogens are abundantly available from plant-based diets and are considered to be the protective factors in reducing cancer risk in vegetarians (1). Phytoestrogens used in this study (genistein, quercetin, kaempferol, daidzein and biochanin A) have been reported to reduce cancer cell growth of various origins in experimental studies (2-5). In breast cancer cells, the action of these compounds varies with the estrogen receptor (ER) status. In my previous report, we showed that genistein, quercetin, kaempferol, biochanin A and daidzein bind to the MCF-7 cellular and recombinant ER with varying affinities compared to estradiol, as determined by sucrose gradient analyses. Genistein was found to have the highest affinity to both cellular and recombinant ER among all the compounds.

Estradiol is known to induce and promote cancer growth, particularly of breast tissue (6). ER binds to estradiol with high affinity, although it binds to other structurally related compounds with low affinity (7). Estradiol binding induces a conformational change in the ER, allowing it to form a dimeric complex which is capable of binding to the consensus estrogen response element (ERE) and regulate transcription of growth related genes (8, 9). Two subtypes of human ER, ER α and ER β , encoded by two distinct genes have been described (10, 11). These proteins have 95% homology in their DNA binding domain and 55% homology in the ligand binding domain. In addition, each protein has specific tissue based expression and has been reported to bind DNA either as homo- or heterodimers. Phytoestrogens with differential affinity to ER α and ER β appear to produce ligand- and tissue-specific responses (7). Phytoestrogens are thought to interfere with any of the cascade of events starting with estradiol binding to ER, in generating their estrogenic (growth stimulatory) or antiestrogenic (growth inhibitory) effects in breast cancer cells.

In the estrogen-induced breast cancer development, it is proposed that a critical event is the dysregulated gene expression, which confers a proliferative advantage to the ER-positive cells. Estradiol stimulates a rapid and transient increase in both c-*myc* and ornithine decarboxylase (ODC) transcription in ER-positive MCF-7 cells (12, 13). The c-*myc* oncogene is involved in the stimulation of cell proliferation and is amplified in 30-45% of the breast tumors (14). ODC is the first enzyme in the biosynthesis of polyamines, the cellular cations involved in cell growth and differentiation. Increased levels of ODC and polyamines are observed in several types of cancers (15).

In order to understand the role of genistein as ligand for ER, we studied the effects of genistein binding on ER structure and ER-ERE binding. Ligand receptor interactions were examined by sucrose density gradients using [¹⁴C]-genistein. ER-ERE interactions were evaluated by electrophoretic mobility shift assays (EMSA). In addition, we also measured the effect of genistein on expression of the *c-myc* and ODC mRNA using Northern blot analysis.

In order to understand the mechanism of growth inhibition of phytoestrogens independent of ER, we determined the effects of these compounds on growth, cell cycle kinetics, cell cycle regulatory proteins and apoptotic cell death of MDA-MB-468 cells.

Our efforts in the past two years revealed specific effects for each phytoestrogen on the growth, cell cycle regulatory mechanisms and apoptotic cell death of estrogenreceptor (ER)-negative MDA-MB-468 breast cancer cells. Based on our results, we submitted a manuscript for publication (copy attached). Also, we gained some insight into the estrogenic effects of genistein. The chemical structure of estradiol and phytoestrogens used in this study is given in Figure 1.

(6) BODY

A. METHODS

1. Cell Culture and Chemicals. MCF-7 cells are maintained in Dulbecco's Modified Eagles Medium (DMEM) with 100 μ g/ml penicillin, 100 μ g/ml streptomycin, 2 μ g/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 (16). FBS was treated with dextran coated charcoal (DCC) to remove endogenous estrogens and added to the medium as described previously (17). MDA-MB-468 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, Virginia). The cells were maintained in Improved minimum essential medium (IMEM) with 10% fetal bovine serum (FBS), 4 mM glutamine, 0.4 mM sodium pyruvate, 40 μ g/ml gentamycin, and 100 μ g/ml each of penicillin and streptomycin.

Genistein, quercetin, daidzein, kaempferol and biochanin A were purchased from Sigma Chemical Co. (St. Louis, MO). Stock solutions were made in dimethylsulfoxide (DMSO), aliquoted, and frozen until use. The APO-BRDUTM assay kit was purchased from Pharmingen (San Diego, CA). The antibodies for cyclin B1, C-terminal domain of cdc2 and β -actin were obtained from Neomarkers (Union City, CA).

2. Electrophoretic Mobility Shift Assay to Determine Binding of Genistein-ER

Complex with ERE. The consensus sequence of ERE (5'

CCAGGTCAGAGTGACCTGAGCTAAAATAACACATTCAG) from vitellogenin gene was purchased from Oligos, Etc. (Wilsonville, OR). The oligonucleotide and its complementary strand were dissolved in 10 mM Tris-HCl (pH 8.0), 200 mM NaCl and equimolar solutions were boiled for 10 min and allowed to anneal for 2 h at room temperature. Then the oligonucleotide solution was dialyzed thrice against the same buffer and end-labeled with ³²P- γ -ATP using an end labeling kit from Promega (Madison, WI). Human recombinant ER α or ER β , 500 ng each was incubated with different concentrations of genistein for 2 h. About 20,000 cpm of labeled probe and 4 X binding buffer were added to the ER-genistein mixture to reach a final concentration of 10 mM Tris-HCl, 10% glycerol, 1 mM dithiothreitol and 10 µg/ml poly(dl-dC).poly(dl-dC), (Pharmacia, Piscataway, NJ). The binding reaction was allowed to proceed for 1 h at 4°C and 30 min at room temperature. Then the samples were loaded in a 6% polyacrylamide gel and electrophoresed for 3 h at 100 V. The gel was dried and exposed to Kodak Biomax MR-1 film for autoradiography for 3 to 6 h. Intensity of the DNA-protein complex was quantified using a scanning densitometer.

3. Sucrose Density Gradient Analysis of ER α and ER β bound to[¹⁴C]-genistein.

Purified ER α or ER β was incubated at 4°C for 3 h with either [¹⁴C]-genistein or [³H]-E₂.

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. 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 with the receptor incubated with [¹⁴C]-genistein or [³H]-E₂, with 100-fold excess cold genistein or 200-fold excess cold E_2 , respectively.

<u>4. [³H]-Thymidine Incorporation Assay.</u> Cells (0.5 x 10⁶/dish) 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 μ Ci/ml of [³H]-thymidine was added to the cells. The radioactivity incorporated into the cellular DNA was measured by liquid scintillation counting.

5. Northern Blot Analysis of ODC and c-*myc* mRNA. Total RNA was extracted using the TRI reagent (Molecular Research Inc., Cincinnati, OH). RNA concentration was determined by measuring absorbance at 260 nm and converted to μ g/ml, using the equation, one Absorbance unit (A260) = 40 μ g/ml. Twenty μ g of RNA was loaded into each well and RNA molecules were separated under denaturing conditions on 1% agarose gel, electrophoresed for 24 h. RNA from the gel was transferred onto a nylon membrane (Oncor, Gaithersburg, MD). Prior to hybridization, the membrane was preconditioned with hybrisol for 1 h at 42°C. Overnight hybridization was carried out with cDNA probe for c-*myc* or ODC labeled with ³²P- α -dCTP (~10⁶ cpm/ml of hybridization solution). The membranes had 3 x 15 min washes in 0.1% SSC/0.1%SDS at room temperature and 1 h in 0.1 X SSC/0.1% SDS at 52°C. Membranes were then exposed to Kodak X-OMAT AR film at -70°C for 48-72 h. Relative intensities of bands were quantified using a scanning densitometer.

<u>6. Reversal of Cell Growth Inhibition.</u> MDA-MB-468 cells (0.5 x 10^6 cells/dish) were plated in 60 mm culture dishes in triplicate and were allowed to adhere for 24 h. Groups of cultures were treated with genistein for different periods (2, 4, 6, 8, 12, 16, and 24 h), and media changed to drug-free medium. Twenty four hours later, the cells were dosed with 1 µCi/ml of [³H]-thymidine for 1 h, and the radioactivity incorporated into cellular DNA was measured by liquid scintillation counting.

7. Flow Cytometric Determination of Cell Cycle Analysis. MDA-MB-468 cells (2 x 10^{6} cells/dish) were allowed to adhere to plate for 24 h and then dosed with genistein, or other phytoestrogens. After 24, 48 or 72 h of treatment, media was removed, 2 ml citrate buffer (40 mM Citrate-Trisodium, 250 mM sucrose and 5% DMSO) added and cells frozen at -70°C until further analysis. For cell cycle analysis, cells were thawed and harvested to collect the pellet. The cell pellet was treated with trypsin for 10 min and trypsin inhibitor and RNase (Sigma, St. Louis, MO) were added for 10 min and stained with 750 µl propidium iodide in citrate buffer (30 µg/ml). Cells were analyzed by

an Epics Profile-II Flow Cytometer (Coulter Corp., Miami, FL). Distribution of cells in different phases of cell cycle was calculated using CytoLogic software.

8. APO-BRDU[™] **Labeling Studies.** MDA-MB-468 cells (2 x 10⁶ cells/dish) were plated and dosed with genistein for 24 h. Floating and adherent cells were harvested in PBS and fixed in 1% paraformaldehyde. After two washings with PBS, the cell pellet was fixed in 70% ethanol and frozen at -20°C until further use. Apoptosis was quantified using the APO-BRDU[™] kit according to the manufacturer's instructions. Briefly, cell pellet was incubated with bromolated deoxyuridine triphosphate (BRDU) for 24 h in a 28°C water bath. After the incubation period, cells were treated with fluorescein-labeled anti-BRDU monoclonal antibody for 1 h, stained with propidium iodide/RNase solution for 30 min in dark, and analyzed by flow cytometry.

9. Analysis of Apoptosis by Hoechst 33342 Dye Staining. Cells $(3.5 \times 10^4/\text{dish})$ were plated in 4-well LabTek chambered cover glass plates (Nalge Nunc Int., IL). After allowing for adherence of cells for 48 h, different concentrations of genistein were added to the plates. After 24 h treatment, cells were fixed in 4% paraformaldehyde with 4 µg/ml Hoeschst 33342 dye and incubated at 37°C for 30 min. Stained nuclei were observed under Zeiss ICM 405 inverted microscope (magnification 1000 X) using a UV filter in the range of 395-450 nm. Apoptotic cells, characterized by nuclear shrinkage and fragmentation, were counted from 3 random fields with at least 100 cells per field.

10. Western Blot Analysis. MDA-MB-468 cells (2 x 10⁶ cells/dish) were plated in 100 mm dishes and were allowed to adhere for 24 h. Twenty four hours after treatment with genistein, cells were harvested in PBS, centrifuged at 500 x g for 10 min and stored at -70°C until further analysis. Cells were solubulized in 300 μ l of a buffer containing 50 mM Tris, 50 mM NaCl, 50 mM NaF, 0.2% SDS, 1% NP40, 2 mM EDTA, 100 μ M Na₃Po₄. Total protein was determined using the Bio-Rad kit (Bio-Rad, Hercules, CA) and 30 μ g was electrophoretically separated on a 10% polyacrylamide gel. The proteins were transferred to PVDF immobilon membrane. After blocking overnight with 2% nonfat dry milk, blots were incubated for 3 h with purified monoclonal mouse or polyclonal rabbit antibodies, followed by horseradish-peroxidase labeled anti-mouse/anti-rabbit secondary antibody. Protein was visualized with a chemiluminiscence based detection system.

Statistical Analysis. Statistical analyses were performed using Sigma Plot 3.0 software. Means and standard deviations were calculated for each treated group and the significant difference between the groups were determined using Student's t-test. p value <0.05 was considered significant.

B. RESULTS

I. Results of Task 4. Binding of phytoestrogen-ER complex to consensus ERE, months 13-16. In my previous experiments, I observed that phytoestrogens bind to the MCF-7 cellular and recombinant ER with varying affinities compared to estradiol. Genistein was the most effective compound among all phytoestrogens used. To determine if this binding can lead to potential estrogenic or antiestrogenic effects for genistein, we measured the binding of genistein-ER complex to the consensus ERE in EMSA experiments. We have used both recombinant ER α and ER β in our experiments. An increase in the intensity of the band corresponding to the ER β -ERE was observed in the presence of 500 nM genistein (Figure 2). In contrast, there was no significant binding of ER α -ERE in the presence of different concentrations of genistein (data not shown).

Binding of [¹⁴**C]-genistein to ER** α and ER β , months 13-16. To understand the differences in the structural and conformational state of ER bound to estradiol and genistein, we conducted sucrose density gradients in the presence of these two ligands. [³H]-E₂ and [¹⁴C]-genistein were used to monitor the sedimentation profile of ER. As shown in Figures 3A and 4A, both ER α and ER β sedimented as the dimeric 6S form in the presence of [³H]-E₂. In contrast, genistein bound to ER α or ER β sedimented as the monomeric 4S form on the sucrose gradient (Figures 3B and 4B). We also examined the sedimentation profile of a 1:1 mixture of ER α and ER β . In this case also ER bound to [³H]-E₂ sedimented as the dimeric as the dimeric form, while ER bound to [¹⁴C]-genistein sedimented as the 4S form (Figure 5).

II. Results of Task 5. Cell proliferation studies with phytoestrogens,

months 17-19. We determined the effects of the phytoestrogens on DNA synthesis of MCF-7 and MDA-MB-468 cells. Cells were treated with 0, 10, 25, 50 and 100 μ M phytoestrogens for 24 h. DNA synthesis was determined by [³H]-thymidine incorporation assay. Effect of genistein and quercetin on DNA synthesis of MCF-7 cells is presented in Figures 6A and B, respectively. Genistein treatment of 10 and 25 μ M for 24 h caused a 27% and 39% increase in DNA synthesis of MCF-7 cells, respectively. By 72 h of treatment, all doses of genistein were growth inhibitory. Biochanin A, daidzein and kaempferol increased DNA synthesis at 10 μ M concentration. However, quercetin did not have a stimulatory effect on DNA synthesis, and inhibited growth of these cells with an IC₅₀ value of 17.3 μ M. Genistein, biochanin A and kaempferol inhibited MCF-7 DNA synthesis with IC₅₀ value of 37, 40 and 50 μ M, respectively, whereas, daidzein was ineffective even at 100 μ M concentration (Table 1).

Effect of genistein and quercetin on DNA synthesis of MDA-MB-468 cells is given in Figures 7A and B, respectively. 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 IC₅₀ value for the phytoestrogens in inhibiting the growth of MCF-7 and MDA-MB-468 cells are presented in Table 1. Genistein was more effective than quercetin in inhibiting the growth of ER-negative cells. Biochanin A and kaempferol exhibited similar effects on growth inhibition in ER-positive as well as ER-negative cells, where as daidzein did not have a significant effect in decreasing the growth of these breast cancer cells at the concentrations used.

III. Results of Task 6. Expression of growth regulating genes by

phytoestrogens, months 21-24. We also examined whether the effect of genistein on cell proliferation in MCF-7 cells is mediated by its effects on growth regulatory genes such as c-*myc* and ODC. At all concentrations studied, genistein had no significant effect on c-*myc* mRNA levels after 24 h of treatment (Figure 8), compared to untreated cells. However, when mRNA level of ODC was determined after treatment with 100 μ M genistein for 2, 4 and 8 h, there was a decrease in ODC mRNA level at 8 h of treatment (Figure 9). Since the experiments were conducted with the addition of 4 nM estradiol, there was an induction of ODC mRNA at 4 h in the control cells. Detailed experiments on the time course and dose-response of genistein treatment on c-*myc* and ODC mRNA levels are currently in progress.

IV. 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 (59-63%) phase, and only a small percentage of cells in the G_2/M phase (10-18%) 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 10). The increase in the population of G_2/M cells was associated with a corresponding decrease in the percentage of cells in G_0/G_1 phase of the cell cycle. Genistein treatment (100 μ M) for 24 h caused 70% of the MDA-MB-468 cells and 42% of the MCF-7 cells to arrest at the G_2/M phase (Tables 2 and 3 respectively). Quercetin treatment also caused G_2/M block, although to a lesser extent compared to genistein, in both the cell lines. The effects of G_2/M block by genistein and quercetin were much more prominent in MDA-MB-468 cells. Kaempferol, at 100 μ M, arrested 35% of the MDA-MB-468 cells in the G_2/M phase of the cell cycle, while biochanin A and daidzein did not have significant effects on cell cycle distribution in these cells.

V. Results of Task 8. Effects of genistein and quercetin on cell cycle regulatory protein expression, months 29-36. To further understand the genistein-activated pathway that induces cell cycle arrest, the expression of proteins that function during G₂/M phase transition was examined by Western blot analysis. Figure 11 shows the effect of genistein on the expression of cyclin B1 and cdc2 in MDA-MB-468 cells. β actin was used as a control to confirm that the observed differences in cyclin B1 and cdc2 are not due to differences in protein levels. Cyclin B1 levels were dramatically altered by genistein treatment. There was a biphasic response to genistein treatment, at 25 µM, there was a 70% increase in cyclin B1 level compared to control. However, a 40 and 52% lower cyclin B1 levels than control was found at 50 and 100 µM genistein. Western blot analysis of cdc2 showed no change at 10, 25, and 50 µM treatment. However, at 100 µM genistein, the upper band corresponding to the phosphorylated form of cdc2 showed a marked decrease. The β-actin levels were comparable in all lanes, showing that the observed differences are not due to altered levels of proteins.

Figure 12 shows the effect of quercetin on cyclin B1 and cdc2 in MDA-MB-468 cells. Quercetin treatment showed a dose-dependent increase in cyclin B1, with 100 μ M treatment resulting in a 30% increase. The level of cdc2 was unchanged after quercetin

treatment. Thus even though cyclin B1 is altered by quercetin, there was a differential response of this protein to genistein treatment.

In MCF-7 cells, there was a 7 and 40% increase in cyclin B1 protein level at 25 and 50 μ M genistein, after 24 h respectively, compared to control. At 100 μ M, however, cyclin B1 was undetectable. The effect of genistein on the expression of cell cycle regulatory proteins in MCF-7 cells is currently being studied in more detail.

VI. Results of additional experiments.

1. Effects of genistein on reversal of cell growth inhibition. We also examined if genistein-induced growth suppression is reversible. Cells were treated with genistein for defined time periods, and then the drug was removed and cells treated with drug-free medium. Genistein treatment at 50 and 100 μ M caused a significant growth arrest (p<0.05) after even 2 h of exposure (Figure 13A). After 8 h, genistein treatment with 25 μ M concentration was cytotoxic to these cells, with growth inhibition comparable to cells which had continuous exposure to the drug (Figure 13B). Furthermore, genistein induced irreversible changes in DNA synthesis in MDA-MB-468 cells by 16 h at all concentrations studied.

2. Effects of phytoestrogens on apoptotic cell death. MDA-MB-468 cells were treated with phytoestrogens for 24 h and the percentage of apoptotic cells was assessed by the APO-BRDUTM kit (Pharmingen, CA). In this assay, induction of apoptosis is detected by an increase in DNA fragments that are labeled with bromolated-dUTP and a fluorescent tagged antibody using flow cytometry. Figure 14 shows a representative picture of cytograms (panel A) and histograms (panel B) of MDA-MB-468 cells treated with genistein (0-100 μ M). Treatment of cells with 10, 25, 50, and 100 μ M genistein resulted in the presence of significant amount of apoptotic cells, corresponding to 19, 34, 64, 86%, respectively, compared to 1.6% in the control cells (p<0.01, n=4). The effect of phytoestrogen treatment on the percentage of apoptotic cells, whereas Biochanin A treatment caused 11% apoptosis. In contrast, there was no significant level of apoptosis by treatment with kaempferol and daidzein.

3. Confirmation of genistein-induced apoptosis by Hoechst 33342 staining and fluorescence microscopy. To confirm genistein-induced apoptosis, cells were also treated with genistein for 24 h and stained with Hoechst 33342. Stained nuclei were then visualized under a fluorescence microscope. A representative view of dye-stained nuclei is presented in Figure 15. Nuclear condensation and apoptosis was observed even at 10 to 25 μ M concentrations of genistein. Majority of the cells appeared to undergo apoptosis at 50 μ M genistein. With 100 μ M genistein treatment, very few cells remained adherent to the cells, making the evaluation difficult. The percentage of apoptosis as determined by counting the apoptotic cells in three different fields were comparable to those obtained from flow cytometry (data not shown).

C. DISCUSSION

Our results show that genistein has stimulatory effects on DNA synthesis in MCF-7 cells up to 10 μ M. Genistein binds to the recombinant ER α , however, this did not result in increased affinity to the ERE. In contrast, genistein-bound ER β showed significant interaction with ERE. Genistein was reported to have a higher relative binding affinity to ER β than ER α in a previous study (7). Genistein bound to the recombinant ER α and ER β and sedimented the protein as 4S form, in contrast to estradiol, which sedimented it as 6S form. These data indicate that the dimerization potential of ER is deficient when it is bound to genistein. It is known for some time that ER functions in concert with several associated proteins to modulate gene transcription. Some of these accessory proteins have been identified, such as ERAP 160, RIP 140, SRC-1, SPT6 and T1F1 (19-23). These adaptor proteins have been shown to activate the receptor by binding to it (24), or enhance DNA binding and transcriptional activation (19, 20). It is likely that accessory proteins may help in the dimerization of the genistein bound ER and induce genistein mediated gene transcription in vivo. However, in the case of ER β , ERE may be providing allosteric conformational change to ER β in the presence of genistein, thus showing increased affinity binding in our EMSA experiments. Indeed modulation of ER conformation by ERE has been reported (18). Genistein-mediated increase in DNA synthesis of MCF-7 cells did not persist at 72 h. A possible reason for this may be that prolonged treatment may cause changes in the ratio of ER versus co-activators and therefore estrogenic effects may be lost.

The other phyoestrogens, daidzein, biochanin A and kaempferol stimulated DNA synthesis of MCF-7 cells at 10 μ M. However, biochanin A and kaempferol exhibited growth suppressive effects at higher concentrations with IC₅₀ value of 40 and 50 μ M, respectively. Quercetin had only inhibitory effects on DNA synthesis in these cells.

In a previous study, genistein was reported to stimulate pS2 mRNA expression at 10 μ M, a concentration that results in proliferation of ER-positive breast cancer cells (25). Experiments are in progress to provide insight into the dose-dependent effects of genistein on time-course of ODC and c-*myc* mRNA expression in MCF-7 cells.

Our results confirm the findings of G_2/M phase arrest by genistein in MCF-7 breast cancer cells (26, 27) and extend these to MDA-MB-468 cells. Furthermore, we show that the G_2/M block is associated with disturbances in cell cycle regulation. Thus, in contrast to the classic estrogens and antiestrogens which exert their effects in the G_1 phase of the cell cycle in breast cancer cells (28), genistein and quercetin induce a G_2/M block (26, 27, 29-34). The order of potency of genistein > quercetin > kaempferol remains the same with antiproliferative effects as with the efficacy of cell cycle arrest.

Our studies link the action of genistein and quercetin with the regulation of cyclin B1 in both MCF-7 and MDA-MB-468 cells, suggesting that the growth inhibitory mechanism of these compounds may be independent of ER status. However, the mode of action of these two compounds on the regulation of cyclin B1 appears to be different in MDA-MB-468 cells. With genistein, increase in cyclin B1 was observed at lower concentration and was followed by a dramatic decrease at higher concentrations. With quercetin, G_2/M arrest is associated with an increase in cyclin B1.

In eukaryotic cells, cyclin B1 accumulates during the late S and G₂ phases and allows entry of cells into the M phase and is rapidly degraded at the end of mitosis,

allowing the cells to divide (35). Inappropriate accumulation of cyclin B1 or its untimely degradation, have been reported to induce a G_2/M arrest in cells (36-40). Taxol treatment was reported to increase cyclin B1 protein in epidermoid carcinoma KB cells, parallel to mitotic arrest and programmed cell death (36). Similarly, treatment of HeLa S3 cells with X-irradiation was associated with G_2/M arrest and accelerated accumulation of cyclin B1 (37).

Alternately, decreased amount of cyclin B1 protein and G₂ arrest were reported after colcemid treatment (38) and high doses of ionizing radiation (39) in HeLa cells. In our study, lower concentrations of genistein treatment resulted in the accumulation of cyclin B1 compared to untreated cells. However, 100 μ M genistein treatment resulted in a progressive decrease of cyclin B1 protein level. This result is consistent with a recent report showing a decrease in cyclin B1 at 100 μ M genistein in MDA-MB-231 breast cancer cells (32). However, the effect of genistein at lower concentrations on cyclin B1 levels was not examined in this study (32). Our results suggest that accumulation of cyclin B1 occurs early in G₂/M phase, whereas strong apoptotic signals generated at 100 μ M genistein may lead to a degradation of the protein.

In contrast to genistein, 100 μ M quercetin treatment for 24 h increased cyclin B1 levels compared to untreated controls in MDA-MB-468 cells, similar to the microtubule inhibitor nocodazole (41). These results suggest that the mechanism of G₂/M arrest by quercetin may follow a similar pathway as low doses of genistein. However, at higher concentrations, genistein may be interacting with other pathways to generate more potent apoptotic effects.

In mammalian cells, the levels of both mRNA and protein of cyclin B1 oscillate between the initiation and completion of mitosis. Growth inhibitory agents alter one of these parameters to deregulate the levels of these proteins, and cause cell cycle perturbations. For example, treatment of HeLa cells with camptothecin was reported to result in cyclin B1 accumulation, due to reduced rate of degradation of the protein (40). Also, irradiation of HeLa cells was reported to decrease cyclin B1 availability for G_2/M transition by delaying its mRNA synthesis during S phase or increase the degradation of the protein in the G_2/M phase, leading to cell cycle arrest (39). It is not clear if the changes seen in cyclin B1 level with genistein and quercetin treatment are due to alterations in the synthesis or degradation of the protein or mRNA.

Accumulation of cells in the G₂/M phase and apoptotic cell death were prominent features of the mechanism of action of genistein and quercetin in MDA-MB-468 cells. However, with genistein, inhibition of DNA synthesis appears not to be dependent of G₂/M arrest because 10 μ M genistein was able to suppress DNA synthesis by 53% whereas G₂/M arrest was not apparent at this concentration. Effects of genistein were manifested as irreversible inhibition of DNA synthesis at 50 and 100 μ M concentrations, with as little as 2 h of exposure in MDA-MB-468 cells. Interestingly, other structurally related phytoestrogens, such as biochanin A and kaempferol only had minor effects on apoptosis, even though these compounds had growth inhibition by molecular pathways different from that of genistein and quercetin. Genistein and quercetin appear to alter additional targets of signal transduction pathway, leading to their diverse effects on DNA synthesis, G₂/M arrest and apoptosis of MDA-MB-468 cells.

I completed the second task proposed for the second year of my grant period and made major progress in the other two tasks proposed. In our EMSA experiments we found an increased binding of ER β to ERE in the presence of genistein, however, sucrose gradient analysis showed a monomeric ER β with genistein unlike a dimer obtained with estradiol. These seemingly paradoxical results will be studied in depth by using ERE in our sucrose gradient studies. In addition to the tasks proposed in the second year, I completed some of the work proposed for the third year of my study. Additional research related to this work was also performed and the data consolidated for publication.

(7) KEY RESEARCH ACCOMPLISHMENTS

Та	sks for this report	Status	
•	Months 13-16	ER-ERE interactions in the presence of phytoestrogens- partially completed and these data were presented as an abstract at the Annual Meeting of the American Association of Cancer Research, 1999, Philadelphia.	
•	Months 17-19	Cell proliferation studies - completed and this data was used in the manuscript.	
•	Months 20-26	Expression of c- <i>myc</i> , ODC and pS2 genes by phytoestrogens-partially completed.	
Та	isks proposed for the	third year	
•	Months 27-28	Cell cycle kinetics - completed.	
•	Months 29-36	Cell cycle regulatory protein expression - completed with ER- negative cells. Studies will be continued with ER-positive cells. The data from the above two tasks were presented in the Annual Meeting of the American Association of Cancer Research, 1999, Philadelphia and also used in the preparation of the manuscript.	
*A	dditional tasks	1. Binding of [¹⁴ C]-genistein to ER α and ER β - completed 2. Growth reversal experiments with genistein-completed. 3. Apoptotic cell death of MDA-MB-468 cells - completed.	
*These experiments were not proposed, but are conducted to understand in			

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

*These experiments were not proposed, but are conducted to understand i depth, the ER-dependent and -independent effects of phytoestrogens.

(8) **REPORTABLE OUTCOMES**

1. Presented two abstracts titled "Genistein induces apoptosis and biphasic changes in cyclin B1 in MDA-MB-468 breast cancer cells" and "Interaction of genistein and other phytoestrogens with estrogen receptor (ER)" to the Annual Meeting of the American Association of Cancer Research, 1999, Philadelphia, PA (COPY ATTACHED).

2. Submitted a manuscript titled "Effects of Genistein and Structurally Related Phytoestrogens on Cell Cycle Kinetics and Apoptosis in MDA-MB-468 Human Breast Cancer Cells" (COPY ATTACHED).

(9) CONCLUSIONS.

- (i) Genistein did not significantly alter ER α and ERE binding in EMSA. In contrast, at 500 nM concentration, genistein facilitated ER β -ERE binding. Our sucrose gradient experiments showed that genistein at 10 μ M was unable to dimerize ER α or β protein, a step that offers conformational advantage for binding of ER to ERE. In contrast, estradiol treatment resulted in the sedimentation of the protein in the dimeric 6S form.
- (ii) Genistein, biochanin A, daidzein and kaempferol have growth stimulatory effects in ER-positive MCF-7 cells, although at higher concentrations, genistein, biochanin A and kaempferol were growth inhibitory. In contrast, quercetin had growth inhibitory effects at all concentrations studied.
- (iii) Genistein and quercetin were potent growth inhibitors of ER-negative MDA-MB-468 breast cancer cells. Accumulation of cells at the G2/M phase and apoptotic cell death were prominent effects of these two compounds.
- (iv) The effect of genistein on cyclin B1 protein level was biphasic in both MCF-7 and MDA-MB-468 cells, with lower concentrations increasing the level of the protein and higher concentrations decreasing it.

SO WHAT.

Our results may have practical applications for the treatment of ER-negative breast cancer, as ER-positive cells acquire ER-negative phenotype that is more aggressive and resistant to antiestrogen therapy (42). Under these conditions, genistein and quercetin may be useful to inhibit breast cancer cell growth as their growth inhibitory effects are ER-independent. In this context, it is also important to note that genistein suppressed the growth of nude mice xenografts in ER-positive and -negative breast cancer cells (43). Investigations on the mechanism of action of phytoestrogens and their combination in chemo- and radiation therapy might be a fruitful approach to the design of improved breast cancer therapies.

(10) REFERENCES

- 1. Murkies, A. L., Wilcox, G., and Davis, S. R. Phytoestrogens. Clinical Review. J. Clin. Endocrinology and Metabolism., 83: 297-303, 1998.
- Phang, J. M., Poore, C. M., Lopaczynska, J., Yeh, G. C. Flavonol-stimulated efflux of 7,12-dimethylbenz(a)anthracene in multidrug-resistant breast cancer cells. Cancer Res., 53: 5977-5981, 1993.
- 3. Ciolino, H. P., Daschner, P. J., Yeh, G. C. Dietary flavonols quercetin and kaempferol are ligands of the aryl hydrocarbon receptor that affect CYP1A1 transcription differentially. Biochem. J., 340: 715-722, 1999.
- Wang, W., Liu, L. Q., Higuchi, C. M., Chen, H. Induction of NADPH:quinone reductase by dietary phytoestrogens in colonic Colo205 cells. Biochem. Pharmacol., 56: 189-195, 1998.
- 5. Adlercretz, H., Mousavi, Y., Clark, J., Hockerstedt, K., Hamaleinen, E., Wahala, K., Makela, T., Hase, T.J. Dietary phytoestrogens and cancer- in *vitro* and *in vivo* studies. Steroid Biochem. Mol. Biol., 41: 331-337, 1992.
- Jensen, E. V. Steroid hormones, receptors and antagonists. In: Basis for cancer management, (Eds., Castagnetta, L., Nenci, I., and Bradlow, H. L.), vol.784, pp1-17., Annals of NY Acd. Sci., New York, NY, 1996.
- Kuiper, G. G. J. M., Carlsson, B. O., Grandien, K., Enmark, E., Haggblad, M., Nilsson, S., Gustafson, J. A. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β. Endocrinology, 138: 863-870, 1997.
- 8. Gordon, M. S., and Notides, A. C. Computer modeling of estradiol interactions with the estrogen receptor. J. Steroid Biochem. Molec. Biol., 25: 177-181, 1986.
- Elliston, J. F., Fawell, S. E., Klein-Hitpass, L., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. Mechanism of estrogen receptor-dependent transcription in a cell-free system. Mol. Cell Biol., 10: 6607-6612, 1990.
- Green, S., Walter, P., Kumar, V., Krust, A., Bornet, J-M., Argos, P., and Chambon, P. Human oestrogen receptor cDNA: sequence, expression and homology to v-erb-A. Nature, 320: 134-139, 1986.
- 11. Kuiper, G. G. J. M., Enmark, E., Pelto-Huikko, M., Nilsson, S., and Gustafson, J. A. Cloning of a novel estrogen receptor expressed in rat prostate and ovary. Proc. Natl. Acad. Sci. U. S. A, 93: 5925-5930, 1996.

- van der Burg, B., van Selm-Mittenburg, A. J. P., de Laat, S. W., and van Zoelen, E. J. J. Direct effects of estrogen on c-fos and c-myc proto-oncogene expression and cellular proliferation in human breast cancer cells. Mol. Cell Endocrinol., 64: 223-228, 1989.
- 13. 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.
- 14. Nass, S. J., and Dickson, R. B. Defining a role for c-myc in breast tumorigenesis. Breast Cancer Res. Treatment, 44: 1-22, 1997.
- Wright, P. S., Cooper, J. R., Cross-Doersen, D. E., Miller, J. E., Chmielewski, P. A., Wagner, R. L., Streng, K. A., and Flanagan, M. A. Regulation of ornithine decarboxylase mRNA levels in human breast cancer cells: pattern of expression and involvement of core enhancer promoter element. Cell Growth & Differen., 6: 1097-1102, 1995.
- 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. U. S. A, 83: 2496-2500, 1986.
- 17. Wiese, T. E., Kral, L. J., Dennis, K. E., Butler, W. B., and Brooks, S. C. Optimization of estrogen growth response in MCF-7 cells. In Vitro Cell Develop. Biol., 28A: 595-602, 1992.
- Wood, J. R., Greene, G. L., and Nardulli, A. M. Estrogen response elements function as allosteric modulators of estrogen receptor conformation. Molec. Cell. Biol., 18: 1927-1934, 1998.
- 19. Halachmi, S., Marden, E., Martin, G., Mackay, H., Abbondanza, C. and Brown, M. Estrogen receptor-associated proteins: possible mediators of hormone-induced transcription. Science, 264: 1455-1458, 1994.
- 20. Cavailles, V., Dauvois, S., L'Horst, F., Lopez, G., Hoare, S., Kushner, P. J., and Parker, M. G. Nuclear factor RIP 140 modulates transcriptional activation by the estrogen receptor. EMBO. J., 14: 3741-3751, 1995.
- 21. Onate, S. A., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. Science, 270: 1354-1357, 1995.
- 22. Baniahmad, C., Nawaz, Z., Baniahmad, A., Gleeson, M. A. G., Tsai, M. J., and O'Malley, B. W. Enhancement of human estrogen receptor activity by SPT6: a potential coactivator. Molec. Endocr., 9: 34-43, 1995.

- LeDouarin, B., Zechel, C., Garnier, J. M., Lutz, Y., Tora, L., Pierrat, B., Heery, D., Gronemeyer, H., Chambon, P., and Losson, R. The N-terminal part of TIF1, a putative mediator of the ligand-dependent activation function (AF-2) of nuclear receptors, is fused to B-raf in the oncogenic protein T18. EMBO. J., 14: 2020-2033, 1995
- Anolik, J. H., Klinge, C. M., Brolly, C. L., Bambara, R. A., and Hilf, R. Stability of the ligand-estrogen receptor interaction depends on estrogen response element flanking sequences and cellular factors. J. Steroid Biochem. Molec. Biol., 59: 413-429, 1996.
- 25. Sathyamoorthy, N., Thomas, T., and Phang, J. M. Stimulation of pS2 expression by diet-derived compounds. Cancer Res., 54: 957-961, 1994.
- 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. Cancer, 30A: 1675-1682, 1994.
- 27. Shao, Z-M., Alpaugh, M. L., Fontana, J. A., and Barsky, S. F. Genistein inhibits proliferation similarly in estrogen receptor-positive and negative human breast carcinoma cell lines characterized by p21^{WAF1/C1P1} induction, G2/M arrest, and apoptosis. J. Cell Biochem., 69: 44-54, 1998.
- Prall, O. W., Rogan, E. M., Sutherland, R. L. Estrogen regulation of cell cycle progression in breast cancer cells. J. Steroid Biochem. Mol. Biol., 65: 169-174, 1998.
- 29. Traganos, F., Ardelt, B., Halko, N., Bruno, S., and Darzynkiewicz, Z. Effects of genistein on the growth and cell cycle progression of normal human lymphocytes and human leukemic MOLT-4 and HL-60 cells. Cancer Res., 52: 6200-6208, 1992.
- Matsukawa, Y., Marui, N., Sakai, T., Satomi, Y., Yoshida, M., Matsumoto, K., Nishini, H., and Aoike, A. Genistein arrests cell cycle progression at G₂-M. Cancer Res., 53: 328-331, 1993.
- 31. Fioravanti, L., Cappelletti, V., Miodini, P., Ronchi, E., Brivio, M., and Di Fronzo, G. Genistein in the control of breast cancer cell growth: insights into the mechanism of action in vitro. Cancer Lett., 30: 43-52, 1998.
- 32. Choi, Y. H., Zhang, L., Lee, W. H., Park, K-Y. Genistein induced G2/M arrest is associated with the inhibition of cyclin B1 and the induction of p21 in human breast carcinoma cells. Int. J. Oncology, 13: 391-396, 1998.
- Constantinou, A. L., Kamath, N., Murley, J. S. Genistein inactivates bcl-2, delays the G2/M phase of the cell cycle and, induces apoptosis of human breast adenocarcinoma MCF-7 cells. Eur. J. Cancer, 34: 1927-1934, 1998.

- 34. Lian, F., Bhuiyan, M., Li, Y-W., Wall, N., Kraut, M., and Sarkar, F. H. Genisteininduced G₂/M arrest, p21^{WAF1} upregulation, and apoptosis in a non-small-cell lung cancer cell line. Nutr. Cancer, 31: 84-91, 1998.
- 35. King, R. W., Deshaies, R. J., Peters, J. M., and Kirschner, M. W. How proteolysis drives the cell cycle. Science, 274: 652-659, 1996.
- Ling, Y-H., Consoli, U., Tornos, C., Andreeff, M., Perez-Soler, R. Accumulation of cyclin B1, activation of cyclin B1-dependent kinase and induction of programmed cell death in human epidermoid carcinoma KB cells treated with taxol. Int. J. Cancer, 75: 925-932, 1998.
- Ianzini, F., Mackey, M. A. Delayed DNA damage associated with mitotic catastrophe following x-irradiation of HeLa S3 cells. Mutagenesis, 13: 337-344, 1998.
- Sherwood, S.W., and Schimke, R. T. Cell cycle analysis of apoptosis using flow cytometry. In: L. M. Schwartz, and B. A. Osborne (eds.)., Methods in cell biology, Vol. 46, pp. 77-97. San Diego: Academic Press, Inc., 1995.
- 39. Kao, G. D., McKenna, W. G., Maity, A., Blank, K., Muschel, R. J. Cyclin B1 availability is a rate-limiting component of the radiation-induced G2 delay in HeLa cells. Cancer Res., 57: 753-758, 1997.
- 40. Maity, A., Hwang, A., Janss, A., Phillips, P., McKenna, W. G., Muschel, R. J. Delayed cyclin B1 expression during the G2 arrest following DNA damage. Oncogene, 13:1647-1657, 1996.
- 41. 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 Res., 55: 6021-6025, 1995.
- Petrangeli, E., Lubrano, C., Ortolani, F., Ravenna, L., Vacca, A., Sciacchitano, S., Frati, L., Gulino, A. Estrogen receptors: new perspectives in breast cancer management. J. Steroid Biochem. Mol. Biol., 49: 327-331, 1994.
- 43. Shao, Z. M., Wu, J., Shen, Z., Z., and Barsky, S. H. Genistein exerts multiple suppressive effects on human breast carcinoma cells. Cancer res., 58: 4851-4857, 1998.

Table 1. IC₅₀ values for phytoestrogens needed for cell growth inhibition

Cells were treated with each of the phytoestrogens at 0, 10, 25, 50, 100 μ M concentrations for 24, 48 or 72 h. Cell growth was determined by [³H]-thymidine incorporation assay. IC₅₀ value was calculated from the growth curves as the concentration that inhibited 50% of cell growth. The values are an average of two separate experiments conducted in triplicate.

Phytoestrogen	IC ₅₀ , μ M (concentration ± SD)			
	MCF-7 cells	MDA-MB-468 cells		
Genistein	37 ± 3.8	8.8 ± 1.6		
Quercetin	17.3 ± 2.7	18.1 ± 1.6		
Biochanin A	40 ± 4.1	44.0 ± 10.1		
Kaempferol	50 ± 3.9	47.0 ± 2.9		
Daidzein	>100	>100		

Table 2. Effect of phytoestrogens on cell cycle distribution of MDA-MB-468 cells

Flow cytometric analysis was performed and the percentage of cells in the G_0/G_1 , S and G_2/M phases of the cell cycle was calculated using the *CytoLogic* software, after treatment of MDA-MB-468 cells with different phytoestrogens for 24 h. *Significantly different from control, p<0.05, (n = 6).

Phytoestrogen	Concentration	G₀/G₁	S	G ₂ /M
Control	0	59.0 ± 1.7	22.4 ± 1.1	17.5 ± 1.1
Genistein	10 μΜ 25 μΜ 50 μΜ 100 μΜ	$65.5 \pm 1.6^{*}$ $47.9 \pm 0.9^{*}$ $27.7 \pm 0.3^{*}$ $27.3 \pm 3.2^{*}$	$18.3 \pm 1.6 \\ 23.3 \pm 1.3 \\ 17.0 \pm 0.9 \\ 3.0 \pm 0.9^*$	$\begin{array}{c} 16.2 \pm 1.9 \\ 33.2 \pm 0.7^* \\ 49.5 \pm 0.7^* \\ 70.0 \pm 4.3^* \end{array}$
Quercetin	10 μΜ 25 μΜ 50 μΜ 100 μΜ	$52.1 \pm 2.1^*$ $50.5 \pm 0.7^*$ $48.4 \pm 1.3^*$ $35.0 \pm 5.1^*$	$\begin{array}{c} 32.6 \pm 4.3^{*} \\ 27.7 \pm 1.2 \\ 24.7 \pm 2.5 \\ 5.0 \pm 2.1^{*} \end{array}$	$15.2 \pm 2.5 \\ 21.7 \pm 0.7 \\ 41.2 \pm 2.2^* \\ 60.0 \pm 5.2^*$
Kaempferol	10 μΜ 25 μΜ 50 μΜ 100 μΜ	$58.8 \pm 0.2 \\ 58.2 \pm 0.5 \\ 46.4 \pm 4.2^* \\ 45.6 \pm 2.7^*$	20.9 ± 0.9 20.6 ± 1.3 19.5 ± 0.9 19.2 ± 1.1	20.3 ± 1.1 21.2 ± 1.7 $34.1 \pm 3.3^*$ $35.1 \pm 1.7^*$
Biochanin A	10 μΜ 25 μΜ 50 μΜ 100 μΜ	$59.7 \pm 0.4 \\ 60.9 \pm 0.1 \\ 60.3 \pm 1.2 \\ 59.7 \pm 1.6$	$22.9 \pm 1.1 \\ 20.7 \pm 1.1 \\ 20.9 \pm 1.0 \\ 20.9 \pm 0.1$	17.4 ± 0.8 18.3 ± 1.2 18.7 ± 0.5 19.3 ± 1.5
Daidzein	10 μΜ 25 μΜ 50 μΜ 100 μΜ	57.1 ± 1.8 52.6 ± 1.2 53.8 ± 5.7 52.2 ± 5.7	$19.5 \pm 1.1 \\ 20.7 \pm 1.1 \\ 19.1 \pm 0.7 \\ 22.5 \pm 3.9$	$\begin{array}{c} 20.0 \pm 2.7 \\ 23.6 \pm 2.5 \\ 22.8 \pm 5.4 \\ 21.8 \pm 2.3 \end{array}$

Table 3. Phytoestrogen treatment on cell cycle distribution of MCF-7 cells

MCF-7 cell cycle distribution was determined after treatment with genistein or quercetin for 24 h using flow cytometric analysis. *Significantly different from control at p<0.05.

% Cells at 24 h after treatment

Treatment	G ₀ /G ₁	S	G₂/M		
Control	62.2 ± 1.4	21.9 ± 1.9	9.9 ± 1.6		
Genistein					
10 μM	62.7 ± 8.2	25.9 ± 0.01	17.2 ± 0.07		
25 μΜ	61.1 ± 0.2	25.21 ± 1.6	13.6 ± 1.6		
50 μM	55.4 ± 0.2	27 ± 1.4	17.6 ± 1.6		
100 μM	40.4 ± 1.2	15.5 ± 1.8	$42.4 \pm 0.9^{*}$		
Quercetin					
10 μM	56.64 ± 1.0	33.9 ± 4	9.46 ± 1.1		
25 µM	53.49 ± 2.0	23.91 ± 3.6	22.6 ± 1.7		
50 μM	50.65 ± 1.8	14.8 ± 0.2	34.55 ± 1.6		
100 μ Μ	50.5 ± 1.6	12.3 ± 1.5	$37.2 \pm 2.3^{*}$		

Table 4. Effect of phytoestrogens on percentage apoptosis of MDA-MB-468 cells

APO-BRDUTM analysis for apoptosis was determined after treatment of MDA-MB-468 cells with different phytoestrogens for 24 h. DNA fragments in apoptotic cells are end-labeled with BRDU and incubated with fluorescent tagged BRDU-specific antibody. Fluorescence intensity generated is proportional to the percentage apoptosis as determined by flow cytometry. *Significantly different from control, p<0.01, (n = 6).

Phytoestrogen

% apoptosis by 24 h treatment with:

0 μΜ	10 μM	25 μΜ	50 μ Μ	100 μM
1.6±0.6	19.5 ± 9.8*	34.7 ± 8.3*	64.3 ± 15.0*	86.0 ± 4.0*
1.6 ± 0.6	3.3 ± 0.4	12.3 ± 1.5*	18.1 ± 5.6*	47.2 ± 1.5*
1.5 ± 0.6	1.5 ± 0.1	3.8 ± 1.3	6.1 ± 0.3*	11.2 ± 1.2*
1.5 ± 0.8	1.3 ± 0.1	2.4 ± 0.1	2.9 ± 0.1	1.7 ± 0.6
1.9 ± 0.1	2.5 ± 1.7	3.4 ± 0.7	3.7 ± 0.7	$4.8 \pm 0.4^{**}$
	0 μ M 1.6 \pm 0.6 1.6 \pm 0.6 1.5 \pm 0.6 1.5 \pm 0.8 1.9 \pm 0.1	$0 \ \mu M$ $10 \ \mu M$ 1.6 ± 0.6 $19.5 \pm 9.8^*$ 1.6 ± 0.6 3.3 ± 0.4 1.5 ± 0.6 1.5 ± 0.1 1.5 ± 0.8 1.3 ± 0.1 1.9 ± 0.1 2.5 ± 1.7	$0 \mu M$ $10 \mu M$ $25 \mu M$ 1.6 ± 0.6 $19.5 \pm 9.8^*$ $34.7 \pm 8.3^*$ 1.6 ± 0.6 3.3 ± 0.4 $12.3 \pm 1.5^*$ 1.5 ± 0.6 1.5 ± 0.1 3.8 ± 1.3 1.5 ± 0.8 1.3 ± 0.1 2.4 ± 0.1 1.9 ± 0.1 2.5 ± 1.7 3.4 ± 0.7	$0 \mu M$ $10 \mu M$ $25 \mu M$ $50 \mu M$ 1.6 ± 0.6 $19.5 \pm 9.8^*$ $34.7 \pm 8.3^*$ $64.3 \pm 15.0^*$ 1.6 ± 0.6 3.3 ± 0.4 $12.3 \pm 1.5^*$ $18.1 \pm 5.6^*$ 1.5 ± 0.6 1.5 ± 0.1 3.8 ± 1.3 $6.1 \pm 0.3^*$ 1.5 ± 0.8 1.3 ± 0.1 2.4 ± 0.1 2.9 ± 0.1 1.9 ± 0.1 2.5 ± 1.7 3.4 ± 0.7 3.7 ± 0.7

FIG. 1. CHEMICAL STRUCTURES OF 17-β-ESTRADIOL AND PHYTOESTROGENS USED IN THIS STUDY





HO

HO

H

GENISTEIN





HO

17-B-ESTRADIOL

FIGURE 1

24



ER β +ERE

-ER β-ERE



-Free Probe

FIG. 2. Electrophoretic mobility shift assay to determine the ability of genistein in facilitating ER β -ERE interaction. ER β (500 ng) was incubated with 4 nM E₂ or different concentrations of genistein at 4°C for 2 h. A 4 X binding buffer (final concentration reaching 10 mm Tris-HCl, 10% glycerol, 1 mm dithiothreitol and 10 µg/ml poly(dI-dC). poly(dI-dC)) was added for 15 min. Labeled ERE was then added to this reaction mixture and incubated for an additional 1 h at 4°C and 30 min at room temperature and loaded on to a 6% polyacylamide gel.











FIG. 5. Sucrose density gradient profile of a 1:1 mixture of recombinant ER α and ER β bound to [¹⁴C]-genistein. ER α and β (500 fmoles each) were incubated with [¹⁴C]-genistein for 3 h and treated with dextran coated charcoal to remove free ligand. [¹⁴C]-labeled proteins γ -globulin (7S) and bovine serum albumin (4.6S) were used as markers of sedimentation constants. Binding in the presence of 10 μ M [¹⁴C]-genistein (\circ) and 10 μ M [¹⁴C]-genistein with 0.5 nM unlabeled estradiol (\bullet) are shown.





FIG. 6. Effects of genistein and quercetin on cell proliferation. MCF-7 cells were treated with 0 (\Box), 10 (\Box), 25 (\boxtimes), 50 (\boxtimes) and 100 μ M (\equiv) concentrations of (A) genistein or (B) quercetin for 24, 48 or 72 h. DNA synthesis was measured by pulse labeling for 1 h with 1 μ g/ml [³H]-thymidine. *Significant difference from control (p<0.05). Data are the mean +/- SD from two experiments.





FIG. 7. Effects of genistein and quercetin on cell proliferation. MDA-MB-468 cells were treated with 0 (\Box), 10 (\Box), 25 (\boxtimes), 50 (\equiv) and 100 μ M (\blacksquare) concentrations of (A) genistein or (B) quercetin for 24, 48 or 72 h. DNA synthesis was measured by pulse labeling for 1 h with 1 μ g/ml [³H]-thymidine. *Significant difference from control (p<0.05). Data are the mean +/- SD from two experiments.



FIG. 8. Effect of genistein on the level of ODC mRNA in MCF-7 cells. Cells were treated with 100 μ M genistein for 2, 4, or 8 h. Total RNA was extracted, separated on 1% agarose gel, transferred to a nylon membrane and probed with labeled ODC cDNA.



FIG.9. Effect of genistein on the level of c-myc mRNA in MCF-7 cells. Cells were treated with 10, 25, 50 or 100 μ M genistein for 24 h. Control cells had 40 μ l of DMSO. Total RNA was extracted, separated on a 1% agarose gel, transferred to a nylon membrane and probed with labeled c-myc cDNA.

Cells were treated with 0, 10, 25, 50, and 100 µM concentrations of (A) genistein, FIG. 10. Flow cytometry profile of cell cycle distribution of MDA-MB-468 cells. (B) quercetin or (C) kaempferol for 24 h. RNase treated and propidium iodide **DNA CONTENT** stained cells were sorted by flow cytometry.



FIGURE 10

33



GENISTEIN, µM:

FIG. 11. Western blot analysis of cyclin B1, cdc2 and β -actin protein expression in MDA-MB-468 cells treated with 0, 10, 25, 50 and 100 μ M concentrations of genistein for 24 h. Protein lysates were electrophoresed, transferred onto a PVDF immobilon membrane and probed with an antibody against cyclin B1 cdc2 or β -actin. Similar results were obtained in three independent experiments.



FIG. 12. Western blot analysis of cyclin B1 and cdc2 protein expression in MDA-MB-468 cells treated with 0, 10, 25, 50 and 100 μ M concentrations of quercetin for 24 h. Protein lysates were electrophoresed, transferred onto a PVDF immobilon membrane and probed with an antibody against cyclin B1 or cdc2. Similar results were obtained in two separate experiments.

35



TIME OF TREATMENT, HOURS




mm

100

50 LLM

ILLM

25

10 µM

CONTROL

0001

FIGURE 14

fluorescein BRDU) on Y axis. Panel B: histograms showing percentage of apoptotic

cells. X axis, fluorescence intensity; Y axis, cell number.

cytograms with cell number on X axis vs. relative fluorescence intensity (log green

FIGURE 15



FIG. 15. Genistein induced apoptotic cell death of MDA-MB-468 cells as determined by 33342 Hoechst staining. Cells were treated with genistein (0, 10, 25 and 50 μ M, panels A through D, respectively) for 24 h and fixed in 4% paraformaldehyde. Then the cells were treated with Hoechst 33342 dye and the stained nuclei were visualized under fluorescence microscope. Irregular and condensed or fragmented nuclei were observed as characteristic features of apoptosis. To quantify apoptosis, cells from three different fields were counted, with about 100 cells per field.

APPENDIX 1

Acronym and symbol definitions in alphabetical order

Dextran coated charcoal
Diethyl stilbestrol
Dulbecco's minimal essential medium
Dimethyl sulfoxide
Electrophoretic mobility shift assay
Estradiol
Estrogen receptor
Estrogen response element
Fetal bovine serum
Improved minimum essential medium
Ornithine decarboxylase

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Raf-1 and Bcl-2 hyperphosphorylation following treatment with microtubuleactive agents is associated with mitotic arrest. Here we show that phosphorylation of Raf-1 by paclitaxel (PTX) does not activate the MAPK pathway in leukemia cells. PD98059, a MEK inhibitor, when added simultaneously or after PTX, does not abrogate Bcl-2 phosphorylation nor apoptosis. In contrast to PTX, phorbol ester (TPA) activates the MAPK pathway causing p21^{WAP1/CIP1} induction, Rb dephosphorylation and growth arrest without Bcl-2 phosphorylation or apoptosis, whereas inhibition of MEK abolishes the induction of p21 and growth arrest in leukemia cells. Although recently implicated in Bcl-2 phosphorylation, cAMP, like TPA, induces p21 but not Bcl-2 hyperphosphorylation. Coincident with the beginning of apoptosis, PTX-induced cleavage of Bcl-2 protein yields a pro-apoptotic 22 kDa product. It should be stressed that mitotic arrest by PTX requires cell cycling prior to PTX exposure. Although the MAPK pathway does not play role in PTX-induced apoptosis, modulation of the MAPK pathway may induce cell-type selective G1- or G2 arrest precluding from PTX-induced mitotic arrest/apoptosis. Thus, PTX induces neither Bcl-2 phosphorylation nor apoptosis in TPA-arrested HL60 cells. Similarly, inhibitors of the MAPK pathway induce G1 arrest in sensitive cells protecting from PTX cytotoxicity.

#76 Taxane-mediated gene induction in human cells. Moos, P.J., and Fitzpatrick, F.A. Huntsman Cancer Institute, University of Utah, Salt Lake City, UT 84108.

Taxol and taxotere are potent and efficacious chemotherapeutics in breast, ovarian, non-small cell lung, head and neck, and malignant melanoma cancers. Their efficacy appears disproportionate to the recognized mode of action as anti-mitotic agents. We have begun examining taxane effects on gene induction along the host-defense axis. We initially used a genomic approach to identify and broaden the scope of taxane-mediated gene induction in the murine model system. We identified genes whose expression was potentiated by taxol in primarily three categories: inflammatory, transcription factors and novel genes whose functions have not been identified. We have extended the initial observation from the murine system that PGHS₂ is induced by taxol to primary human monocytes where both taxol and taxotere could induce the expression of this gene product. PGHS₂ production could be completely inhibited in the human monocytes by inhibiting p38 MAPK activity. These results indicate that a signal transduction pathway that is activated in the murine model system is also activated in primary human cells. We feel it is unlikely that the activation of this signal transduction system would be limited to inducing only this gene product so we are presently identifying the scope of taxane-mediated gene induction utilizing microarrays with expressed-tagged sequences as targets. We are utilizing both primary human cells and human cell lines to profile the changes in transcription as a consequence of these compounds to clarify their mechanism of action.

#77 The rapamycin target, mTOR kinase, may link IGF-1 signaling to terminal differentiation. Shu, L., Liu, L.N., Dilling, M.B., and Houghton, P.J. St. Jude Children's Research Hospital, Memphis, TN 38105.

Signals transduced through the type 1 insulin-like growth factor receptor (IGF-1R) are involved in growth and survival of malignant myogenic cells (rhabdomyosarcoma). However, the role of IGF-1R in terminal differentiation is less clear. Rapamycin is a specific inhibitor of mTOR, a protein kinase downstream of IGF-1R known to activate translation through both ribosomal p70S6 kinase (p70S6K) and eIF4E pathways. Using C2C12 murine myoblasts we have found that rapamycin arrests cells in G1, as anticipated, but completely inhibits terminal myogenesis. Stable expression of a mutant rapamycin-resistant mTOR (S2035→I) allows myogenesis in rapamycin-treated myoblasts, confirming that inhibition of myogenesis is mTOR dependent. We next established stable C2C12 clones expressing a constitutively active p70S6K enzyme (D3E-E389). These clones were ~10-fold resistant to rapamycin-induced growth inhibition, but remained sensitive to inhibition of myogenesis by rapamycin. These results suggest that mTOR controls myogenic differentiation through a pathway independent of either p70S6K or eIF4E. Results will be presented that indicate mTOR kinase positively regulates the function of the myogenic transcription factor MyoD, and links IGF-1R signaling to terminal myogenesis. Supported by CA23099 and by American Lebanese Syrian Associated Charities.

#78 Calcitriol inhibits p21^{War1/Cip1} (p21) expression and sensitizes SCC cells to cisplatin cytotoxicity in vitro and in vivo. Hershberger, PA, Modzelewski, RA, Trump, DL, and Johnson, CS. Depts of Pharmacology and Medicine, University of Pittsburgh Cancer Institute, Pittsburgh, PA 15213.

We demonstrated that calcitriol pre-treatment enhances the cytotoxicity of cisplatin in the murine squamous cell tumor (SCC) model *in vitro* and *in vivo*. These effects are schedule dependent. No enhancement of cisplatin-mediated cytotoxicity occurs with concurrent administration. Since inhibition of cdk inhibitor p21 expression sensitizes tumor cells to DNA damaging agents, we initiated studies to determine whether calcitriol inhibits p21 expression and thereby enhances cisplatin cytotoxicity in SCC. Western blot analysis of tumor cell systems revealed that *in vivo* administration of calcitriol (0.75 μ g/day×3) to SCC tumor bearing mice results in a statistically significant decrease in p21 expression (103 ± 63 OD units) relative to saline-injected controls (325 ± 162 OD units). These studies are the first to show *in vivo* modulation of cell cycle regulators in SCC by calcitriol. Similarly, *in vitro* treatment of SCC with calcitriol for 24h resulted in 3-fold inhibition of p21 protein expression which was associated with

a reduction in the steady-state level of p21 transcripts. P21 down-modulation correlated with a significant increase in anti-tumor activity as measured by an actual decrease in tumor volume. Studies are in progress to more precisely define the relationship between calcitriol-mediated inhibition of p21 and enhanced cisplatin cytotoxicity. Supported by NIH grant CA67267 and CaPCURE.

#79 Elevated level of cdc25A and C in human tumor cell lines. Robinson, S.P., Perron, D., Miller, R., Parker, L., Johnston, C., Wei, F-Y., Jin, P., Tam, S., Worland, P., Wickramasighe, D. and Voss, J. BASF Bioresearch Corporation 100 Research Drive, Worcester, MA 01564 and Mitotix Inc. Cambridge, MA 02139.

Elevated mRNA levels for cdc25A and B has been reported in a variety of human tumors. When over expressed in embryonic fibroblasts cdc25A and B have been shown to be oncogenic. In the present studies, comparison in HeLa cells of mRNA through the cell cycle indicated only slight fluctuations in cdc25A, B and C levels. In contrast protein levels for cdc25 A and B. were strongly cell cycle dependent. Cdc25C levels, however, changed only slightly through the cell cycle with little change in phosphatase activity. Cdc25A, B and C mRNA and protein levels were determined in human tumor cell lines (lung, renal, prostate, cervical) and compared to primary epithelial cells of the same tissue origin. Tumor lines consistently had elevated mRNA levels of both cdc25A and C. This was matched by elevated levels of cdc25 A and C protein in the tumor lines compared to the primary epithelial cells. In contrast cdc25B mRNA levels were similar in all cell lines tested and the protein levels were below the level of detection in asynchronous cells. These studies are consistent with cdc25A and B being regulated post-transcriptionally and cdc25A and C being aberrant in some tumors.

#80 Vitamin K₃ induces cell death by inhibiting dual specificity phosphatases. Chyan, C.-L. and Wu, F.Y.-H. Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan, R.O.C.

We have shown that synthetic vitamin K_3 (VK₃) exhibits antitumor activity in animal and various cancer cell lines and induces two main effects - cell cycle arrest and apoptosis. VK₃ inhibited the growth of HepG2 cells via a cell cycle delay at the G1/S and S/G2 phases as monitored by flow cytometry. In VK_3treated HepG2 cells, Cdc2 kinase was mainly populated in a hyperphosphorylated inactive form. Dephosphorylation at Thr-14 and Tyr-15 of Cdc2 kinase by a dual specificity phosphatase (DSP), Cdc25 phosphatase, activates Cdc2 kinase. To determine the effect of VK_3 on the activity of Cdc25, the recombinant wild type Cdc25A, its catalytic C-domain (Cdc25AC), and the catalytic site mutant (C430S) were produced and assayed for their abilities to hydrolyze pNPP substrate in the absence or presence of VK₃. The mutant (C430S) lost its activity. When Cdc25A and Cdc25AC were preincubated with VK₃, their activities were abolished. Since all the PTPases and DSPs including Cdc25 possess a highly conserved Cys residue at their active sites, we propose that the modification of active sites of DSPs by VK₃ subsequently reduces or even abolishes their dephosphorylation abilities. Using two-dimensional NMR spectroscopy, an *in vitro* model study on the complex of VK_3 and a synthetic 11-residue peptide containing the active site of a DSP (MKP-1) was achieved, and the largest chemical shift deviation of the crosspeaks in the finger print region was found in the Cys residue. This suggests that VK_3 binds to the Cys residue of the MKP-1 peptide. Our data indicate that VK_3 is a potent inhibitor of the DSPs by binding to their active sites. Thus, VK_3 prevents tumor cells from entering mitosis, leading to cell death.

#81 1 α ,25-dihydroxyvitamin D₃ inhibits the proliferation of the human bladder carcinoma cell lines T24 and TCC-SUP which express specific VDR receptor. Gautier, C., Mourah, S., Le Brun, G., Cussenot, O., Villette, J.M., Desgrandchamps, F., Teillac, P., Le Duc, A., Fiet, J., and Soliman, H. Departments of Hormonal Biology, Urology, and Laboratory of Genetics and Pathobiology of Prostate Tumors, Saint Louis Hospital, University Paris VII, 75010 Paris, France.

Calcitriol (1 α ,25 dihydroxyvitamin D₃ (1,25-(OH)₂D₃)) is the biologically active form of vitamin D which has been reported to be a potent inhibitor of proliferation and a promoter of differentiation in various normal and cancer cell lines. 1,25-(OH)₂D₃—responsive cells express specific vitamin D receptor (VDR). The human bladder cancer cell lines T24, TCC-SUP were examined for the presence of specific VDR. Both of the cell lines were found to express VDR as assessed by RT-nested PCR methodology. In the present study, we further evaluated the effect of 1,25-(OH)₂D₃ on the proliferation of both cell lines. When bladder cancer cell lines (T24, TCC-SUP) were treated in culture with 0.1 μ M concentration of 1,25-(OH)₂D₃ for 6 days, it induced proliferation inhibition by 47% and 30% respectively, versus those treated with vehicle only. This is the first study, to our knowledge, demonstrating the presence of VDR in human bladder cancer cell lines and the inhibition of their proliferation by 1,25-(OH)₂D₃. Accordingly, thes findings might suggest a potential role for 1,25-(OH)₂D₃ or analogues with m hypercalcemic effect in the chemoprevention and/or therapy of bladder cancer

#82 Genistein induces apoptosis and biphasic changes in cyclin B1 MDA-MB-468 breast cancer cells. Balabhadrapathruni, S., Thomas, TJ Yurkow, E., and Thomas, T. UMDNJ-Robert Wood Johnson Medical School, Ne Brunswick, NJ 08903, and Rutgers University, Piscataway NJ 08544.

The isoflavanoid genistein, available from soybeans, is known to have chemo preventive effects. We examined its effects on cell growth, cell cycle progression

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apoptosis, and on cell cycle regulatory proteins in MDA-MB-468 breast cancer cells. Genistein inhibited cell growth with an IC₅₀ of 8.8 ±1.8 μ M and it caused G2/M arrest of these cells (16 to 70% at 10 to 100 μ M) by 24 h. The percentage of apoptosis was analyzed by APO-BRDU kit. Genistein (10 to 100 μ M) caused apoptosis of 27 to 90% of the cells by 24 h. Apoptosis was also confirmed by Hoechst 33342 staining and fluorescence microscopy. Western blot analysis of cyclin B1 showed a biphasic response, with an increase at 25 μ M genistein, but a 90% decrease at 50 and 100 μ M concentrations. There was no significant changes in the level of cyclin D1 or β -actin. As little as 1 h of exposure with 100 μ M genistein was sufficient to induce irreversible suppression of DNA synthesis. Our results indicate that breast cancer cell growth suppression by genistein includes G2/M arrest, disruption of cyclin B1 regulation and the induction of apoptosis for the level of cyclin Changes in the level of cyclin Change is the suppression of DNA synthesis.

#83 Doxycycline-regulated over-expression of spermidine/spermine N¹acetyltransferase (SSAT) inhibits cell growth and enhances sensitivity to the polyamine analog, N¹, N¹¹-diethylnorspermine (DENSPM). Vujcic, S., Halmekytö, M., Jänne, J., Porter, C.W. Roswell Park Cancer Institute, Buffalo, NY, 14263 and A.I. Virtanen Institute, University of Kuopio, Kuopio Finland.

DENSPM is a polyamine analog currently undergoing Phase II clinical evaluation. In addition to down-regulating polyamine biosynthesis, the analog potently upregulates the polyamine catabolic enzyme SSAT. To further address the role of SSAT induction in the regulation of polyamine pools and cell growth, we have established MCF-7 human breast adenocarcinoma cells containing a SSAT gene under the control of a tetracyline-regulatable system. Removal of doxycycline for 4 days caused a 5-fold increase in SSAT RNA and a 10-fold increase in enzyme activity. Putrescine, spermidine and spermine pools were depleted by ~50-75% while N1-acetylated spermidine and spermine pools increased significantly. Cell growth was inhibited by 40% relative to cells grown in presence of doxycycline indicating that SSAT overexpression itself is sufficient to reduce cell growth rate. Treatment of SSAT overexpressing cells with 10 µM DENSPM caused a much more rapid cytotoxic response than in cells, which were not overexpressing the enzyme. The results indicate that induction of SSAT is an important determinant of DENSPM drug action and that therapeutic strategies, which increase SSAT expression, might be useful in cancer therapy. (Supported by CA-76429).

#84 Is inhibition of histone deacetylase responsible for phenylbutyrate (PB)-induced cytostasis and differentiation in myeloid neoplasms? Fu, S. and Gore, S.D. *The Johns Hopkins Oncology Center, Baltimore, MD 21287-8963.*

Sodium PB induces cell cycle arrest, terminal differentiation, and apoptosis in myeloid leukemia cells and has demonstrated clinical activity in Phase I trials in myeloid disorders. The cellular actions of PB appear more similar to sodium butyrate (SB) than phenylacetate. Prominent among the numerous molecular activities of PB may be inhibition of histone deacetylase, similar to SB. We have compared the dose response characteristics of PB for changes in histone acetylation to its pharmacodynamic effects, and examined the pharmacodynamic effects of the histone deacetylase inhibitor Trichostatin A (TSA) in the ML-1 myeloid leukemia cell model. ML-1 cells were cultured in the presence or absence of TSA or PB for 1 – 3 days before evaluation of differentiation, cell cycle parameters, apoptosis, and induction of p21^{WAF1/CIP1}. Unlike PB, TSA did not lead to induction of CD1 lb. Like PB, TSA led to rapid induction of expression of p21, accompanied by G2/M arrest in the case of TSA in contrast to PB treatment which is associated with G1 arrest. TSA was a strong inducer of apoptosis (ED50 \sim 0.2 nM); in contrast the ED50 for apoptosis induction by PB is twice that for CD1 1b induction and cell cycle arrest. Incubation of ML-1 cells with PB induced detectable histone acetylation at doses ranging from 1 to 5 mM; all measured pharmacodynamic endpoints are induced within this dose range. While PB clearly leads to induction of histone acetylation in ML-1 cells, these data suggest that inhibition of histone deacetylase in myeloid leukemia cells may not account for all of the pharmacodynamic actions of PB.

#85 Inhibitors of arachidonic acid metabolism block migration of human glioma cells. Giese A., Zapf S., Kürzel F., Berens M.E.* Dept. Neurosurgery, University Hospital Eppendorf, 20246 Hamburg, Germany *Dept. Neuro-Oncology, Barrow Neurological Institute, Phoenix, Arizona, USA.

Differential mRNA display previously identified thromboxane synthase (Thxsyn) as differentially expressed in migratory glioma cells. We have characterized the expression of cyclooxygenase I and II as well as Thxsyn in glioma cell lines and tissue from various glial tumors. In addition inhibitors of these enzymes were used to block migration of glioma cell lines in vitro. RT-PCR demonstrated Thxsyn expression in 9 out of 11 cell lines, in contrast astrocytes did not express Thxsyn. COX I and COX II were expressed in 8 of 11 and 10 of 11 cell lines respectively, expression in astrocytes was present at low levels. Immunohistochemistry demonstrated COX and Thxsyn expression in gliomas of various grads with high expression in low grade gliomas and decreased and focal expression in the most malignant glioblastomas which also demonstrated the highest proliferation rates. Glioma cell lines showed a wide range of thromboxane-B2 expression by ELISA. Expression correlated with migration rates. Inhibitors of Thxsyn and COX were tested in migration assays, inhibitors such as indomethacin and imidazole had the or no effect. The highly specific Thxsyn inhibitors furegrelate and dazmegrel blocked glioma migration in a dose dependent manner to levels of non-specific notility. In addition cell attachment to matrix was slightly enhanced in a substrate

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independent manner. In contrast cell-cell adhesion was strongly decreased. Thromboxane synthase plays a crucial role in the signal transduction of glioma motility, possibly regulating migration as well as detachment from neighboring cells.

#86 Progressive delayed cardiovascular toxicity in doxorubicin-but not in MEN10755-treated rats. G. Sacco, R. Cirillo, M. Bigioni, S. Venturella, C.A. Maggi, F. Animati and S. Manzini. *Menarini Ricerche, 00040 Pomezia, Italy.*

MEN 10755 is a new disaccharide anthracycline with a broad spectrum of antitumoral activity against a wide panel of human xenografts (Arcamone et al., JNCI, 1997, 16, 1217-1223; Pratesi et al., Clinical C. R., in press). The risk of delayed cardiomyopathy is a major burden in clinical use of anthracycline (R. D. Olson et al., FASEB J., 1990, 4, 3076-3086). The delayed functional cardiotoxic effects (ECG analysis, haemodynamics and isoprenaline-induced cardiac responses) of treatment with equimyelotoxic regimen of MEN 10755 and doxorubicin (1.5 mg/kg i.v. weekly for five times) were investigated (at 4 and 13 weeks after the last treatment) in anaesthetized rats. Doxorubicin induced significant ECG alterations (prolongation of Q α T and S α T intervals) starting from 2 days after last treatment. These cardiotoxic effects were progressive being further worsened after 4 and 13 weeks of recovery period. MEN 10755 induced ECG alterations similar in nature but of lesser severity compared to doxorubicin and in addtion with no progression at 13 as compared to 4 weeks. Significative reduction in mean arterial pressure, heart rate and adrenergic stimulation of contractile performance (LVSP and dP/dt) was also observed, at week 4 and 13, in animals receiving doxorubicin but not MEN10755. In conclusion, with an equimyelotoxic regimen, MEN 10755 produced, as compared to doxorubicin, a minor ECG alterations and cardiac performance impairment. In addition a progressive delayed deterioration (at 13 weeks) of cardiac toxicities was evident in doxorubicinbut not in MEN 10755-treated rats.

#87 Potentiation of doxorubicin (DOX)-induced apoptosis by N-benzyladriamycin-14-valerate (AD198). Koseki Y, Roaten JB, Savranskaya L, Lothstein L, Rodrigues PJ, Israel M, and Sweatman TW. University of Tennessee College of Medicine, Memphis, TN 38163.

AD 198, a novel lipophilic anthracycline, is capable of circumventing multiple forms of DOX resistance (P-gp, MRP and BCI-2 overexpression; at-MDR). Recent studies have shown that AD 198 is a potent PKC inhibitor while its principal metabolite, N-benzyladriamycin (AD 288) and DOX are virtually devoid of this activity. In light of these findings, we have re-examined our previous observation that low doses of AD 198 potentiate the cytotoxicity of DOX (clonogenic assay) in human ovarian (OVCAR-3) carcinoma cells (Koseki et al Proc. AACR 37: 334, 1996). The effects of AD 198 and AD 288 on DOX-induced apoptosis (bisbenz-amide staining) in these cells were determined using conditions [DOX (4.0 μ m) \pm AD 198 or AD 288 (0.3 μ m): 3-hr exposure] identical to those used earlier. At 48-hr and 72-hr following drug exposure, apoptotic indices were control (1.7% vs. 1.9%), DOX alone (2.7% vs. 3.5%), AD198 alone (1.7% vs. 3.2%) and DOX+AD198 (8.5% vs. 11.2%). By contrast, AD 288 (2.9% and 3.2% apoptotic cells at 48-hr and 72-hr, resp.) was without additional effect when admixed with DOX. Thus, the previously reported synergistic effects of AD 198 appear to correlate with the PKC-modulatory activity of this novel compound.

#88 Low doses of anticancer agents induce a senescence-like phenotype and mitotic death in human tumor cells. Chang, B.-D., Broude, E.V., Zhu, H., Xuan, Y., Dokmanovic, M., Ruth, A., Kandel, E.S., Lausch, E., Christov, K., and Roninson, I.B. Depts. of Molecular Genetics and Surgical Oncology (K.C.), University of Illinois at Chicago, Chicago, IL 60607.

Exposure of human HT1080 fibrosarcoma cells to moderate doses of different anticancer drugs and ionizing radiation induces two major non-exclusive responses in the treated cells. The first response, induced in 45-66% of the cells after 4 day exposure to ${\rm ID}_{\rm 85}$ doses of all the tested agents, is mitotic death characterized by the formation of multiple micronuclei. The second response, induced by most agents in 15-79% of the cells, was the appearance of cells that express senescence-associated β -galactosidase (SA- β -gal) and morphologic features of senescent fibroblasts. SA- β -gal+ cells that survive doxorubicin treatment show increased DNA ploidy and proliferative changes associated with senescence, including decreased DNA replication, growth arrest after a small number of cell divisions, and loss of long-term clonogenic potential. Doxorubicin treatment induced features of senescence in 10 of 13 tested cell lines derived from different types of human solid tumors, including all the tested lines with wild-type p53 and half of p53-mutated lines. SA-β-gal expression was also observed in a breast cancer xenograft model after in vivo retinoid treatment. Induction of tumor cell senescence may provide an important component of treatment response in clinical cancer.

#89 Activation of MAP kinase during apoptosis mediated by vinorelbine in MCF-7 cells. Wang, LG., Liu, XM., Kreis, W., Budman, DR., Adams, LM. Don Monti Division of Med. Oncol., NYU School of Medicine, North Shore University Hosp., Manhasset, New York 11030, and Glaxo Wellcome Inc., 5 Moore Drive, Research Triangle Park, NC, 27709.

Vinorelbine (Navelbine) is a third-generation vinca alkaloid frequently used alone or in combination with other anticancer drugs. Our recent study established

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in less than 50 μ M nicotinamide, following DNA damage, ADP-ribose polymer metabolism was abrogated, NAD was consumed and never resumed normal content, and elevations in p53 levels observed in controls as measured by Western blot analyses did not occur. Furthermore, cells with low NAD content showed greatly decreased entry into apoptosis and an increased occurrence of necrosis. These results show that human mammary epithelial cells with suboptimal NAD content have altered DNA damage response pathways and suggest that optimization of niacin status may be preventive in mammary carcinogenesis. (Supported in part by NIH Grant CA65579.)

#4298 Interaction of genistein and other phytoestrogens with estrogen receptor (ER). Balabhadrapathruni, S., Thomas, T.J., Ghosh, S., Gallo, M.A., and Thomas, T. UMDNJ-Robert Wood Johnson Medical School, New Brunswick, NJ 08903

Genistein, the phytoestrogen abundant in soy products, exerts estrogenic effects on ER-positive breast cancer cells. Genistein also has preventive effects on the incidence of human breast cancer. In order to understand these paradoxical effects, we analyzed the binding of estradiol, genistein, and other phytoestrogens to MCF-7 cell ER and to recombinant human ER α . The ability of phytoestrogens to displace [³H]estradiol from ER was determined by competition assays. IC_{50} for displacing [³H]estradiol from recombinant ER α by genistein was 60 nM compared to 900 nM for ER from MCF-7 cells. Other phytoestrogens also showed 10- to 50- fold differences in their ability to displace [³H]estradiol from recombinant ER and cellular ER. Sucrose density gradient analysis of ER α bound to [14C]-genistein showed a monomeric 4S form compared to a dimeric 6S form when bound to [³H]estradiol. These results suggest that ER-associated proteins in MCF-7 cellular ER modulate the affinity of ER and phytoestrogens. ER bound to genistein appears to be deficient in dimerization and therefore coactivators of ER may play a dominant role in genistein-induced changes in cell growth.

#4299

Mechanism of melatonin oncostatic activity in MCF-7 breast tumor cells. Scott, A., Cosma, G., Frank, A., and Wells, R. Dept. Environmental Health, Dept. Pathology, and Dept. Radiological Health Sciences, Colorado State

Clinical and laboratory studies have provided evidence of oncostatic activity by the pineal neurohormone, melatonin. However, these studies have not elucidated its mechanism of action. The following series of MCF-7 breast tumor cell studies, conducted in the absence of exogenous steroid hormones, provide evidence for a novel mechanism of oncostatic activity by this endogenous hormone. We observed a 40-60% loss of MCF-7 cells after 20 hr treatment with 100 nM melatonin, which confirmed and extended previous reports of its oncostatic potency. Interestingly, there were no observed changes in tritiated thymidine uptake, suggesting a lack of effect on cell cycle/nascent DNA synthesis. Further evidence of a cytocidal effect came from morphologic observations of degenerative changes in mitochondria, accompanied by acute necrosis and autophagocytosis. Studies of mitochondrial function via standard polarography revealed a significant increase in oxygen consumption in melatonin treated MCF-7 cells. Enzyme-substrate studies of electron transport chain activities in detergent permeabilized cells demonstrated a 53% increase (p<0.01) in cytochrome c oxidase activity (complex IV). Finally, there was a 64% decrease (p<0.05) in cellular ATP levels in melatonin treated cells, as measured by chemiluminescence. These studies demonstrate an uncoupling of oxidative phosphorylation in melatonin treated tumor cells, whereby this agent exerts its cytotoxicity, and which may represent a novel mechanism of tumor prevention.

#4300 Dual roles for cyclin D1 in human breast premalignancy. Zhou, Q., Fukushima, P., De Graff, W., Miller, F.R., Mitchell, J., Stetler-Stevenson, M., and Steeg, P.S. WCS, Lab. of Path., National Cancer Institute, National Institutes of Health, Bethesda, MD 20892; Karmanos Cancer Institute, Detroit, MI 48201

Cyclin D1 is overexpressed in many human in situ breast carcinoma (DCIS). To test the role of cyclin D1 in early neoplastic progression, we transfected it into human premalignant breast cell line, MCF-10A. The overexpression of cyclin D1 resulted in increased colonization in vitro, suggesting that it may functionally contribute to neoplastic progression. Interestingly, the cyclin D1 transfectants were more sensitive to γ -radiation inhibition of colonization, which was accompanied by increased apoptosis. Apo-2L, but not $TNF-\alpha$, also preferentially inhibited the colonization of cyclin D1 transfected MCF-10A cells. Our results suggest that cyclin D1 overexpression may contribute to human breast neoplastic progression through promotion of colonization; However, colonization remains sensitive to induction of apoptosis by radiation or specific apoptosis signaling pathways, which may be relevant to breast cancer prevention and treatment

#4301 Diets containing whey protein or soy protein isolate protect against DMBA-induced mammary cancer in rats. Badger, T., Hakkak, R., Korourian, S., Shelnutt, S., Ronis, M., and Lensing S. Arkansas Children's Nutrition Center, Little Rock, AR 72202.

Proteins from milk and soybeans are reported to reduce the risk of some cancers. The present study was conducted to determine the possible preventive effects of soy protein isolate (SPI) and whey proteins on 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary cancers in rats. Adult male and female

Sprague-Dawley rats were fed diets made according to the AIN-93G diet formula except that corn oil replaced soy bean oil and the protein source was eithe casein, whey or soy protein isolate. Amino acids were added to equalize the essential amino acids among diets. After 3 weeks, rats fed the same diets were mated and the offspring were weaned to the same diets as the dams. At 50 days of age, female offspring (n = 19-59/group) were orally gavaged with sesame of containing 80 mg/kg DMBA. All rats were killed when at least one palpatable mammary mass was present in 100% of the casein-fed rats. While rats fed either soy or whey had lower (P< 0.05) mean incidence of mammary adenocarcinoma (AC) than casein-fed rats, whey diets provided greater protection (P<0.05). Both soy-fed and whey-fed rats had delayed onset of mammary tumors (P <0.05): These results demonstrate that diets made with soy or whey protein partially prevent chemically-induced mammary AC. Diets formulated with whey protein provided significantly more protection than casein or soy-based diets. Our data further suggest that whey may be the most effective dietary protein source yet identified for mammary cancer prevention. SPI provided by Protein Technology International. Supported by USDA CRIS6251-51000-001-02S.

Inhibitory effect of dibenzoylmethane (DBM) on 7, 12-dimethyl-#4302 benz[a]anthracene (DMBA)-induced mammary tumorigenesis in rodents. Lou Y-R., Lu, Y-P., Xie, J-G., Zhu, B.T., Thomas, P.E., Newmark, H.L., and Huang, M-T. Laboratory for Cancer Research, College of Pharmacy, Rutgers University,

DBM is a naturally occurring substance in licorice. Feeding DBM in the diet inhibited DMBA-induced mammary tumorigenesis in rodents (Carcinogenesis, 19 1697, 1998). Oral intubation of 10 mg of DMBA to Sprague-Dawley rats produced an average of 3.4 mammary tumors per rat, and 92% of rats had mammary tumors at 17 weeks. Feeding 0.5% DBM in the diet to rats only during the initiation period inhibited the number of DMBA-induced mammary tumors by 64%, and the % tumor incidence was inhibited by 41%. Feeding 0.5% DBM in the diet during the post-initiation period inhibited the number of mammary tumors per rat by 25%, and the % tumor incidence was inhibited by 7%. There was little or no effect of dietary DBM on size per tumor when DBM was given during the initiation period. However, the average size per tumor was decreased by 36%, and tumor volume per rat was reduced by 52% when DBM diet was given during the post-initiation. Feeding DBM in the diet to mice or rats increased liver weight, decreased uterine weight, increased levels of certain hepatic cytochrome P-450 enzymes and in vitro DBM inhibited the binding of [³H]estradiol to estrogen receptors. The above actions of DBM may play important roles for its inhibitory actions on breast tumorigenesis in rodents. (Supported by NIH grants CA49756,

#4303

Chemopreventive effects of slow release medroxyprogesterone acetate (MPA) on methylnitrosourea (MNU)-induced mammary carcinogenesis. Hill, D.L., Grubbs, C.J., Lubet, R.A., Eto, I., Kelloff, G.J., Cohen, L.A. and Steele, V.E. University of Alabama at Birmingham, Birmingham, AL 35294, National Cancer Institute, Bethesda, MD 20892, and American Health Foundation,

The progestin medroxyprogesterone acetate (Depo-Provera®) has demonstrated therapeutic activity in breast cancer patients who have failed tamoxifen therapy. When a slow release formulation of MPA (10 or 3 mg/Kg BW) was administered once (subcutaneous) to female Sprague-Dawley rats either 7 days prior to or 7 days following MNU, it profoundly decreased mammary carcinogenesis. In contrast, MPA (10 mg/Kg BW) given 28 days after MNU minimally decreased tumor multiplicity although it did decrease the volume of tumors that were palpable. Therefore, various doses of MPA (30, 10, 3 or 1 mg/Kg BW) were administered beginning 5 days post MNU. The two highest doses of MPA decreased tumor multiplicity > 90%, the 3 mg/Kg BW dose decreased multiplicity 66% while the lowest dose had minimal effects. The effects of this agent on hormonal levels and mammary gland morphology were also examined. The striking efficacy of MPA in rats more closely paralleled human efficacy in contrast to studies in beagle dogs and mice in which MPA reportedly caused early preneoplastic changes. Supported in part by NCI-CN-75101.

#4304

Chemoprevention of hormonal-induced carcinogenesis by antioxidants. Sharma, M. Roswell Park Cancer Institute, Buffalo, NY 14263.

The estrogens (17 β -estradiol and estrone) and the antiestrogen tamoxifen are both known to induce DNA damage by oxidation of some of their hydroxylated metabolites to highly electrophilic quinone derivatives. As observed with other carcinogens, metabolism of estrogens and tamoxifen may occur in the liver and accumulate in susceptible tissues. If unrepaired, DNA adducts induced by such oxidation pathways have potential for endogenous tumor initiators, especially in those tissues with high peroxidase activity. Diethylstilbestrol (Des) which undergoes metabolic redox cycling to generate free radicals and form reactive quinone/ semiquinone intermediate contribute to diethylstilbestrol-induced carcinogenesemiquinone intermediate contribute to diethylstilbestroi-induced carcinogene-sis. Initially, using Des-induced DNA adduct as an indicator, we tested the inhibitory effect of small molecule antioxidants, such as ascorbic acid and Nacetylcysteine on DNA damage induced by Des. Both these agents inhibit the formation of Des quinone in a dose-dependent manner. Extension of this study to inhibit the formation of quinone derivative of 3,4-estrogen catechol and 4-hydroxy tamoxifen will be described. Since antioxidant nutrients are inexpensive and high doses are remarkably nontoxic, their combined use with estrogens in postmeno-

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APPENDIX 4

Effects of Genistein and Structurally Related Phytoestrogens on Cell Cycle Kinetics and Apoptosis in MDA-MB-468 Human Breast Cancer Cells¹

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Running title: Effects of phytoestrogens on MDA-MB-468 cells

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Abstract

We have studied the effects of phytoestrogens, (genistein, quercetin, daidzein, biochanin A and kaempferol), on proliferation, cell cycle kinetics, and apoptosis of MDA-MB-468 breast cancer cells. Genistein and guercetin inhibited cell growth with IC₅₀ values of 8.8 and 18.1 µM, respectively, while the other phytoestrogens were less effective. Flow cytometric analysis showed G₂/M cell cycle arrest with 25 µM and higher concentrations of genistein. At 100 µM, genistein, quercetin and kaempferol caused accumulation of 70, 60 and 35% of cells, respectively, in G₂/M phase by 24 h. In contrast, biochanin A and daidzein were ineffective. APO-BRDU™ analysis revealed apoptosis with 10 μ M genistein (19.5%), reaching 86% at 100 μ M. Apoptosis by genistein was confirmed by Hoechst 33342 staining and fluorescence microscopy. With 100 µM quercetin, 47% of the cells were apoptotic, while the other bioflavonoids had little effect. Genistein treatment resulted in a biphasic response on cyclin B1: 70% increase in cyclin B1 level at 25 μ M, and 50 and 70% decrease at 50 and 100 μ M, respectively. In contrast, the action of guercetin involved an increase in cyclin B1 level. Genistein had no effect on cdc2 level up to 50 µM concentration; however, there was a decrease in the phosphorylated form of the protein at 100 µM. Quercetin had no effect on cdc2 levels. Our results suggest that the action of genistein and guercetin involves G₂/M arrest and apoptosis in MDA-MB-468 cells. Biochanin A and daidzein, although structurally related to genistein, did not share this mechanism. Effects of genistein and quercetin on cyclin B1 level also appear to involve different modes of action. Thus, structurally related phytoestrogens have discrete target sites and mechanisms in their growth inhibitory action on breast cancer cells.

Introduction

Phytoestrogens, such as genistein, quercetin, biochanin A, kaempferol and daidzein are naturally occurring phytochemicals with significant biological activities, including potential estrogenic or growth promoting, and/or anticarcinogenic effects (1-4). The soy derived isoflavonoid genistein, was shown to have important chemotherapeutic and preventive effects in most (5-9), but not all (2, 10, 11), of experimental breast cancer models. In breast cancer cells, the effects of genistein are dependent on the estrogen receptor (ER) status. While low concentrations of genistein (<25 μ M) are growth stimulatory in ER-positive breast cancer cells (11), it is growth suppressive at all concentrations in ER-negative cell lines (8, 9). Quercetin, a flavonoid abundantly present in apples, red wine and onions, has antiproliferative activity against several cancer cell lines including breast cancer cells (12, 13) and has entered Phase I clinical trials (14). Despite the general protective effect of phytoestrogens against breast cancer, their mechanism of growth inhibition and intracellular targets of action remain largely unknown.

The anti-cancer effects of several drugs are mediated by cell cycle arrest and involve modulation of the action of cyclins and cyclin dependent kinases that regulate cell cycle progression (15). Previous studies suggest G_2/M cell cycle arrest after genistein treatment (16-23). Cyclin B1 protein is the catalytic partner of cyclin dependent kinase, p34^{cdc2} kinase (cdc2), that promotes microtubule spindle formation and nuclear membrane breakdown during G_2/M transition (15). Therefore, alterations in cyclin B1 and cdc2 proteins may play an important role in the effects of genistein and other phytoestrogens in breast cancer cells.

Apoptosis has been reported as critical cellular responses to a variety of chemotherapeutic agents, including genistein (23, 24). Apoptosis or programmed cell death is a genetically and metabolically controlled mechanism of cell death, and is characterized by plasma membrane blebbing, shrinkage, chromatin condensation, nuclear fragmentation and formation of membrane bound apoptotic bodies that are removed by phagocytosis (25). Furthermore, apoptosis, following treatment with a wide range of anti-cancer agents, was reported to be associated with cyclin B1 deregulation (26-32) and cdc2 dephosphorylation (33-38).

ER-negative breast cancer patients are challenged with debilitating chemotherapy and a 5-year survival rate of 53% (39). Therefore, new alternatives and additions to traditional chemotherapy are urgently needed. In this context, a better understanding of the mechanisms of action of phytoestrogens on mediators of cell cycle and apoptosis is particularly useful in that they could be used in combination with chemotherapy or radiation therapy against ER-negative tumors. Indeed, genistein and quercetin have been shown to induce radiation sensitivity in H35 hepatoma cells (40).

In the present study, we examined the effects of genistein and a group of structurally related phytoestrogens on estrogen receptor (ER)-negative MDA-MB-468 breast carcinoma cells to understand the pathways involved in phytoestrogen-mediated growth inhibition. Our results indicate G₂/M arrest and apoptosis in the action of genistein and quercetin, but these processes seem to be less important in the action of kaempferol and biochanin A.

Results

Growth inhibitory effects of phytoestrogens. The chemical structure of phytoestrogens used in this study are shown in Fig. 1. In order to determine the effects of phytoestrogens on cell proliferation, MDA-MB-468 cells were treated with 0, 10, 25, 50 and 100 µM concentrations of genistein, guercetin, kaempferol, biochanin A and daidzein. After 24, 48 or 72 h of treatment, cells were pulsed with [³H]-thymidine for 1 h to measure the level of DNA synthesis. Figs. 2A and 2B show the effects of genistein and guercetin treatment on [³H]-thymidine incorporation of MDA-MB-468 cells. Twenty four hour treatment with 10 µM genistein or quercetin significantly reduced [³H]thymidine incorporation by 47 and 32%, compared to the control, respectively (p<0.01, n=6). At 100 µM, genistein suppressed DNA synthesis by 90%, while 70% reduction was found with guercetin. To guantify the effects of these and other phytoestrogens, we determined the IC₅₀ values, the mean concentration of phytoestrogens required for 50% growth inhibition (Table 1). Among the phytoestrogens studied, genistein was the most potent growth inhibitor with an IC₅₀ value of 8.8 \pm 1.6 μ M, followed by quercetin (IC₅₀ = $18.1 \pm 1.6 \mu$ M). Genistein exhibited a 5-fold higher potency than its methoxy derivative, biochanin A. Kaempferol, which differs from quercetin by lacking an -OH group on the 5' position of its B ring, is 2.6-fold less effective than quercetin. Interestingly, a major phytoestrogen in soy, daidzein did not inhibit the growth of MDA-MB-468 cells even at 100 µM concentration.

Effect of phytoestrogens on cell cycle kinetics. To assess the effects of phytoestrogens on cell cycle, cells were treated with these compounds (0-100 μ M) for

24, 48 or 72 h, and stained with propidium iodide. Flow cytometry profiles of cells treated for 24 h with genistein, quercetin and kaempferol are shown in Fig. 3. Genistein and guercetin induced cell cycle arrest by 24 h of treatment. Interestingly, arrest of cells in G_2/M phase reached significant levels only at 25 μ M concentration, although inhibition of DNA synthesis was seen at 10 µM genistein. At 100 µM genistein, 70% of the cells were found in G₂/M phase of the cell cycle. The G₂/M arrest was associated with a corresponding decrease in the percentage of cells in the G₀/G₁ phase of the cell cycle. The percentage of cells in each phase of the cell cycle after 24 h of treatment with phytoestrogens are shown in Table 2. Quercetin treatment at 50 and 100 μ M concentrations showed an accumulation of cells in G₂/M phase leading to 41% and 60% by 24 h, respectively. Kaempferol was less effective than quercetin in inducing a G₂/M arrest. In contrast, biochanin A and daidzein did not have significant effects on cell cycle progression. Similar results were obtained after 48 and 72 h of treatment. Treatment of cells with 100 µM genistein, guercetin, kaempferol, daidzein or biochanin A for 72 h resulted in G₂/M phase accumulation of cells corresponding to 87, 67, 35, 21, 9%, respectively. These results show that inhibition of DNA synthesis occurred with genistein and guercetin, at lower concentration than those required for G_2/M arrest.

Effects of genistein on reversal of cell growth. We next examined if genisteininduced inhibition of DNA synthesis is reversible. If inhibition of DNA synthesis results from moderate changes in the level of certain mediators without irrepairable damage to DNA, recovery of the cells can be expected when the damaging agent is removed.

Cells were treated with genistein for defined time periods, and then the drug was removed and cells treated with drug-free medium. Genistein treatment at 50 and 100 μ M caused irreversible inhibition of DNA synthesis (p<0.05) even after 2 h of exposure (Fig. 4A). After 8 h, genistein treatment with 25 μ M concentration was cytotoxic to these cells, with growth inhibition comparable to cells which had continuous exposure to the drug (Fig. 4B). Furthermore, genistein induced irreversible changes in DNA synthesis of MDA-MB-468 cells by 16 h even at 10 μ M concentration. Thus, inhibition of DNA synthesis observed after 24 h treatment with 10 μ M genistein may be the result of irreversible damage to DNA or other critical components of cell cycle machinery.

Effects of phytoestrogens on apoptotic cell death. MDA-MB-468 cells were treated with phytoestrogens for 24 h and the percentage of apoptotic cells was assessed by the APO-BRDU[™] kit (Pharmingen, CA). In this assay, induction of apoptosis is detected by an increase in DNA fragments that are labeled with bromolated-dUTP and a fluorescent tagged antibody using flow cytometry. Fig. 5 shows a representative view of the cytograms (panel A) and histograms (panel B) obtained after treatment of cells with increasing concentrations of genistein. Treatment of cells with 10, 25, 50, and 100 μM genistein resulted in significant increase in apoptotic cells, 19, 34, 64, 86%, respectively, compared to 1.6% in the control cells (p<0.01, n=4). The effect of phytoestrogen treatment on the percentage of apoptotic cells is presented in Table 3. Quercetin exposure at 100 μM resulted in 47% apoptotic cells, whereas Biochanin A treatment caused 11% apoptosis. In contrast, there was no significant level of apoptosis by treatment with kaempferol and daidzein.

To confirm genistein-induced apoptosis, cells were treated with genistein for 24 h and stained with Hoechst 33342. Stained nuclei were then visualized under a fluorescence microscope. A representative view of dye-stained nuclei is presented in Fig. 6. Nuclear condensation and apoptosis was observed even at 10 to 25 μM concentrations of genistein. Quantification of apoptotic cells by counting non-apoptotic and apoptotic cells yielded values comparable to that of APO-BRDU analysis at 10-25 μM genistein. At 50 to 100 μM genistein, percentage of apoptotic cells counted after Hoechst 33342 staining were not as quantitative as those from APO-BRDU assay probably due to loss of apoptotic cells during staining and washing.

Effects of genistein and quercetin on the expression of cell cycle regulatory proteins. To further understand the genistein-activated pathway that induces cell cycle arrest and apoptosis, the expression of proteins that function during G_2/M phase transition was examined by Western blot analysis. Fig. 7 shows the effect of genistein on the expression of cyclin B1 and cdc2. β actin was used as a control to assess differences in the amount of protein loading and transfer during the Western analysis. Cyclin B1 levels were dramatically altered by genistein treatment. There was a biphasic response with a 70% increase in cyclin B1 level at 25 μ M genistein, compared to the control. In contrast, at 50 and 100 μ M genistein, cyclin B1 level was reduced to 50 and 30% of the control, respectively. This biphasic response of cyclin B1 may represent the ability of genistein to act at multiple levels of gene expression such as transcription, mRNA stability, and/or protein synthesis/degradation (29, 31).

Western blot analysis of cdc2 showed no change at 10, 25, and 50 μ M genistein treatment. However, at 100 μ M genistein, the upper band corresponding to the phosphorylated form of cdc2 showed a marked decrease. This decrease corresponded with a decrease in total phosphorylation levels also, as determined from Western blot analysis using phosphotyrosine antibody (not shown). In contrast, β -actin levels were not altered.

We also examined the effect of genistein on cyclin D1 and cyclin E, the G1 cyclins known to mediate the progression of cells through G1 to S phase. The Western blot analysis using anti-cyclin D1 antibody and anti-cyclin E showed that genistein at 10-100 μ M concentrations did not have significant effects on these cyclins (results not shown).

Fig. 8 shows the effect of quercetin on cyclin B1 and cdc2. Quercetin treatment showed an increase in cyclin B1, with 100 μM treatment resulting in a 30% increase. Even though 100 μM quercetin caused 47% of the cells to undergo apoptosis, unlike genistein, quercetin did not cause a decrease in cyclin B1. Furthermore, the level of cdc2 was unchanged after quercetin treatment. Thus genistein and quercetin elicited different responses on the control of cyclin B1 and cdc2 although both agents caused a significant level of apoptosis in MDA-MB-468 cells.

Discussion

Our results show that genistein and guercetin are highly efficient in inhibiting the growth of MDA-MB-468 cells. Accumulation of cells in the G₂/M phase and apoptotic cell death were prominent features of the mechanism of action of both compounds. However, with genistein, inhibition of DNA synthesis appears not to be dependent of G₂/M arrest because 10 µM genistein was able to suppress DNA synthesis by 53% whereas G₂/M arrest was not apparent at this concentration. Both genistein and guercetin altered cyclin B1 levels, although, the mode of action of these two compounds on the regulation of cyclin B1 appears to be different. With genistein, an increase in cyclin B1 was observed at 25 µM concentration and was followed by a dramatic decrease at higher concentrations. With quercetin, G₂/M arrest and apoptosis are associated with an increase in cyclin B1 protein level. Interestingly, other structurally related phytoestrogens, such as biochanin A and kaempferol only had minor effects on apoptosis, even though these compounds had growth inhibitory effects with IC_{50} of ~45 μ M. Thus, biochanin A and kaempferol may exert growth inhibition by molecular pathways different from that of genistein and quercetin.

Our results extend the findings of G_2/M phase arrest by genistein in MCF-7 (19, 20) and MDA-MB-231 cells (21-23) to MDA-MB-468 breast cancer cells and further link this blockade to dysregulation of cell cycle associated genes and apoptosis. Thus, in contrast to the classic estrogens and antiestrogens which exert their effects in the G_1 phase of the cell cycle in breast cancer cells (41), genistein and quercetin induce a G_2/M block (13, 19-23). The order of potency of genistein > quercetin > kaempferol remains the same with antiproliferative effects as with the efficacy of cell cycle arrest.

Cell cycle arrest by genistein and quercetin suggests that these phytoestrogens may interfere with the synthesis or degradation of cyclins and cyclin dependent kinases (CDKs) or their inhibitors. Our studies link the action of genistein and quercetin with the regulation of cyclin B1. 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 (42). Inappropriate accumulation of cyclin B1 or its untimely degradation, have been reported to induce a G_2 /M arrest in cells (26, 30-32). Taxol treatment was reported to increase cyclin B1 protein in epidermoid carcinoma KB cells, parallel to mitotic arrest and programmed cell death (30). Similarly, treatment of HeLa S3 cells with X-irradiation was associated with G_2 /M arrest and accelerated accumulation of cyclin B1 (32).

Alternately, decreased amount of cyclin B1 protein and G₂ arrest were reported after colcernid treatment (26) and high doses of ionizing radiation (31) in HeLa cells. In our study, 25 μ M genistein treatment resulted in the accumulation of cyclin B1 compared to untreated cells. However, 50 and 100 μ M genistein treatment resulted in a progressive decrease of cyclin B1 protein level. This result is consistent with a recent report showing a decrease in cyclin B1 at 100 μ M genistein in MDA-MB-231 breast cancer cells (22). However, the effect of genistein at lower concentrations on cyclin B1 levels was not examined in this study (22). Our results suggest that accumulation of cyclin B1 occurs early in G₂/M phase, whereas strong apoptotic signals generated at 50 and 100 μ M genistein may lead to a degradation of the protein.

In contrast to genistein, 100 μ M quercetin treatment for 24 h increased cyclin B1 levels compared to untreated controls, similar to the microtubule inhibitor nocodazole

(43). These results suggest that the mechanism of G_2/M arrest by quercetin may follow a similar pathway as low doses of genistein. However, at higher concentrations, genistein may be interacting with other pathways to generate more potent apoptotic effects.

In mammalian cells, the levels of both mRNA and protein of cyclin B1 oscillate between the initiation and completion of mitosis. Growth inhibitory agents alter one of these parameters to deregulate the levels of these proteins, and cause cell cycle perturbations. For example, treatment of HeLa cells with camptothecin was reported to result in cyclin B1 accumulation, due to reduced rate of degradation of the protein (29). Also, irradiation of HeLa cells was reported to decrease cyclin B1 availability for G₂/M transition by delaying its mRNA synthesis during S phase or increase the degradation of the protein in the G₂/M phase, leading to cell cycle arrest (31). It is not clear if the changes seen in cyclin B1 level with genistein and quercetin treatment are due to alterations in the synthesis or degradation of the protein or mRNA.

Premature dephosphorylation of cdc2-tyr 15, has been implicated in apoptotic cancer cell death induced by chemotherapeutic agents such as GL331 (33), 7-hydroxy-staurospaurine (UCN-01) (34), caffeine (36) and taxol (37). It has been suggested that cdc2 elicits a protein phosphorylation cascade that could lead to aberrant mitotic mechanisms that play an important part in apoptotic morphology (33). In our studies, no change in the cdc2 protein level was observed with genistein treatment at 10, 25 and 50 μ M concentrations. However, at 100 μ M genistein, the phosphorylated form of the protein was drastically reduced. Use of phosphorylated tyrosine kinase antibodies also showed similar results in that a decrease in the level of phosphorylation was observed

with 100 μ M genistein (results not shown). Thus, the role of genistein as an inhibitor of tyrosine phosphorylation plays a part in its mechanism of action only at the highest concentration examined. This lack of tyrosine kinase inhibition with low concentrations of genistein is consistent with the observation by Barnes et al (44). In contrast to genistein, quercetin treatment did not have an effect on cdc2 protein in MDA-MB-468 cells.

Quercetin is known to inhibit cdc2 kinase (45) thereby exerting growth inhibitory effects. In addition, quercetin was shown to down-regulate mutant p53 protein in MDA-MB-468 cells, thus leading to significant growth inhibition (13). Genistein has been reported to increase the expression of the CDK inhibitor, p21^{WAF1/CIP1} at the mRNA and protein levels (46), even in a p53 negative cell line (22), thus leading cells through a p21-dependent apoptotic pathway.

It has been known for a long time that genistein and quercetin are potent inhibitors of DNA topoisomerases (47, 48). The ability of genistein and quercetin to inhibit the ATPase component of the topomerization reaction and enhance the topoisomerase II-mediated DNA cleavage has been reported (49). Other topoisomerase II inhibitors were shown to induce DNA double strand breaks leading to a major mitotic arrest (G₂ delay) and, cell death by apoptosis (50). Furthermore, several topoisomerase II inhibitors were reported to preferentially damage cell cycle regulatory genes, such as c-*myc* resulting in decreased DNA synthesis, and increased apoptosis (51, 52). Therefore, it is possible that genistein may inhibit topoisomerases and trigger DNA damage.

Mammalian DNA damage response includes a set of protein kinases such as DNA-dependent protein kinase (DNA-PK), ataxia telangiectasia mutated (ATM), and ataxia telangiectasia and RAD 3 related (ATR) protein kinase (53). Cells defective in these proteins are hypersensitive to DNA damaging agents, as they are unable to mediate repair processes during DNA-damage. In mammalian cells, DNA-PK levels are relatively high, however, in cells undergoing apoptosis, the activity of DNA-PK is lost, and this prevents the repair of damaged DNA (54). Recently, quercetin was reported to inhibit the activity of DNA-PK with a Ki of 110 μ M, suggesting a role for DNA-PK in quercetin-induced apoptosis (55). Inhibition of DNA synthesis as an early response suggests that G₂/M arrest and apoptosis may be the result of DNA-damage. Inhibition of DNA-PK or related processes by genistein and quercetin may facilitate apoptosis at 50 and 100 μ M concentrations.

Our results show that cyclin B1 is altered by genistein and quercetin, although it is not clear at present whether it is a primary or secondary event. Effects of genistein were manifested as irreversible inhibition of DNA synthesis at 50 and 100 μ M concentrations, with as little as 2 h of exposure. Other structurally related phytoestrogens; biochanin A, and kaempferol are less effective in mediating the growth inhibition of these cells. Genistein and quercetin appear to alter additional targets of signal transduction pathway, leading to their diverse effects on DNA synthesis, G₂/M arrest and apoptosis.

Our results may have practical applications for the treatment of ER-negative breast cancer, as ER-positive cells acquire ER-negative phenotype that is more aggressive and resistant to antiestrogen therapy (56). Under these conditions, genistein

and quercetin may be useful to inhibit breast cancer cell growth as their growth inhibitory effects are ER-independent. In this context, it is also important to note that genistein suppressed the nude mice xenografts in ER-positive and -negative breast cancer cells (57). Investigations on the mechanism of action of phytoestrogens and their combination in chemo- and radiation therapy might be a fruitful approach to the design of improved breast cancer therapies.

Materials and Methods

Cell Culture and Chemicals. MDA-MB-468 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, Virginia). The cells were maintained in Improved minimum essential medium (IMEM) with 10% fetal bovine serum (FBS), 4 mM glutamine, 0.4 mM sodium pyruvate, 40 mg/ml gentamycin, and 100 µg/ml each of penicillin and streptomycin.

Genistein, quercetin, daidzein, kaempferol and biochanin A were purchased from Sigma Chemical Co. (St. Louis, MO). Stock solutions were made in dimethylsulfoxide (DMSO), aliquoted, and frozen until use. The APO-BRDUTM kit was purchased from Pharmingen (San Diego, CA). The antibodies for cyclin B1, cyclin D1 and cyclin E were obtained from Neomarkers (Union City, CA). The antibody for C-terminal domain of cdc2 and phosphotyrosine antibody were purchased from Upstate Biotechnology (Lake Placid, NY).

[³H]-Thymidine Incorporation Assay. MDA-MB-468 cells (0.5×10^{6} /dish) were plated in 60 mm culture dishes in triplicate and were allowed to adhere for 24 h. Cells were dosed with phytoestrogens at 0, 10, 25, 50 and 100 µM concentrations for 24, 48 or 72 h. Control group received DMSO, equal to the volume used in drug studies (40 µl/dish). After the treatment period, the cells were pulsed with 1 µCi/ml of [³H]-thymidine for 1 h. Cells were then washed twice in ice-cold PBS and two times in 5% ice-cold trichloroacetic acid and later lysed in 1 N NaOH. Lysates were transferred into vials containing liquid scintillation fluid and the radioactivity was quantitated by scintillation

counting, using a Beckman Scintillation Counter, LS 5000 TD (Beckman Instruments, CA).

Reversal of Cell Growth Inhibition. Cells were plated as described above. Groups of cultures were treated with genistein for different periods, (2, 4, 6, 8, 12, 16, and 24 h), and media changed to drug-free medium. Twenty four hours later, the cells were dosed with 1 μ Ci/ml of [³H]-thymidine for 1 h, and the radioactivity incorporated into cellular DNA was measured by liquid scintillation counting.

Flow Cytometric Determination of Cell Cycle Analysis. MDA-MB-468 cells (2×10^{6} cells/dish) were allowed to adhere to plate for 24 h and then dosed with genistein, or other phytoestrogens. After 24, 48 or 72 h of treatment, media was removed, 2 ml citrate buffer (40 mM Citrate-Trisodium, 250 mM sucrose and 5% DMSO) added and cells frozen at -70°C until further analysis. For cell cycle analysis, cells were thawed and harvested to collect the pellet. The cell pellet was treated with trypsin for 10 min, and trypsin inhibitor and RNase (Sigma, St. Louis, MO) were added for 10 min and stained with 750 µl propidium iodide in citrate buffer (30 µg/ml). Cells were analyzed by an Epics Profile-II Flow Cytometer (Beckman Coulter Inc., Fullerton, CA). Distribution of cells in different phases of cell cycle was calculated using *CytoLogic* software.

Western Blot Analysis. Cells were plated as described above for flow cytometry. Twenty four hours after treatment with genistein, cells were harvested in PBS, centrifuged at 500 x g for 10 min and stored at -70°C until further analysis. Floating

cells were also harvested and added to the adherent cells in each treatment group. Cells were solubulized in 300 µl of a buffer containing 50 mM Tris, 50 mM NaCl, 50 mM NaF, 0.2% SDS, 1% NP40, 2 mM EDTA and 100 µM Na₃Po₄. Total protein was determined using the Bio-Rad kit (Bio-Rad, Hercules, CA) and 30 µg 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 or polyclonal rabbit antibodies, followed by horseradish-peroxidase labeled anti-mouse/anti-rabbit secondary antibody. Protein was visualized with a chemiluminiscence based detection system.

APO-BRDU[™] Labeling Studies. MDA-MB-468 cells (2 x 10⁶/dish) were plated and dosed with genistein for 24 h. Floating and adherent cells were harvested in PBS and fixed in 1% paraformaldehyde. After two washings with PBS, the cell pellet was fixed in 70% ethanol and frozen at -20°C until further use. Apoptosis was quantified using the APO-BRDU[™] kit with minor modifications to the manufacturer's instructions. Briefly, cell pellet was incubated with bromolated deoxyuridine triphosphate (BRDU) for 24 h in a 28°C water bath. After the incubation period, cells were treated with fluorescein-labeled anti-BRDU monoclonal antibody for 1 h, stained with propidium iodide/RNase solution for 30 min in dark, and analyzed by flow cytometry.

Analysis of Apoptosis by Hoechst 33342 Dye Staining. Cells (3.5 x 10⁴/dish) were plated in 4-well LabTek chambered cover glass plates (Nalge Nunc Int., IL). After

allowing for adherence of cells for 48 h, different concentrations of genistein were added to the plates. After 24 h treatment, cells were fixed in 4% paraformaldehyde with 4 μ g/ml Hoeschst 33342 dye and incubated at 37°C for 30 min. Stained nuclei were observed under Zeiss ICM 405 inverted microscope (magnification 1000 X) using a UV filter in the range of 395-450 nm. Apoptotic cells, characterized by nuclear shrinkage and fragmentation, were counted from 3 random fields with at least 100 cells per field.

Statistical Analysis. Statistical analyses were performed using Sigma Plot 3.0 software. Means and standard deviations were calculated for each treated group and the significant difference between the groups were determined using Student's t-test. p value <0.05 was considered significant.

References

- Miksicek, R. J. Commonly occurring plant compounds have estrogenic activity. Mol. Pharmacol., 44: 37-43, 1993.
- 2. Hsieh, C-Y., Santell, R. C., Haslam, S. Z., and Helferich, W. G. Estrogenic effects of genistein on the growth of estrogen receptor-positive human breast cancer (MCF-7) cells *in vitro* and *in vivo*. Cancer Res., 58: 3833-3838, 1988.
- Adlercretz, H., Mousavi, Y., Clark, J., Hockerstedt, K., Hamaleinen, E., Wahala,
 K., Makela, T., and Hase, T. Dietary phytoestrogens and cancer- in *vitro* and *in vivo* studies. J. Steroid Biochem. Molec. Biol., 41: 331-337, 1992.
- Adlercreutz, H., Honjo, H., Higashi, A., Fotsis, T., Hamaten, E., and Hasegawa, T., 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.
- Lamartiniere, C. A., Moore, J., Holland, M., and Barnes, S. Neonatal genistein chemoprevents mammary carcinogenesis. Proc. Soc. Exptl. Biol. Med., 208: 120-123, 1995.
- Constantinou, A. I., Krygier, A. E., and Mehta, R. R. Genistein induces maturation of cultured human breast cancer cells and prevents tumor growth in nude mice. Am. J. Clin. Nutr., 68 (6 Suppl): 1426S-1430S, 1998.
- 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 Lett., 112: 127-133, 1997.

- 8. 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. Biophys. Res. Commun., 179: 661-667, 1991.
- Monti, E., and Sinha, B. K. Antiproliferative effect of genistein and adriamycin against estrogen-dependent and independent human breast carcinoma cell lines. Anticancer Res., 4: 1221-1226, 1994.
- 10. Sathyamoorthy, N., Thomas, T., and Phang, J. M. Stimulation of pS2 expression by diet-derived compounds. Cancer Res., 54: 957-961, 1994.
- Wang, T. T. Y., Sathyamoorthy, N., and Phang, J. M. Molecular effects of genistein on estrogen receptor mediated pathways. Carcinogenesis, 17: 271-275, 1996.
- Singhal, R. L., Yeh, Y. A., Prajda, N., Olah, E., Sledge, G. W. Jr., and Webber,
 G. Quercetin down-regulates signal transduction in human breast carcinoma cells. Biochem. Biophys. Res. Commun., 208: 425-431, 1995.
- 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 Res., 54: 2424-2428, 1994.
- Ferry, D. R., Smith, A., Malkhandi, J., Fyfe, D. W., deTakats, P. G., Anderson,
 D., Baker, J., and Kerr, D. J. Phase I clinical trial of the flavonoid quercetin:
 Pharmacokinetics and evidence for in vivo tyrosine kinase inhibition. Clin.
 Cancer Res., 2: 659-668, 1996.
- 15. Sherr, C. J. Cancer cell cycles. Science, 274: 1672-1677, 1996.

- Traganos, F., Ardelt, B., Halko, N., Bruno, S., and Darzynkiewicz, Z. Effects of genistein on the growth and cell cycle progression of normal human lymphocytes and human leukemic MOLT-4 and HL-60 cells. Cancer Res., 52: 6200-6208, 1992.
- Matsukawa, Y., Marui, N., Sakai, T., Satomi, Y., Yoshida, M., Matsumoto, K., Nishini, H., and Aoike, A. Genistein arrests cell cycle progression at G₂-M. Cancer Res., 53: 1328-1331, 1993.
- Lian, F., Bhuiyan, M., Li, Y-W., Wall, N., Kraut, M., and Sarkar, F. H. Genisteininduced G₂/M arrest, p21^{WAF1} upregulation, and apoptosis in a non-small-cell lung cancer cell line. Nutr. Cancer, 31: 84-91, 1998.
- 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. Cancer, 30A: 1675-1682, 1994.
- 20. Shao, Z-M., Alpaugh, M. L., Fontana, J. A., and Barsky, S. F. Genistein inhibits proliferation similarly in estrogen receptor-positive and negative human breast carcinoma cell lines characterized by p21^{WAF1/C1P1} induction, G2/M arrest, and apoptosis. J. Cell Biochem., 69: 44-54, 1998.
- Fioravanti, L., Cappelletti, V., Miodini, P., Ronchi, E., Brivio, M., and Di Fronzo,
 G. Genistein in the control of breast cancer cell growth: insights into the
 mechanism of action in vitro. Cancer Lett., 130: 143-152, 1998.
- 22. Choi, Y. H., Zhang, L., Lee, W. H., and Park, K-Y. Genistein induced G2/M arrest is associated with the inhibition of cyclin B1 and the induction of p21 in human breast carcinoma cells. Int. J. Oncology, 13: 391-396, 1998.

- 23. Constantinou, A. L., Kamath, N., and Murley, J. S. Genistein inactivates bcl-2, delays the G2/M phase of the cell cycle and, induces apoptosis of human breast adenocarcinoma MCF-7 cells. Eur. J. Cancer, 34: 1927-1934, 1998.
- 24. Li, Y., Upadhyay, S., Bhuiyan, M., and Sarkar, F. H. Induction of apoptosis in breast cancer cells MDA-MB-231 by genistein. Oncogene, 18: 3166-3172, 1999.
- Allen, R. T., Hunter III, W. J., and Agrawal, D. K. Morphological and biochemical characterization and analysis of apoptosis. J. Pharmacol. Toxicol. Methods, 37: 215-228, 1997.
- Sherwood, S.W., and Schimke, R. T. Cell cycle analysis of apoptosis using flow cytometry. In: L. M. Schwartz, and B. A. Osborne (eds.)., Methods in Cell Biology, Vol. 46, pp. 77-97. San Diego: Academic Press, Inc., 1995.
- 27. Katayose, Y., Kim, M., Rakkar, N. S., Li, Z., Cowan., K. H., and Seth, P.
 Promoting apoptosis: a novel activity associated with the cyclin-dependent kinase inhibitor p27. Cancer Res., 57: 5441-5445, 1997.
- Wuerberger, S. M., Pink, J. J., Planchon, S. M., Byers, K. L., Bornmann, W. G., and Boothman, D. A. Induction of apoptosis in MCF-7:WS8 breast cancer cells by β-lapachone. Cancer Res., 58: 1876-1885, 1998.
- 29. Maity, A., Hwang, A., Janss, A., Phillips, P., McKenna, W. G., and Muschel, R. J. Delayed cyclin B1 expression during the G2 arrest following DNA damage. Oncogene, 13:1647-1657, 1996.
- 30. Ling, Y-H., Consoli, U., Tornos, C., Andreeff, M., and Perez-Soler, R. Accumulation of cyclin B1, activation of cyclin B1-dependent kinase and

induction of programmed cell death in human epidermoid carcinoma KB cells treated with taxol. Int. J. Cancer, 75: 925-932, 1998.

- 31. Kao, G. D., McKenna, W. G., Maity, A., Blank, K., and Muschel, R. J. Cyclin B1 availability is a rate-limiting component of the radiation-induced G2 delay in HeLa cells. Cancer Res., 57: 753-758, 1997.
- Ianzini, F., and Mackey, M. A. Delayed DNA damage associated with mitotic catastrophe following X-irradiation of HeLa S3 cells. Mutagenesis, 13: 337-344, 1998.
- Huang, T-S., Shu, C-H., Yang, W. K., and Whang-Peng, J. Activation of CDC 25 phosphatase and CDC 2 kinase involved in GL331-induced apoptosis. Cancer Res., 57: 2974-2978, 1997.
- Wang, Q., Fan, S., Eastman, A., Worland, P. J., Sausville, E. A., and O'Connor,
 P. M. UCN-01: a potent abrogator of G2 checkpoint function in cancer cells with disrupted p53. J. Natl. Cancer Inst., 88: 956-965, 1996.
- 35. Leach, S. D., Scatena, C. D., Keefer, C. J., Goodman, H. A., Song, S. Y., Yang,
 L., and Pietenpol, J. A. Negative regulation of Wee1 expression and Cdc2
 phosphorylation during p53-mediated growth arrest and apoptosis. Cancer Res.,
 58: 3231-3236, 1998.
- 36. Lock, R. B., Galperina, O. V., Feldhoff, R. C., and Rhodes, L. J. Concentrationdependent differences in the mechanisms by which caffeine potentiates etoposide cytotoxicity in HeLa cells. Cancer Res., 54: 4933-4939, 1994.
- 37. Ibrado, A. M., Kim, C. N., and Bhalla, K. Temporal relationship of p34cdc2 activation and mitotic arrest to cytosolic accumulation of cytochrome C and

caspase-3 activity during taxol-induced apoptosis of human AML HL-60 cells. Leukemia, 12: 1930-1936, 1998.

- Shi, L., Nishioka, W. K., Th'ng, J., Bradburg, E. M., Litchfield, D. W., and
 Greenberg, A. H. Premature p34^{cdc2} activation required for apoptosis. Science, 263, 1143-1145, 1994.
- International breast cancer study group. Duration and reintroduction of adjuvant chemotherapy for node-positive premenopausal breast cancer patients. J. Clin. Oncol., 14:1885-1894, 1996.
- 40. van Rijn, J., and van den Berg, J. Flavonoids as enhancers of X-ray induced cell damage in hepatoma cells. Clin. Cancer Res., 3: 1775-1779, 1997.
- 41. Prall, O. W., Rogan, E. M., and Sutherland, R. L. Estrogen regulation of cell cycle progression in breast cancer cells. J. Steroid Biochem. Molec. Biol., 65: 169-174, 1998
- 42. King, R. W., Deshaies, R. J., Peters, J. M., and Kirschner, M. W. How proteolysis drives the cell cycle. Science, 274: 1652-1659, 1996.
- 43. 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 Res., 55: 6021-6025, 1995.
- 44. Peterson, G., and Barnes, S. Genistein inhibits both estrogen and growth factorstimulated proliferation of human breast cancer cells. Cell Growth Differ., 7: 1345-1351, 1996.

caspase-3 activity during taxol-induced apoptosis of human AML HL-60 cells. Leukemia, 12: 1930-1936, 1998.

- Shi, L., Nishioka, W. K., Th'ng, J., Bradburg, E. M., Litchfield, D. W., and Greenberg, A. H. Premature p34^{cdc2} activation required for apoptosis. Science, 263, 1143-1145, 1994.
- International breast cancer study group. Duration and reintroduction of adjuvant chemotherapy for node-positive premenopausal breast cancer patients. J. Clin. Oncol., 14:1885-1894, 1996.
- 40. van Rijn, J., and van den Berg, J. Flavonoids as enhancers of X-ray induced cell damage in hepatoma cells. Clin. Cancer Res., 3: 1775-1779, 1997.
- 41. Prall, O. W., Rogan, E. M., and Sutherland, R. L. Estrogen regulation of cell cycle progression in breast cancer cells. J. Steroid Biochem. Molec. Biol., 65: 169-174, 1998
- 42. King, R. W., Deshaies, R. J., Peters, J. M., and Kirschner, M. W. How proteolysis drives the cell cycle. Science, 274: 1652-1659, 1996.
- 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 Res., 55: 6021-6025, 1995.
- 44. Peterson, G., and Barnes, S. Genistein inhibits both estrogen and growth factorstimulated proliferation of human breast cancer cells. Cell Growth Differ., 7: 1345-1351, 1996.

- 45. Losiewicz, M. D., Carlson, B. A., Kaur, G., Sausville, E. A., and Worland, P. J.
 Potent inhibition of CDC2 kinase activity by the flavonoid L86-8275. Biochem.
 Biophys. Res. Commun., 201: 589-95, 1994.
- 46. Kuzumaki, T., Kobayashi, T., and Ishikawa, K. Genistein induces
 p21(Cip1/WAF1) expression and blocks the G1 to S phase transition in mouse
 fibroblast and melanoma cells. Biochem. Biophys. Res. Commun., 25: 291-295, 1998.
- Okura, A., Arakawa, H., Oka, H., Yoshinari, T., and Monden, Y. Effect of genistein on topoisomerase activity and on the growth of (VAL 12) Ha-rastransformed NIH 3T3 cells. Biochem. Biophys. Res. Commun., 157: 183-189, 1988.
- Yamashita, Y., Kawada, S., and Nakano, H. Induction of mammalian topoisomerase II dependent DNA cleavage by nonintercalative flavonoids, genistein and orobol. Biochem. Pharmacol., 39: 737-744, 1990.
- 49. Robinson, M. J., Corbet, A. J., and Osheroff, N. Effects of topoisomerase IItargeted drugs on enzyme-mediated DNA cleavage and ATP hydrolysis: evidence for distinct drug interaction domains on topoisomerase II. Biochemistry, 32: 3638-3643, 1993.
- 50. Kizaki, H., and Onishi, Y. Topoisomerase II inhibitor-induced apoptosis in thymocytes and lymphoma cells. Adv. Enzyme Regul., 37: 403-423, 1997
- 51. Gewirtz, D.A., Orr, M. S., Fornari, F. A., Randolph, J. K., Yalowich, J. C., Ritke,
 M. K., Povirk, L. F., and Bunch, R.T. Dissociation between bulk damage to DNA and the antiproliferative activity of teniposide (VM-26) in the MCF-7 breast tumor

cell line: evidence for induction of gene-specific damage and alterations in gene expression. Cancer Res., 53: 3547-3554, 1993.

- 52. Orr, M. S., Fornari, F. A., Randolph, J. K., and Gewirtz, D. A. Transcriptional down-regulation of c-myc expression in the MCF-7 breast tumor cell line by the topoisomerase II inhibitor, VM-26. Biochim. Biophys. Acta., 1262: 139-145, 1995.
- Hoekstra, M. F. Responses to DNA damage and regulation of cell cycle checkpoints by the ATM protein kinase family. Curr. Opin. Genetics & Develop., 7: 170-175, 1997.
- 54. Jackson, S. P. DNA-dependent protein kinase.Int. J. Biochem. Cell Biol., 29: 935-938, 1997.
- 55. Izzard, R. A., Jackson, S. P., and Smith, G. C. M. Competitive and noncompetetive inhibition of the DNA-dependent protein kinase. Cancer Res.,
 59: 2581-2586, 1999.
- Petrangeli, E., Lubrano, C., Ortolani, F., Ravenna, L., Vacca, A., Sciacchitano,
 S., Frati, L., and Gulino, A. Estrogen receptors: new perspectives in breast
 cancer management. J. Steroid Biochem. Molec. Biol., 49: 327-331, 1994.
- 57. Shao, Z. M., Wu, J., Shen, Z. Z., and Barsky, S. H. Genistein exerts multiple suppressive effects on human breast carcinoma cells. Cancer Res., 58: 4851-4857, 1998.

Table 1. IC₅₀ values for phytoestrogens needed for cell growth inhibition

MDA-MB-468 cells were treated with each of the phytoestrogens at 0, 10, 25, 50, 100 μ M concentrations for 24, 48 or 72 h. Cell growth was determined by [³H]-thymidine incorporation assay. IC₅₀ value was calculated from the growth curves as the concentration that inhibited 50% of cell growth. The values are an average of two separate experiments conducted in triplicate.

Phytoestrogen	IC ₅₀ , μ M (concentration ± SD)		
Genistein	8.8 ± 1.6		
Quercetin	18.1 ± 1.6		
Biochanin A	44.0 ± 10.1		
Kaempferol	47.0 ± 2.9		
Daidzein	>100		

Table 2. Effect of phytoestrogens on cell cycle distribution of MDA-MB-468 cells

Flow cytometric analysis was performed and the percentage of cells in the G_0/G_1 , S and G_2/M phases of the cell cycle was calculated using the *CytoLogic* software, after treatment of MDA-MB-468 cells with different phytoestrogens for 24 h. *Significantly different from control, p<0.05, (n = 6).

Phytoestrogen	Concentration	G ₀ /G ₁	S	G₂/M
Control	0	59.0 ± 1.7	22.4 ± 1.1	17.5 ± 1.1
Genistein	10 μΜ 25 μΜ 50 μΜ 100 μΜ	$65.5 \pm 1.6^{*}$ $47.9 \pm 0.9^{*}$ $27.7 \pm 0.3^{*}$ $27.3 \pm 3.2^{*}$	$18.3 \pm 1.6 \\ 23.3 \pm 1.3 \\ 17.0 \pm 0.9 \\ 3.0 \pm 0.9^*$	$\begin{array}{c} 16.2 \pm 1.9 \\ 33.2 \pm 0.7^{*} \\ 49.5 \pm 0.7^{*} \\ 70.0 \pm 4.3^{*} \end{array}$
Quercetin	10 μΜ 25 μΜ 50 μΜ 100 μΜ	$52.1 \pm 2.1^*$ $50.5 \pm 0.7^*$ $48.4 \pm 1.3^*$ $35.0 \pm 5.1^*$	$\begin{array}{c} 32.6 \pm 4.3^{*} \\ 27.7 \pm 1.2 \\ 24.7 \pm 2.5 \\ 5.0 \pm 2.1^{*} \end{array}$	$15.2 \pm 2.5 \\ 21.7 \pm 0.7 \\ 41.2 \pm 2.2^* \\ 60.0 \pm 5.2^*$
Kaempferol	10 μΜ 25 μΜ 50 μΜ 100 μΜ	$58.8 \pm 0.2 \\ 58.2 \pm 0.5 \\ 46.4 \pm 4.2^* \\ 45.6 \pm 2.7^*$	20.9 ± 0.9 20.6 ± 1.3 19.5 ± 0.9 19.2 ± 1.1	20.3 ± 1.1 21.2 ± 1.7 $34.1 \pm 3.3^*$ $35.1 \pm 1.7^*$
Biochanin A	10 μΜ 25 μΜ 50 μΜ 100 μΜ	59.7 ± 0.4 60.9 ± 0.1 60.3 ± 1.2 59.7 ± 1.6	$22.9 \pm 1.1 \\ 20.7 \pm 1.1 \\ 20.9 \pm 1.0 \\ 20.9 \pm 0.1$	$\begin{array}{c} 17.4 \pm 0.8 \\ 18.3 \pm 1.2 \\ 18.7 \pm 0.5 \\ 19.3 \pm 1.5 \end{array}$
Daidzein	10 μΜ 25 μΜ 50 μΜ 100 μΜ	57.1 ± 1.8 52.6 ± 1.2 53.8 ± 5.7 52.2 ± 5.7	$\begin{array}{c} 19.5 \pm 1.1 \\ 20.7 \pm 1.1 \\ 19.1 \pm 0.7 \\ 22.5 \pm 3.9 \end{array}$	20.0 ± 2.7 23.6 ± 2.5 22.8 ± 5.4 21.8 ± 2.3
Table 3. Effect of phytoestrogens on percentage apoptosis of MDA-MB-468 cells

APO-BRDUTM analysis for apoptosis was determined after treatment of MDA-MB-468 cells with different phytoestrogens for 24 h. DNA fragments in apoptotic cells are end-labeled with BRDU and incubated with fluorescent tagged BRDU-specific antibody. Fluorescence intensity generated is proportional to the percentage apoptosis as determined by flow cytometry. *Significantly different from control, p<0.01, (n = 6).

Phytoestrogen

% apoptosis by 24 h treatment with:

	0 μΜ	10 μΜ	25 μΜ	50 μM	100 μM
Genistein	1.6 ± 0.6	19.5 ± 9.8*	34.7 ± 8.3*	64.3 ± 15.0*	86.0±4.0*
Quercetin	1.6 ± 0.6	3.3 ± 0.4	12.3 ± 1.5*	18.1 ± 5.6*	47.2 ± 1.5*
Biochanin A	1.5 ± 0.6	1.5 ± 0.1	3.8 ± 1.3	6.1 ± 0.3*	11.2 ± 1.2*
Daidzein	1.5 ± 0.8	1.3 ± 0.1	2.4 ± 0.1	2.9 ± 0.1	1.7 ± 0.6
Kaempferol	1.9 ± 0.1	2.5 ± 1.7	3.4 ± 0.7	3.7 ± 0.7	4.8 ± 0.4**

Legend to Figures

Fig 1. Chemical structures of $17-\beta$ -estradiol and phytoestrogens used in this study.

Fig 2. Effects of genistein and quercetin on cell proliferation. MDA-MB-468 cells were treated with 0 (口), 10 (四), 25 (函), 50 (目) and 100 μ M (III) concentrations of (A) genistein or (B) quercetin for 24, 48 or 72 h. DNA synthesis was measured by pulse labeling for 1 h with 1 μ g/ml [³H]-thymidine. *Significant difference from control (p<0.05). Data are the mean ± SD from two experiments.

Fig 3. Flow cytometry profile of cell cycle distribution of MDA-MB-468 cells. Cells were treated with 0, 10, 25, 50 and 100 μ M concentrations of (A) genistein, (B) quercetin or (C) kaempferol for 24 h. RNase treated and propidium iodide stained cells were sorted by flow cytometry.

Fig 4. Growth reversal of MDA-MB-468 cells after treatment with genistein. Cells were treated with increasing concentrations of genistein for (A) 2, 4, 6 h and (B) 8, 16 and 24 h. After treatment, media was replaced with fresh media without drug for an additional 24 h and $[^{3}H]$ -thymidine incorporation assay was performed as described in Materials and Methods. *Significant difference from control (p<0.05). The experiment was performed in triplicate. Error bars indicate SD.

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Fig 5. Genistein-induced apoptotic cell death of MDA-MB-468 cells. Cells were treated with 0, 10, 25, 50 and 100 μ M concentrations of genistein for 24 h. Harvested cells were stained with both propidium iodide and fluorescein-BRDU monoclonal antibody and were analyzed by flow cytometry. Panel A: cytograms with cell number on X axis vs. relative fluorescence intensity (log green fluorescein BRDU) on Y axis. Panel B: histograms showing percentage of apoptotic cells. X axis, fluorescence intensity; Y axis, cell number. Similar results were obtained in two separate experiments.

Fig 6. Genistein induced apoptotic cell death of MDA-MB-468 cells as determined by 33342 Hoechst staining. Cells were treated with genistein (0, 10, 25 and 50 μ M, panels A through D, respectively) for 24 h and fixed in 4% paraformaldehyde. Then the cells were treated with Hoechst 33342 dye and the stained nuclei were visualized under fluorescence microscope. Irregular and condensed or fragmented nuclei were observed as characteristic features of apoptosis. To quantify apoptosis, cells from three different fields were counted, with about 100 cells per field.

Fig 7. Western blot analysis of cyclin B1, cdc2 and β -actin expression in MDA-MB-468 cells treated with 0, 10, 25, 50, 100 μ M of genistein for 24 h. Protein lysates were electrophoresed, transferred on to a nitrocellulose membrane and treated with antibodies against cyclin B1, cdc2 or β -actin. Similar results were obtained in three independent experiments. Variations in intensity were less than 10%.

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Fig 8 Western blot analysis of cyclin B1 and cdc2 protein expression in MDA-MB-468 cells treated with 0, 10, 25, 50 and 100 μ M concentrations of quercetin for 24 h. Protein lysates were electrophoresed, transferred onto a nitrocellulose membrane and probed with an antibody against cyclin B1 or cdc2. Similar results were obtained in two separate experiments.



[³H]-THYMIDINE INCORPORATION, % CONTROL



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