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13. ABSTRACT (Maximum 200 Words)

Epidemiological studies suggest that consumption of soy phytoestrgoens, such as genistein, is associated with a decrease risk of breast cancer. Genistein has been shown to exert chemopreventive and antiproliferative in animal models of cancer and breast cancer cells respectively. Accumulating evidence suggest that genistein is oxidatively metabolized by the cytochrome P450 (CYP450) family of enzymes, and that its metabolism is specific to breast cancer cells but not nontransformed breast epithelial cells. We investigated the cellular association and effects of metabolism of genistein by CYP450 in T47D breast cancer cells and MCF10A breast epithelial cells. These studies show that genistein exerts distinct effects on cell cycle progression that is due to its preferential cellular uptake and extent of its metabolism in T47D cells but not MCF10A cells. Few studies describe the cell cycle effects of genistein on nontumorigenic breast epithelial cells. We compared the effects of genistein on cell proliferation, cell cycle progression, cell cycle checkpoints in T47D breast cancer cells and MCF10A nontumorigenic breast epithelial cells. In T47D breast cancer cells, genistein caused G2 arrest with inhibition of cyclin dependent kinase activity at 25 μM or higher with concomitant upregulation of cyclin A and B. Upregulation of cyclin dependent kinase inhibitors, p21 and p27 expressions were not sufficient to inhibit kinase activity. Collectively, these data show that genistein the mechanism for cell cycle arrest involves deregulation of cdc-2/cyclin B complex or cyclin B/cyclin B kinase complex through CDK inhibition. In contrast, there was less G2 arrest in MCF10A nontumorigenic breast epithelial cells with increases in kinase activity.

14. SUBJECT TERMS
Isoflavone, genistein, T47D cells, MCF10A cells, cell cycle, uptake

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A. Introduction

Epidemiological evidence and studies in cancer models suggest that phytoestrogens reduce the risk of breast cancer ((Ingram et al., 1997, Cappelletti et al., 2000). The potential breast cancer preventive effects of phytoestrogens have been attributed to their weak estrogenic or anti-estrogenic effects (Mayr et al., 1992), their ability to reduce the expression of stress response-related genes (Zhou and Lee, 1998), antiangiogenic actions (Fotsis et al., 1995, Fotsis et al., 1997, Fotsis et al., 1998) and their antioxidant activity (Ruiz-Larrea et al., 1997). Further, phytoestrogens have also been demonstrated to exert anti-proliferative effects in breast cancer cells (Dampier et al., 2001, Constantinou et al., 1998). However, the precise mechanism(s) of the antiproliferative effects of phytoestrogens in nontumorigenic breast epithelial cells is not currently known. This, together with the need for development of specific anticancer drugs that target uncontrolled cell cycle machinery, warrants vigorous research on the molecular mechanisms of the action of phytoestrogen in breast cancer. The relevance and goals of this research are summarized in these three critical issues: *(1) The cellular uptake and metabolism of phtyoestrogen into tumorigenic and nontumorigenic breast epithelial cells.* Although genistein has been reported to exert antiproliferative actions in *in vitro* tumor models, the relevance of these studies have been questionable because the high micromolar concentrations are used in these experiments exceed the level of free genistein concentrations in serum. Interestingly, the concentration of free genistein in endocrine-responsive rat tissues is greater than in serum (Chang et al., 2000). However, the uptake of genistein into breast cancer cells has not been determined. Determination of intracellular levels of genistein will increase the relevance of studies using *in vitro* breast cancer models. Because its effective concentration *in vitro* is in the high micromolar range, it is possible that some metabolites of genistein are the actual active compound. Therefore, studying the cellular metabolites of genistein is also important. Growing evidence suggest that genistein is oxidatively metabolized by the cytochrome P450 family of enzymes, and that its metabolism is specific to transformed breast epithelial cells and not nontumorigenic breast epithelial cells (Peterson et al., 1996). The cytochrome P450 (CYP450) superfamily of genes encodes a variety of proteins found in most tissues, and these enzymes catalyze the metabolism of endogenous compounds as well as xenobiotics (Guengerich et al., 1998). The expression of several CYP450 isoforms has been linked to many types of human cancers (Patterson and Murray, 2002). For example, CYP1B1 is overexpressed in breast tumors as well as lung, liver, gastrointestinal tract, prostate, and bladder. Anticancer agents can be designed to exploit this feature Several lines of evidence suggest that genistein may be an effective prodrug that requires specific CYP450 activation in tumor cells: (1) Genistein can be oxidatively metabolized in vitro by human microsomes containing CYP450 (2) Tumor cells, including breast cancer cells, express high levels of CYP450 compared to nontumorigenic cells (3) Existing studies demonstrate that genistein is not metabolized in nontumorigenic breast epithelial cells to the same extent as in breast tumor cells. However, the possible role of CYP450-mediated oxidation of genistein in formation of biologically active metabolite(s) of genistein has not been clearly identified. Therefore, the objectives of this study were: (1) to compare the cell-association of isoflavone

genistein. (2) to identify possible biologically active metabolites of genistein in T47D breast cancer cells and MCF10A breast epithelial cells. *(2) to compare the effects of phytoestrogen on cell cycle components in tumorigenic and nontumorigenic breast epithelial cells.* Studies in breast cancer cells support a role for genistein in the modulation of cell cycle leading to the inhibition of cell proliferation. However, few studies describe the effects of genistein on nontumorigenic breast epithelial cells. In the present study, we compared the effects of genistein on cell proliferation, cell cycle progression, cell cycle checkpoints that included: (i) cyclin B1 expression (ii) cdc-2 and cyclin B kinase activities and, (iii) cyclin dependent kinase inhibitors p21 (WAF1) and p27(kip1) in tumorigenic and nontumorigenic breast epithelial cells. *(3) It addresses the major molecular and cellular mechanisms of action of phytoestrogens involved in signal transduction that is involved in cell proliferation and apoptosis.*

Hypothesis: *Differential uptake and metabolism of genistein into tumorigenic breast epithelial cells accounts for differences in cellular responses between tumorigenic and nontumorigenic breast epithelial cells*

B. The tasks referred to in the Statement of Work aimed at proving this hypothesis are:

1. To determine cellular uptake of phytoestrogens (i.e. genistein) into tumorigenic and nontumorigenic breast epithelial cells

2. To determine biologically metabolites of genistein in tumorigenic and nontumorigenic breast epithelial cells

3. To compare the molecular mechanisms involved in phytoestrogen-mediated modulation of key cell cycle proteins such as cyclin dependent kinase inhibitors in breast tumor cells and the subsequent effects on cell cycle and apoptosis in tumorigenic and nontumorigenic breast epithelial cells.

C. Materials and Methods

Tissue culture

T47D tumorigenic and MCF10A nontumoriegnic breast epithelial cells were used for all the experiments described below. MCF10A nontumorigenic human breast epithelial cells were incubated in DMEM/F12 media supplemented with 5 % (v/v) Horse Serum (Gemini Bioproducts), 2.5 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco), 20 ng/ml EGF, 100 ng/ml cholera toxin, 10 μg/ml bovine insulin, 500 ng/ml hydrocortisone. T47D breast cancer cells were cultured in RPMI media supplemented with 10 % Fetal Bovine Serum (Gemini Bioproducts) and 1 % penicillin/streptomycin (Gibco). Cells were placed in an incubator with 5% carbon dioxide-air at 37^{0}C. RAT 1A and c-myc RAT1A fibroblasts were grown in low glucose DMEM with 10 % FBS and 5 µg/ml G418.

MTT cytotoxicity assay

Cells were seeded at 3000 per well into 96 well plates and cell growth was determined using the Cell Titer 96 Well Non-Radioactive Cell Proliferation kit (Promega). Cells were challenged with genistein (0, 1, 5, 10, 25, 50, 100, 200 μM) for 24, 48, 72 and 96 hours. Briefly, MTT dye was added to the wells for 4 hours prior to the addition of solubilization buffer. The microtiter plate was then read at 590nm.

Association of genistein to cultured cells

The association of genistein to T47D breast cancer cells following incubation was performed as previously described (Spencer et al., 2001). Briefly, genistein will be incubated for 2 and 24 hours with T47D breast cancer cells using various concentrations (5-50 μM). Following this incubation, the cells are washed extensively with ice-cold PBS before lysis in aqueous acidified methanol. The lysed cells are then scraped from the plates, the lysates collected, vortexed, and centrifuged (14000 g for 10 minutes at 4 degree celcius). The supernatant was analyzed by HPLC with photodiode array as previously described (Kuhnle et al., 2000)

Western analysis

Cells were plated on 100mm diameter dishes and were 70 % confluent for all studies. Cells were treated with varying concentrations of genistein for 24 hours (0-100μm) and harvested and lysed with RIPA buffer. Protein concentration was determined by the Bradford method (Biorad). 30μg of total protein from each sample was separated on a 12% SDS-polyacrylamide gel, and then transferred to a nitrocellulose membrane (Amersham). The membrane was then blocked using 5 % casein/tris buffered saline (TBS) (Pierce) for 30 minutes prior to overnight incubation with the appropriate primary antibody at 4° C. Following that, the membrane was washed for 10 minutes for 4 times in TBS/tween and then incubated with secondary antibody (anti-mouse or anti-rabbit). Detection was achieved using electrochemiluminescence (ECL) (Amersham) at 1 and 5 minutes of exposure of radiographic film (Kodak). Western analysis was performed for p21, Apaf-1, caspase 3, caspase 9, cdc2, STAT3, Bcl-2, and Bcl-xl. All antibodies were purchased from Santa Cruz Antibodies. The cdc-2 activity was performed using the histone H1 kinase assay as previously described (Qui et al., 1996).

Apoptosis: TUNEL Assay

Cells ($5X10^5$) were plated onto Labtek two-chambered slides and were treated with genistein (0, 10, 25, 50, 100 μM) for 24 and 48 hours. Apoptotic cells were identified using the DeadEnd™ Colorimetric Apoptosis Detection System (Promega). This assay involves end-labeling the fragmented DNA of apoptotic cells using a modified TUNEL (TdT-mediated dUTP Nick-End Labeling) assay. Biotinylated nucleotide is incorporated at the 3′-OH DNA ends using the enzyme Terminal Deoxynucleotidyl Transferase (TdT). Horseradish-Peroxidase-Labeled Streptavidin (Streptavidin HRP) is then bound to these biotinylated nucleotides, which are detected using the peroxidase substrate, hydrogen peroxide, and the stable chromogen, diaminobenzidine (DAB). Using this procedure, apoptotic nuclei are stained dark brown.

Soft agar assay
C-myc RAT 1A cells were grown in Pour 0.7% agar in appropriate medium without FBS and autoclave 6 well dishes containg 0.7 % agar and DMEM (low glucose) and 10 % FBS. Genistein was added in varying concentrations [5-50 μM] for 7 days. Following seven days, wells were examined using light microscopy and photographed.

Drugs
Genistein was purchased from Sigma and dissolved in DMSO to make a 50mM stock.

Other methods
Other relevant methodologies are discussed in the two manuscripts attached in the appendices.

D. Results and discussions

(i) *Cellular association and metabolism of genistein in T47D tumorigenic and MCF10A nontumorigenic breast epithelial cells: bioreactivity and cytochrome P450 involvement*
-See attached manuscript to be submitted to Int. J. Cancer

(ii) *Contrasting the effects of genistein on cell proliferation and cell cycle arrest in nontumorigenic human breast epithelial cells and human breast cancer cells: involvement of Cdc-2, cyclin B1 kinase, and p27/kip1.*
-See attached manuscript to be submitted to Int. J. Cancer

(iii) *The effects of genistein on cell proliferation and apoptosis in T47D breast cancer cells*

Suppression of the anti-apoptotic members or activation of the pro-apoptotic members of the Bcl-2 family leads to altered mitochondrial membrane permeability resulting in release of cytochrome c into the cytosol. In the cytosol, or on the surface of the mitochondria, cytochrome c is bound by the protein Apaf-1 (apoptotic protease activating factor), which also binds caspase-9 and dATP. Binding of cytochrome c triggers activation of caspase-9, which then accelerates apoptosis by activating other caspases. Interestingly, both Bcl-2 and Bcl-xl, anti-apoptotic proteins were suppression following 24 hours of treatment with 10. μM genistein and higher (fig 1). This suggests that mitochondria are sensitive to physiologically relevant doses of genistein (1-10 μM) and may represent a potential target for phytoestrogens. In addition, Apaf-1 induction was also observed (fig 2). In parallel with this, caspase 9 (fig 3) was activated and procaspase 3 protein expression was upregulated (fig 4). Collectively, these data suggest that genistein may mediate apoptosis via targeting mitochondria at physiologically achievable doses. To confirm whether the activation of these proteins lead to apoptosis or not, TUNEL staining was performed, to measure DNA fragmentation, using T47D cells exposed to genistein for 24 (fig 5). DNA fragmentation was evident in at 25 μM, and to a greater extent with 100 μM genistein. These figures correlate with the relative ability

of these doses to inhibit cell proliferation and cause cell cycle arrest at the similar time courses

(iv) *Genistein and oncogenes*

We have initiated an effort to understand the chemopreventive effects of genistein. We speculate that genistein may have target specific oncogenes, and therefore be more effective in the presence or absence of certain oncogenes. We compared the effects of genistein on cell proliferation in RAT1A fibroblast cells, and RAT1A stably transfected with c-myc oncogenes over 6 days. In this model, genistein affected cell viability, as measured by MTT reduction, to a greater extent in RAT1A c-myc stable transfects (fig .6), suggesting that genistein may have selectivity for the c-myc oncogene. This may be due to either a differential effect on apoptosis or cell proliferation. At present, work is being carried out to determine whether this selective effect on cell viability is due to induction of apoptosis. Fig 7 shows that the genistein induced apoptosis to the same extent in both cell lines, indicating that apoptosis does not account for difference in cell viability between the two cell lines. Future experiment will include an investigation to determine if there is a selective effect of genistein on cell proliferation. The effects of genistein on cellular transformation, using soft agar, was also examined. Approximately 30-70% of breast tumors are c-myc positive C-myc RAT1A cells are reasdily transformable because of the overexpression of the c-myc oncogene. Thus, we explored whether genistein was capable of preventing cellular/oncogenic transformation. Our experiments show that genistein [25 μM] treatment reduced the number of visible colonies using c-myc RAT1A cells (fig 8). Future experiments will include a comparison of the effects on genistein on other oncogenes (i.e. her2/neu, ras) and also to elucidate the effects of genistein on neoplastic transformation and its cellular mechanisms.

E. Key Research Accomplishments

-Cell-associated levels of genistein and of a genistein metabolite in T47D tumorigenic and MCF10A nontumorigenic breast epithelial cells were determined.

-Selective uptake or cell association of genistein in T47D tumorigenic but not MCF10A nontumorigenic breast epithelial cells

-Higher cellular association of metabolite in T47D tumorigenic breast epithelial cells compared with MCF10A nontumorigenic breast epithelial cells

-Inhibition of CYP450 by cimetidine reversed cell cycle arrest in G2-M phase, suggesting that CYP450 oxidation may yield a bioreactive metabolite of genistein in T47D tumorigenic breast epithelial cells

-Genistein elicits antiproliferative effects in both exponentially growing T47D tumorigenic and MCF10A nontumorigenic breast epithelial cells

-Genistein causes G2/M phase cell cycle arrest at 25-100μM after 96 hours of treatment with genistein in T47D cells, but not MCF10A cells.

-Different effects of genistein on cyclin dependent kinase activities, as well as p27/kip1

-Upregulation of cyclin B1 by genistein in T47D cells, with a less pronounced upregulation in MCF10A cells

-Genistein induces apoptosis via a mitochondrial-linked apoptotic pathway. Evidence for this includes: (a) downregulation of antiapoptotic protein bcl-xl (b) upregulation of Apaf-1, caspase 9 and 3 (c) positive TUNEL staining in cells treated with genistein.
-Differential selectivity of genistein towards c-myc stably transfected RAT1A cells. This may represent a potential mechanism for chemoprevention.
-Genistein can prevent cellular transformation of c-myc RAT1A fibroblasts

F. Reportable Outcomes

Abstracts

Nguyen D.T., Garcia J., and Cadenas E. Genistein causes cell cycle arrest via inhibition of p53-independent mechanisms in T47D breast cancer cells. (2000) Free Radical Biology and Medicine 29:S101

Nguyen D.T., Garcia J., and Cadenas E. Genistein cause cell cycle arrest and apoptosis via inhibition of p53-independent mechanisms in T47D breast cancer cells. (2001) Free Radical Biology and Medicine 31: S105

Nguyen D.T., Garcia J., and Cadenas E. Genistein arrests cell cycle and induces apoptosis in T47D breast cancer cells. (2002) Proceedings from the Oxygen Club of California: Oxidants and Antioxidants in Biology p155

Awards

Young Investigator Award by the Oxygen Club, 2000 to Dominique T. Nguyen

Submitted manuscripts

(1) Nguyen D.T., Spencer J.P.E, Rice-Evans C., and Cadenas E. Selective cellular association and metabolism of genistein in T47D tumorigenic but not MCF10A nontumorigenic breast epithelial cells: role for CYP450 in formation of a bioreactive metabolite of genistein. Submission to Int J Cancer for review process
(2) Contrasting the effects of genistein on cell proliferation and cell cycle arrest in nontumorigenic human breast epithelial cells and human breast cancer cells: involvement of Cdc-2, cyclin B1 kinase, and p27/kip1. Submission to Int J Cancer for review process

Degree obtained

The cellular association, metabolism, and cell cycle effects of genistein on T47D breast cancer cells and MCF10A nontumorigenic breast epithelial cells.
Dominique T. Nguyen, Ph.D. (Molecular Pharmacology & Toxicology), July 2003

Employment received based upon research experience supported by this award

Dominique T. Nguyen, Ph.D.
Associate Scientist, Hawaii Biotech

H. Conclusions

Based upon these data, genistein is selectively taken up and metabolized to a bioreactive form via CYP450 oxidation in T47D tumorigenic breast epithelial cells. Contrary to this, little genistein uptake or metabolism was associated with MCF10A cells. Genistein also targeted cyclin dependent kinases (CDK) in T47D cells by inhibiting their activities, but increased CDK activity in nontumorigenic MCF10A cells. Interestingly, mitochondria appear very sensitive to genistein, as evident by its regulatory effects on bcl-xl, Apaf-1, caspase 9 and caspase 3 with treatments of genistein from 1-10μM. However, the effects on these proteins did not necessarily correspond with the ability of genistein to induce apoptosis. After 24 h, positive TUNEL staining was not present in cells treated with doses less the 25 μM. Indeed, this finding is highly relevant in that the anticancer effects of phytoestrogens are believed to be developed after long period of soy consumption in the diets of Asian women. Thus, effects exerted by genistein at these low doses may reflect long-term effects that may contribute to its anticancer abilities. We also have preliminary evidence suggesting that genistein may prevent cellular transformation *in vitro*. Presently, we are in the process of identifying the genistein metabolite, as well as further investigating the effects of genistein on transcriptional of key cell cycle proteins including cyclin A and B1. Given the increasing popularity of soy phytoestrogen, it is important to assess the cellular uptake, metabolism, and effects in both tumorigenic and nontumorigenic breast epithelial cells.

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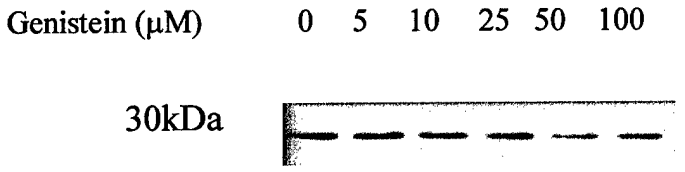
A.



B.

Figure 1. The effects of genistein on (A) Bcl-xl and (B) Bcl-2 expression in T47D breast cancer cells following 24 hour treatment. A representative of three separate western blots. 3(μg of total protein was separated on a 12% SDS-PAGE gel, transferred to a nitrocellulose membrane, and incubated with Apaf-1 antibody.

Figure 2. The effect of genistein on Apaf-1 expression in T47D cells after 24 h treatment. A representative of three separate western blots. 30 μg of total protein was separated on a 12% SDS-PAGE gel, transferred to a nitrocellulose membrane, and incubated with Apaf-1 antibody.

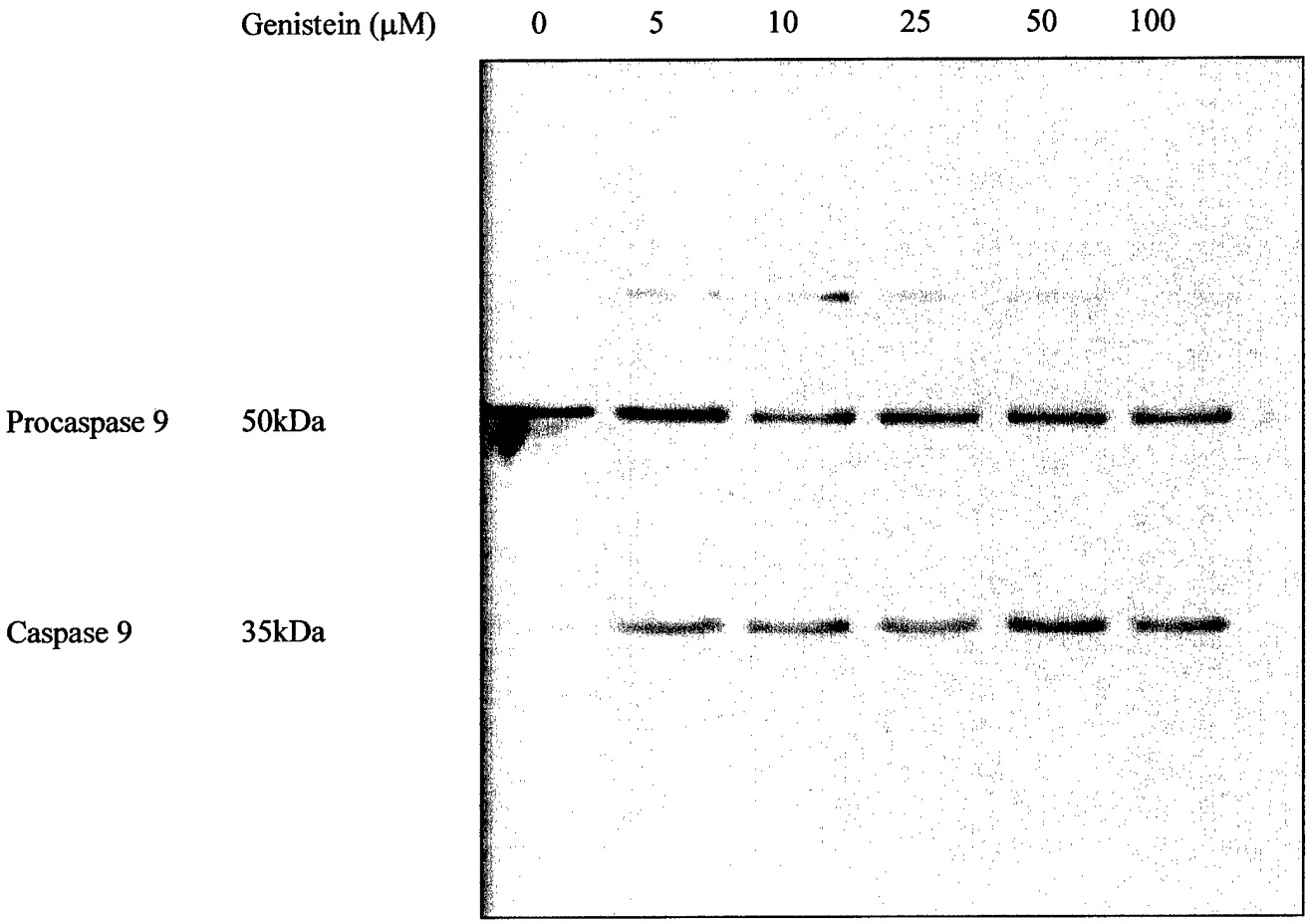


Figure 3. Genistein induces activation of caspase 9 in a dose-dependent manner after 24 hours, as shown by western analysis

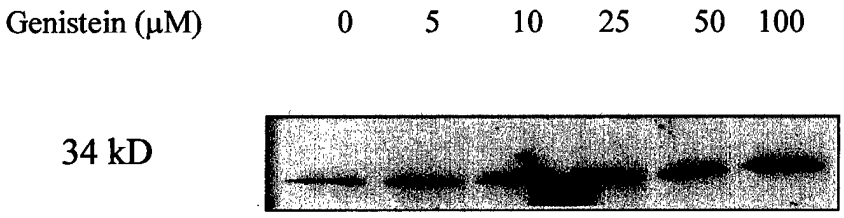
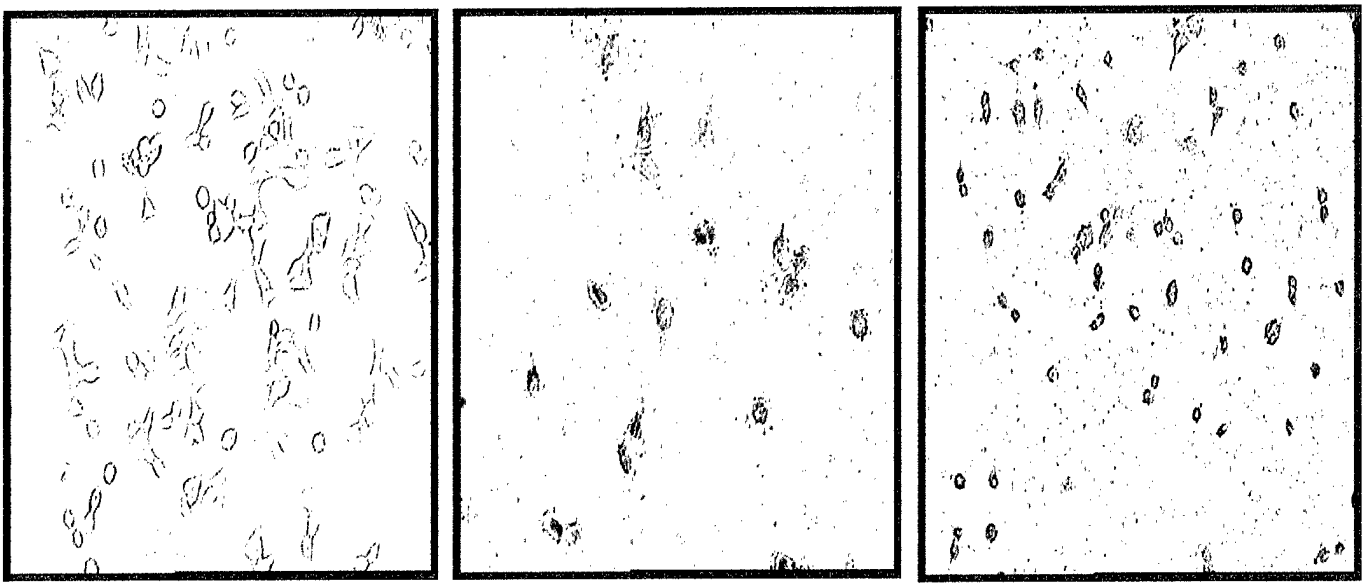


Figure 4. The effect of genistein on procaspase expression in T47D cells after 24 h treatment. A representative of three separate western blots. 30 μg of total protein was separated on a 12% SDS-PAGE gel, transferred to a nitrocellulose membrane, and incubated with Apaf-1 antibody.



Untreated Cells | Genistein (25μM) | Genistein (100 μM)

Figure 5. The effects of genistein on DNA fragmentation in T47D tumorigenic breast epithelial cells after 24 h exposure . Cells were grown in Labtek chambers and stained for TUNEL.

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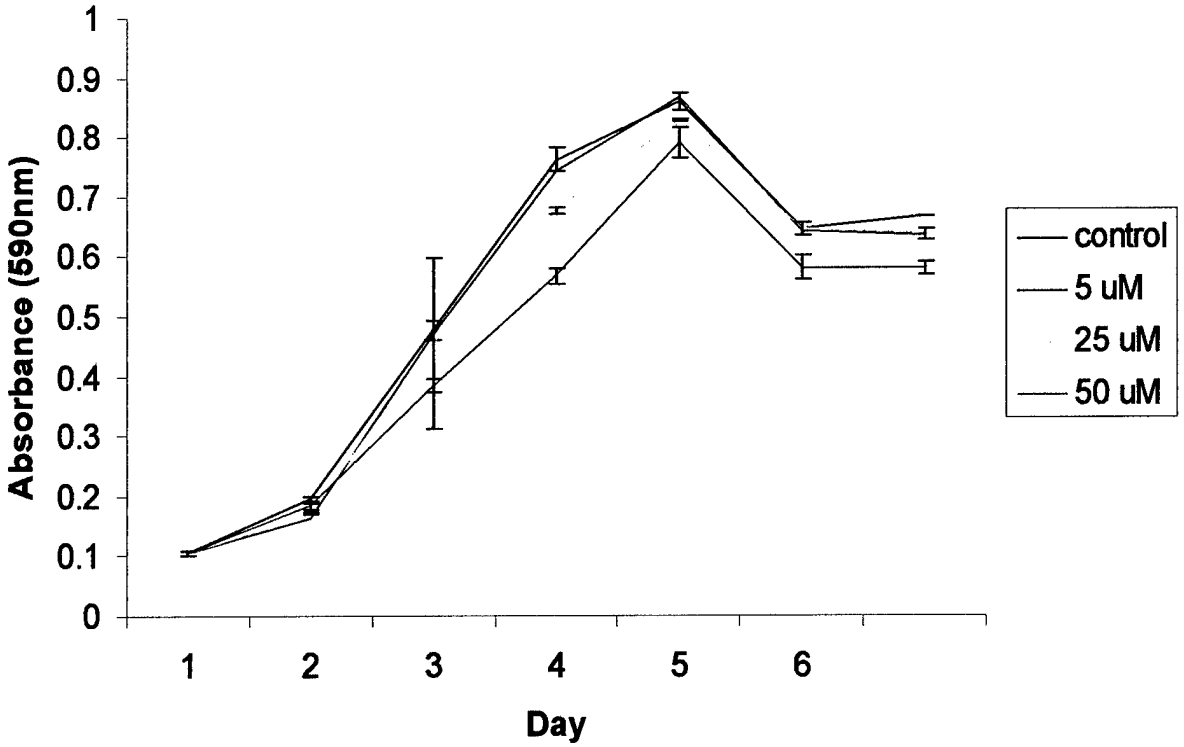

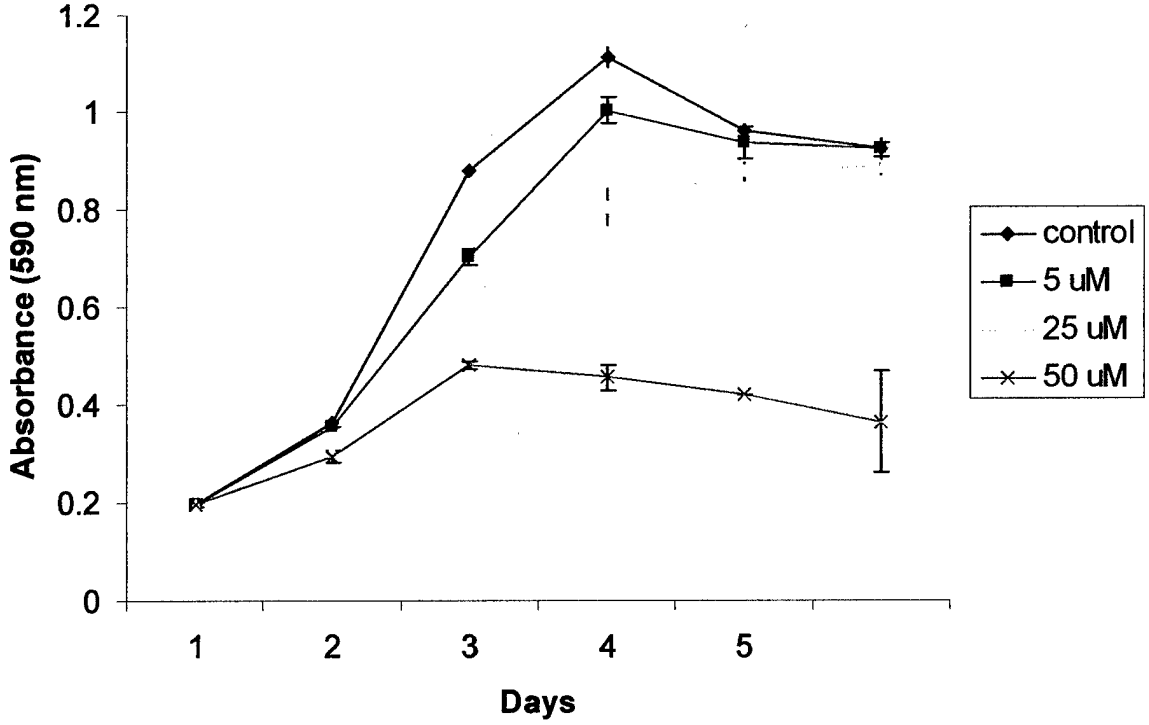

Figure 6. The effects of genistein on MTT reduction of (A) RAT1A fibroblast and (B) c-myc RAT1A fibroblast

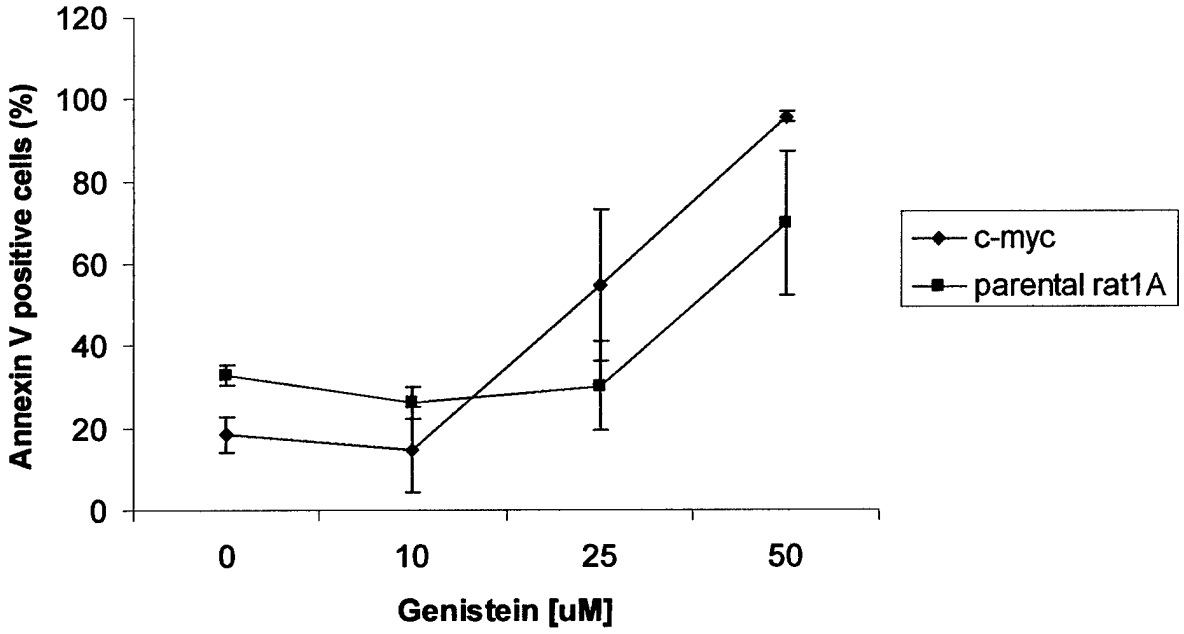

Figure 7. The effects of genistein on annexin V binding in c-myc Rat1A and Rat1A parental cells. Cells were treated with genistein, washed, trypsinized, and stained with Annexin V/propidium iodide, and subjected to FACS analysis

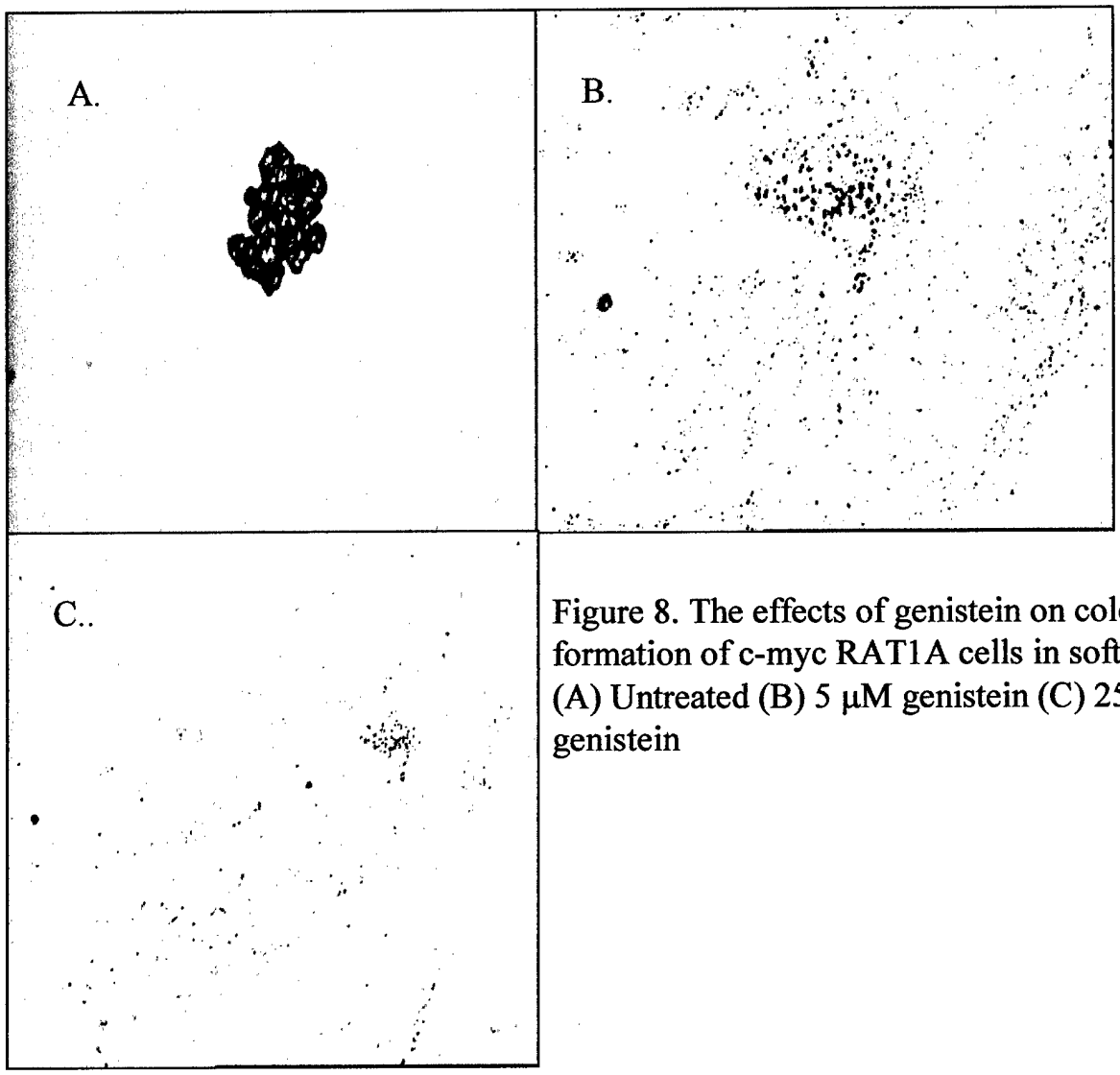

Figure 8. The effects of genistein on colony formation of c-myc RAT1A cells in soft agar. (A) Untreated (B) 5 μM genistein (C) 25 μM genistein

Contrasting the effects of genistein on cell proliferation and cell cycle arrest in nontumorigenic human breast epithelial cells and human breast cancer cells: involvement of Cdc-2, cyclin B1 kinase, and p27/kip1.

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Key words: MCF10A, T47D, genistein, cell cycle, cyclins

Abstract

Studies in many cancer cells, including breast cancer cells, support a role for soy isoflavone, genistein, in the inhibition of cell proliferation and cell cycle progression. However, the effects of genistein on nontumorigenic breast epithelial cells are not well understood. We investigated the effects of genistein on cell proliferation and cell cycle checkpoints in T47D tumorigenic and MCF10A nontumorigenic breast epithelial cells. In exponentially growing cells, genistein inhibited cell proliferation in both MCF10A and T47D cells. However, genistein induced G2 cell cycle arrest in subconfluent/confluent T47D cells but nor MCF10A cells. Furthermore, genistein [25 μM] inhibited cyclin dependent kinase (CDK) activity at 24-48 h, but did not at 96 h. In contrast, genistein treatment in MCF10A cells resulted in an initial decrease in CDK activity [5-25 μM] at 24 h, followed by an increase in CDK activity [5-25 μM] at 96 h. In T47D cells, cyclin B1 and p27/kip1 expressions were increased in response to genistein treatment. However, upregulation of p27/kip1 by genistein [<25 μM] did not inhibit kinase activity in T47D cells. This indicated that p27/kip1 may not be key protein involved in the inhibition CDK activity. In comparison to MCF10A cells, genistein also upregulated cyclin B1 expression, but did not upregulate p27/kip1 to the same extent as with T47D cells. These studies suggest that there are different sensitivities of T47D tumorigenic and MCF10A nontumorigenic breast epithelial cells to genistein-induced cell cycle arrest, which may be explained, in part, by the different effects on CDK activity.

Introduction

Phytoestrogens present in soybean have the subject of intense investigation due to the reported benefits on many diseases, including breast cancer. Genistein is one the principle phytoestrogens contained in soybeans. Although its exact mechanism of action remains unclear, it has been found to be an inhibitor of tyrosine kinases (Akiyama et al., 1987), DNA topoisomerase (Bertrand et al., 1993, Salti et al., 2000), angiogenesis (Fotsis et al., 1995, Fotsis et al., 1998), possess antioxidant activity (Ruiz-Larrea et al., 1997), and targeting stress response elements (Zhou and Lee, 1998), and peroxisome proliferating-activated receptor (Dang et al., 2003). The ability of genistein to inhibit tyrosine kinases and DNA topoisomerases may be contribute to its antiproliferative effects (Cappelletti et al., 2000, Alhasan et al., 2001, Constantinou et al., 1998).

Previous studies have shown that the growth inhibitory effects of genistein in various cancer cell types are accompanied by G2 or G1 cell cycle arrest (Davis et al., 1998, Shen et al., 2000, Lian et al., 1998). Genistein has also been shown to cause a dose-dependent growth inhibition of the different breast cancer cells with accumulation of cells in G2 phase of cell cycle without deregulation of the p34(cdc-2)/cyclin B(1) complex (Cappelletti et al., 2000). Contrary to this, several other studies have shown that genistein-induced cell cycle arrest is related to an inhibition of cyclin B levels, which contributes to the deregulation of the cyclin B1/p34 complex (Choi et al., 2000). In addition, genistein can also exert pronounced antiproliferative effects on both estrogen

receptor-positive and -negative human breast carcinoma cells through G2-M arrest, induction of p21WAF1/CIP1 expression, and apoptosis (Shao et al., 1998).

Although genistein has been reported to inhibit the multiplication of numerous neoplastic cells, including those in the breast, there is limited information on the effects of genistein on nontumorigenic human breast epithelial cells. Whilst one study reported that genistein inhibited proliferation of, and DNA synthesis in MCF10F human nontumorigenic breast epithelial cell line with an IC50 of approximately 19-22 μM, and, with a reversible G2/M block in cell cycle progression. This particular study suggested that genistein also inhibits the growth of nontumorigenic MCF-10F human breast cells by preventing the G2/M phase transition, induces the expression of the cell cycle inhibitor p21(waf/cip1) as well as its interaction with Cdc2, and inhibits the activity of Cdc2 in a phosphorylation-related manner (Frey et al., 2001). Inhibition of cell proliferation of this nontumorigenic breast epithelial cell line is believed to involve MAP kinase signaling (Frey and Singletary, 2003). However, Upadhyay and workers suggested that genistein causes a greater degree of G2-M arrest and induces apoptosis in malignant cell lines compared with normal breast epithelial cells. Interestingly, in these experiments, genistein treatment resulted in a hyperdiploid population in tumorigenic but not nontumorigenic breast epithelial cells (Upadhyay et al., 2001). Therefore, the existing data remains conflicting.

In this present study, we evaluated the effects of genistein on cell proliferation, cell cycle progression, and key proteins involved in the regulation of cell cycle progression in T47D tumorigenic and MCF10A nontumorigenic breast epithelial cells.

Materials and methods

Cell Culture- MCF10A nontumorigenic human breast epithelial cells were incubated in DMEM/F12 media supplemented with 5 % (v/v) Horse Serum (Gemini Bioproducts), 2.5 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco), 20 ng/ml EGF, 100 ng/ml cholera toxin, 10 μg/ml bovine insulin, 500 ng/ml hydrocortisone. T47D breast cancer cells were cultured in RPMI media supplemented with 10 % Fetal Bovine Serum (Gemini Bioproducts) and 1 % penicillin/streptomycin (Gibco). Cells were placed in an incubator with 5% carbon dioxide-air at 37^0C. Cells were continuously exposed to varying concentrations (0, 5, 10, 25, 50, 100 μM) of genistein for 24, 48, 72, and 96 hours depending upon the assay.

Cell growth by MTT reduction- Assessment of cell growth was determined using the Cell Titer 967 Non-Radioactive Cell Proliferation Assay kit (Promega): Cells were seeded at 3000 per well into 96 well plates and cell growth was MTT reduction. Briefly, MTT dye was added to the wells for 4 hours prior to the addition of solubilization buffer. The microtiter plate was then read at 590 nm.

Cell Cycle Analysis- Cells were grown to subconfluence and then DNA content *per* duplicate was analyzed using FACStar flow cytometer as previously described (Qiu et al., 1998). Cells (stained with propidium iodide containing RNase) will be analyzed by flow cytometry. Populations of G0/G1, S, and G2/M will be quantitated using Cellquest software.

Immunoblot and immunoprecipitation analysis for histone H1 kinase- Cells were harvested and lysed with RIPA buffer following treatments. Protein concentration is determined by the Bradford method (Biorad). 30μg of total protein from each sample is separated on a 12% SDS-polyacrylamide gel, and then transferred to a nitrocellulose membrane (Amersham). The membrane is then blocked using 5% casein/tris buffered saline (TBS) (Pierce) for 30 minutes prior to overnight incubation with the appropriate primary antibody at 4° C. Following that, the membrane is washed for 10 minutes for 4 times in TBS/tween and then incubated with secondary antibody (anti-mouse or anti-rabbit). Detection is achieved using electrochemiluminescence (ECL) (Amersham) at 1 and 5 minutes of exposure of radiographic film (Kodak). For histone H1 kinase assay, the cell lysate will be subjected to immunoprecipitation with *cdc2* or cyclin B1 kinase antibody (Santa Cruz Antibodies), as previously described (Qiu et al., 1998). For T47D cells, autoradiographs were obtained after 24 h of exposure of the gel to the film. For MCF10A cells, autoradiographs were obtained after 30 days of exposure of the gel to the film.

BRDU incorporation- Cells were plated at a density of 250 000 in 10 cm culture dishes, and exposed to varying concentration of genistein for 24, 48, 72, and 96 h. Following this, media were removed from plates, and 10 μM BRDU was added with fresh media for 60 min at 37 ^{0}C. Cells were fixed with 70 % ice-cold ethanol, denatured with 2 N HCl with 0.5% Triton-100 for 30 min, and resuspended in 1 ml of 0.1 M Borax/ sodium tetraborate, pH 8.5 and 10 ml water to neutralize the acid. Cells were then incubated with 10 ml anti-BRDU [Promega] for 30 min, washed twice with 1 ml 0.5 % Tween 20/1.0 %

BSA in PBS, and then resuspended in 1 ml of PBS with 5 μg propidium iodide. Samples were analyzed using flow cytometry at laser excitation of 488 nm.

Results

Effects of genistein on MTT reduction, cell proliferation, and cell cycle progression in T47D tumorigenic and MCF10A nontumorigenic breast epithelial cells.

The effect of genistein on MTT reduction was tested over a range of concentrations of 5 to 100 μM in both T47D tumorigenic and MCF10A nontumorigenic breast epithelial cells. As shown in Figure 1, in tumorigenic T47D breast epithelial cells, concentrations of 25 μM was the IC50 for inhibiton of MTT reduction. Concentrations lower than 25 μM had no effect (Fig 1A). However, 100 μM completely inhibited the ability of T47D cells to reduce MTT, thus indicating a toxic response. Similarly, in exponentially growing MCF10A nontumorigenic breast epithelial cells, genistein [>25 μM] also affected the cells' reductive capabilities (Fig 1B).

As determined by bromodeoxyuridine (BRDU) incorporation, DNA synthesis at concentrations of genistein as low as 25 μM was inhibited by 40 % at 96 h in T47D cells. In MCF10A cells, at 25 μM, BRDU incorporation was inhibited by 4 fold (Fig 2).

Incubation with genistein (>25 μM) caused an increase in the number of T47D cells present in G2/M phase of the cell cycle following 96 h exposure. The increase in G2/M population was accompanied by a concomitant decrease in the G1 population. Interestingly following 96 h of exposure, genistein did not cause G2/M phase arrest in MCF10A nontumorigenic cells (Fig 3).

Effects of genistein on cdc-2, cyclin B1 kinase activities in T47D tumorigenic and MCF10A nontumorigenic breast epithelial cells.

Existing evidence supports a role for α-cdc-2-cyclin A/B1 complex in regulating the transition of cells from G2 to M phase. Therefore, we determined whether genistein affected α-cdc-2 or cyclin B1 kinase activities, as well the regulatory effects on cyclin B1. In subconfluent T47D cells, an inhibition of α-cdc-2 histone-associated kinase activity was observed with concentrations of 25 μM or higher at 24 h. This effect was time-dependent from 24 to 48 h. However, by 96 h, the cells overcome this effect (25-50 μM). Similar results are observed with a-cyclin B1 histone-associated kinase activity. In contrast, MCF10A have extremely low levels of cyclin dependent kinase activities. Although the autoradiogram for the CDK activity determined for T47D cells were obtained following 25 h exposure period, detectable levels of cyclin dependent kinase activity were only obtained after 30 days of exposure of the gel to the film. Keeping this in mind, it was possible to see that genistein inhibited α–cdc-2 histone-associated kinase activity following 24 h treatment. However, at 48 and 96 h, there between 5-25 μM of genistein, α–cdc-2 histone-associated kinase activity was increased. This was a part of a biphasic response, with treatments using 50-100 μM genistein inhibited kinase activity (Fig 4).

Effects of genistein on cyclin B expression in T47D tumorigenic and MCF10A nontumorigenic breast epithelial cells.

The mechanism of genistein-induced accumulation of cells in T47D cells in the G2/M phase of the cell cycle was mediated through alterations in the catalytic subunit of p34/α -cdc-2 activity and the expression of cyclin B1 was probed. We noted a time- and dose-dependent increase in cyclin B1 (p62) expression. At 24 h, concentrations lower than 25 μM did not increase cyclin B1 expression at 24-48 h. At 96 h, cyclin B1 expression was

upregulated concentrations of genistein greater than 5 μM (Fig 5A). Interestingly, the upregulation in cyclin B1 expression does not correlate with an inhibition of cyclin dependent kinase activities. Instead, there is a minimal effect on inhibition of α-cdc-2 and cyclin B1 kinases (Fig 4 A). The increase in cyclin B1 expression coupled with no inhibitory effects on cell cycle kinases (at 96 h) appears to be a paradoxical effect. Cell cycle arrest is usually accompanied by a decrease in cyclin levels (quote). Similarly, in MCF10A cells, there was an also an upregulation of cyclin B1 (5-100 μM) at 24 h, which decreases at 48 h, but is returned by 96 h (Fig 5B). However, by 96 h, this is correlated with an increase in a-cdc-2 kinase activity (Fig 4B).

Effects of genistein on p27/kip 1 expression in T47D tumorigenic and MCF10A nontumorigenic breast epithelial cells.

The effects of genistein on cyclin dependent kinase inhibitor, p27, were also investigated. The CDK inhibitor, p27, is a universal inhibitor that is active during both the G1 phase of the cell cycle by binding to the cdk2-cyclin E/A complex to inhibit cdc-2 activity. The kinetics of the effects of genistein on total cellular levels of p27 was examined in both T47D tumorigenic and MCF10A nontumorigenic breast epithelial cells by western analysis, as shown in fig 6. In T47D cells, levels of p27 expression increased in a time- and dose-dependent manner. In contrast to cyclin B1 expression, which was only affected at >25 μM at 24-48 h, p27 expression was sensitive to genistein with 5 μM exposure at 24 h. This suggests that p27, or CDK inhibitors may not play a direct role in regulating the cyclin dependent kinase activity in T47D cells. In contrast, p27 expression was increased with 50 μM exposure at 24 h in MCF10A cells, suggesting that CDK

inhibitors are not as sensitive to genistein challenge as T47D cells. Similar results were obtained for p21 (results not shown).

Discussion

This study was designed to compare the differences in the effects of genistein on the cell cycle components in T47D tumorigenic and MCF10A nontumorigenic human breast epithelial cells. We have demonstrated that exposure of genistein to T47D tumorigenic breast epithelial cells resulted in with concentrations greater than 25 μM for 96 h resulted in G2/M phase cell cycle arrest, but not in MCF10A cells. In agreement with existing studies, genistein inhibited cell proliferation of T47D cells (Dampier et al., 2001) and MCF10A cells by 50 % with a concentration of 25 μM. The differences observed here may be in part related to the confluence of the cell models used in the cell cycle progression studies versus the BRDU incorporation studies. In the cell cycle studies, cells were treated in at subconfluent stage (approximately 80 % confluent). In contrast, for the BRDU incorporation studies, cells in exponential growth were exposed to genistein to measure its effects on DNA synthesis. In a subconfluent model, cells become near confluent after 96 h treatment with genistein. This particular model more closely mimics an in vivo model, where normal breast epithelial cells are in close proximity to each other, MCF10A cells do not undergo cell cycle arrest. In contrast, T47D cells are growth arrested when treated at both the subconfluent phase as well as when they are in exponential growth (data not shown). While nontumorigenic cells do not rapidly divide in vivo and tumorigenic cells rapidly and continuously divide. Therefore, it may be feasible that genistein, similar to other chemotherapeutic drugs, can better target

exponentially growing, rapidly dividing cells. These results are similar to results by Upadhyay and colleagues (Upadhyay et al., 2001), who suggested that there is differential sensitivity of nontumorigenic and tumorigenic breast epithelial cells to genistein. Their investigation showed that genistein induced a greater G2/M phase arrest as well as apoptosis in tumorigenic compared with nontumorigenic breast epithelial cells. Moreover, there is also evidence of a hyperdiploid population of cells associated with the tumorigenic cells which was not present in nontumorigenic cells. Similarly, Peterson et al., reported that normal human mammary epithelial (HME) cells were less sensitive to growth inhibition than MCF-7 tumorigenic cells. The proposed basis for the differential sensitivity of cultured HME cells and a transformed human breast cancer MCF-7 cell line to growth inhibition by genistein was due to a lack of metabolism of genistein by HME cells (Peterson et al., 1996). In contrast, Frey et al., suggested that genistein also exerted antiproliferative effects, induced cell cycle arrest, but did not induce apoptosis in MCF10F nontumorigenic breast epithelial cells (Frey et al., 2001). These effects were correlated with Mitogen-Activated-Protein-Kinase signaling (Frey and Singletary, 2003). However, these studies were conducted for shorter durations (8 and 24 h) compared with longer time-courses for the present studies.

In T47D cells, cell cycle arrest corresponded with a decrease in cyclin dependent kinases, namely α-cdc-2 and cyclin B1 kinases at 24 and 48 h. However, at 96 h, when cell cycle arrest was most prominent, compared with earlier time points (data not shown), at 50 μM treatment, CDK activity was regained. In contrast, MCF10A cells had extremely low and almost undetectable levels of CDK (α-CDK2). Although CDK activity could be detected in T47D cells after 1 day exposure of the gel to film, CDK activity in MCF10A could be

detected without 30 days of exposure. This is reflective of the fact that the overall CDK activity in cancer is much greater than that of noncancer cells which in turn leads to uncontrolled cell proliferation. There was an initial inhibition of α-cdk2 activity after 24 h treatment in MCF10A cells treated with genistein. However, by 48 and 96 h, there was an increase in α-cdk2 activity in response to genistein treatment [5-25 μM]; there was an increase in α-cdk2 activity, whereas 50-100 μM inhibited α-cdk2 activity. The initial decrease in α-cdk2 activity is reflective of the effects of genistein on subconfluent cells that were still proliferating. By 48 and 96 h, the cells had approached confluence whereby the cells are in a similar model to cells in vivo. Thus, genistein can increase α-cdk2 activity. In MCF10A cells once they become confluent. However, in T47D cells, genistein inhibits kinase activity at concentrations greater than 25 μM at 24 and 48 h. At 96 h, despite a large accumulation of cells in the G2/M phase in T47D cells with 50 μM genistein, there is no inhibition of both α-cdk2 and cyclin B1 activities.

In eukaryotic cells, entry into mitosis involves the formation of cyclin B1/α-cdc2 and/or cyclin A-/α-cdc2 complexes, which activates protein kinase activity. As a cell enters the G2 phase of the cell cycle, cyclin B1 levels increase, and cyclin B bind to α-cdc2 to form a complex (Frey et al., 2001). Thus, the effects of genistein on cyclin B1 protein expression were ascertained. In T47D cells, there was an increase in cyclin B1 expression in a time- and dose-dependent manner. The cyclin B1 response of various tumorigenic cells to genistein has been variable. Some existing data suggest that a decrease in cyclin B1 expression paralleled with a decrease in CDK activity in response to genistein underlies cell cycle arrest induced by genistein (Alhasan et al., 2000, Choi et

al., 2000). In addition, another report shows that genistein treatment can result in a biphasic response on cyclin B1: 70% increase in cyclin B1 level at 25 μM, and 50 and 70% decrease at 50 and 100 μM, respectively (Balabhadrapathruni et al., 2000). However, our results are consistent with the work of Capelletti and colleagues (Cappelletti et al., 2000)who demonstrated that genistein caused an increase in cyclin B1 expression in various breast tumorigenic cells, which they hypothesized to be attributed to an accumulation of cyclin B1 in the G2/M phase prior to its degradation. In MCF10A cells, there was also upregulation of cyclin B1 at 24 h in a dose-dependent manner, albeit less pronounced than in T47D cells. However, this upregulation of cyclin B1 decreased at 48 and 96 h. Therefore, cyclin B1 in T47D tumorigenic breast epithelial cells is more sensitive to modulation by genistein than MCF10A nontumorigenic breast epithelial cells.

The activity of CDK can be regulated by cyclin dependent kinase inhibitors (CDKI). p27/kip1 is a CDKI that enters in the G1 phase of the cell cycle and binds to cdk2-cyclin A/E complex to inhibit cdc-2 activity. We, therefore, examined the effects of genistein treatment on p27/kip1 expression in T47D and MCF10A cells. In T47D cells, genistein treatment resulted in a time- and dose-dependent an upregulation of p21/kip1 expression. Although concentrations of genistein of less than 25 μM did not affect cyclin B1 expression and did not inhibit α-cdk2 or cyclin B1 kinase activities, it was effective in upregulating p27/kip1 expression. This may imply that p27/kip1 may not be a crucial element in regulating CDK activity in T47D cells, in response to genistein. Interestingly, another CDKI, p21/cip1, is also upregulated in a similar fashion (results not shown). Our

results suggest that p27/kip1 may be a dispensible factor for genistein-induced G2 arrest. Indeed, it has been shown that p21/cip may be dispensable for the G2 arrest caused by genistein in human melanoma cells (Casagrande and Darbon, 2000). In MCF10 cells, p27/kip1 was also upregulated by genistein [>50 μM] at 24 h. This upregulation was, however, accompanied by a decrease in CDK activity at 24 h with genistein concentrations. At 96 h, there is only a subtle increase in p27/kip1 expression. However, genistein treatment between 5-25 μM increased CDK activity with an absence of cell cycle arrest at 96 h. The upregulation of p27/kip1 in MCF10A cells at 96 h was not sufficient to inhibit CDK activity in MCF10A cells.

In summary, this present study demonstrates that there is a differential sensitivity of T47D tumorigenic and MCF10A nontumorigenic breast epithelial cells to genistein-induced cell cycle arrest. This effect appears to be related to the different effects of genistein on CDK activity. With an increase in soy consumption in health conscious individuals, it is important to conduct further studies to understand and compare the effects of soy phytoestrogen genistein on nontumorigenic as well as tumorigenic breast epithelial cells. Future directions will focus on studying the effects of genistein on the transcriptional activity of cyclin A and B1.

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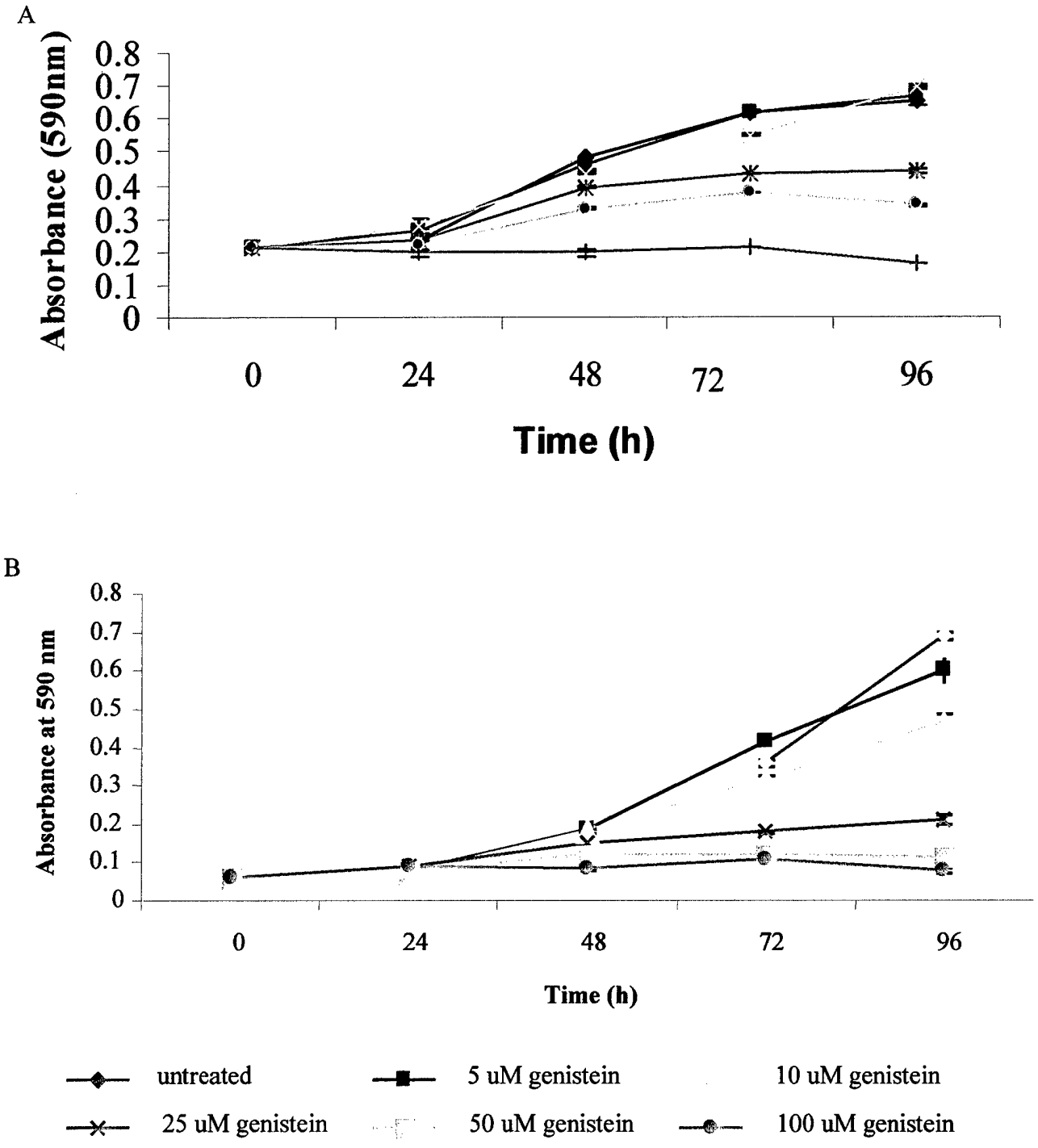
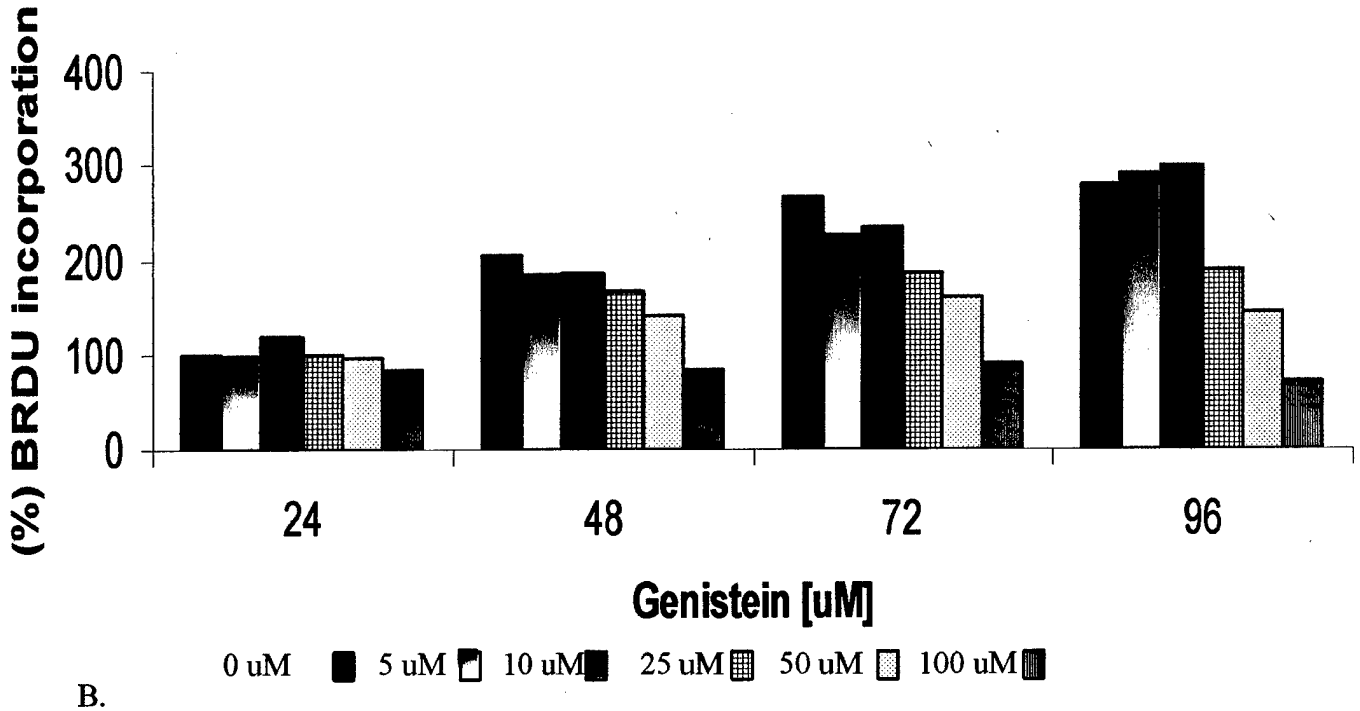


Fig 1. (A) The effects of genistein on MTT reduction in T47D breast cancer cells (A) and MCF10A cells (B) Results are expressed as mean $\pm$ SEM, where n=8. Cells were plated in 96 well culture dishes at a density of 2000 cells/well. The ability to reduce MTT was measured following 4 h of incubation with MTT, followed by addition of solubilization buffer. Samples were measured at 590 nm.

A.



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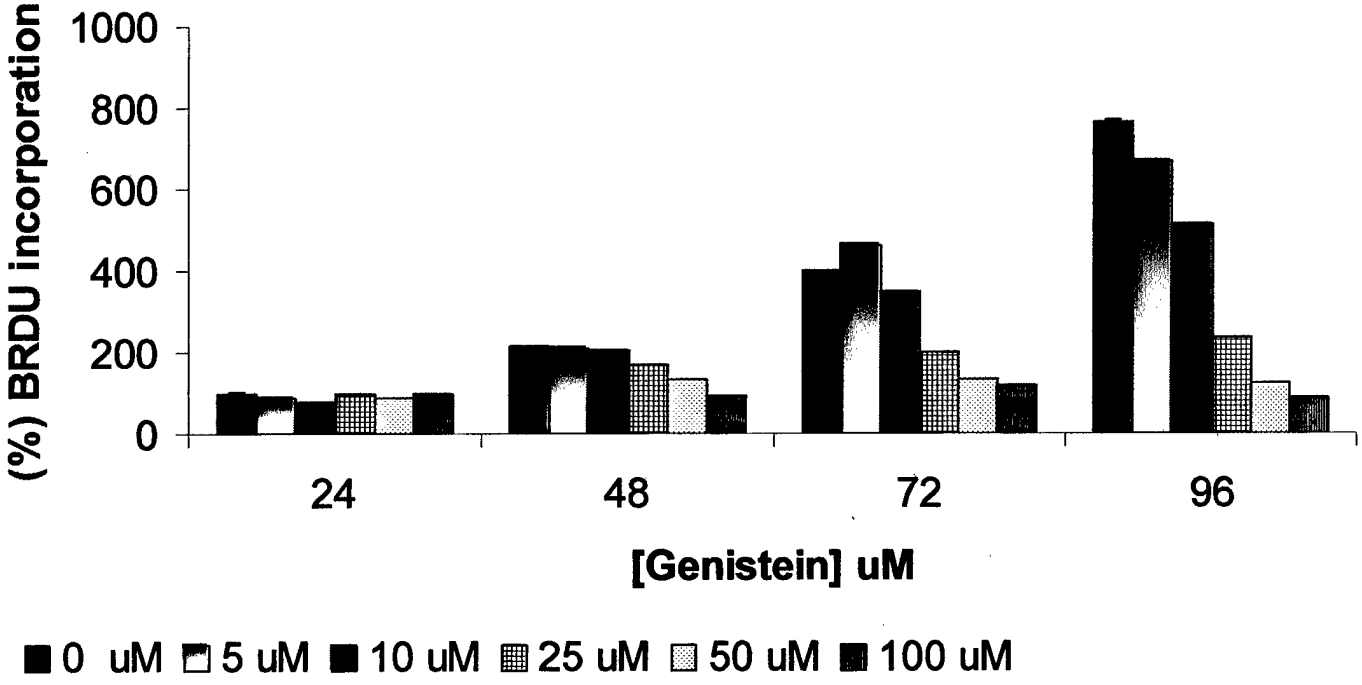
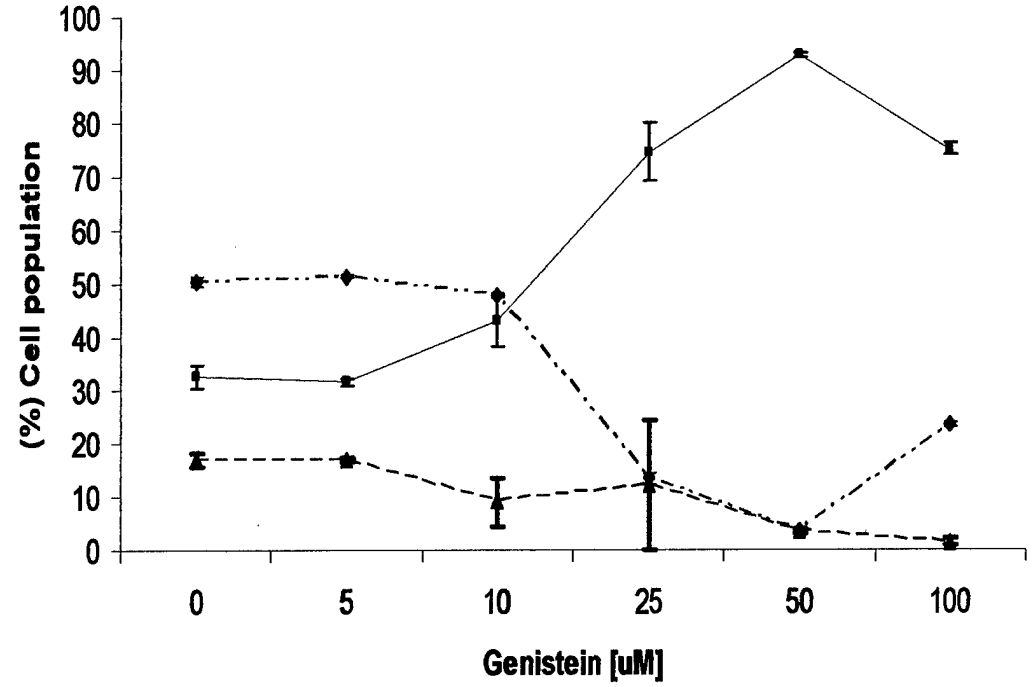


Fig 2. The effects of genistein on BRDU incorporation in (A) T47D tumorigenic breast epithelial cells, and (B) MCF10A nontumorigenic breast epithelial cells. After cells were treated with genistein, 100 μM BDRU was added. Following that, cells were fixed with ethanol, permeabilized, and stained with anti-BRDU conjugated with FITC. Cells were analyzed by flow cytometry.

A.



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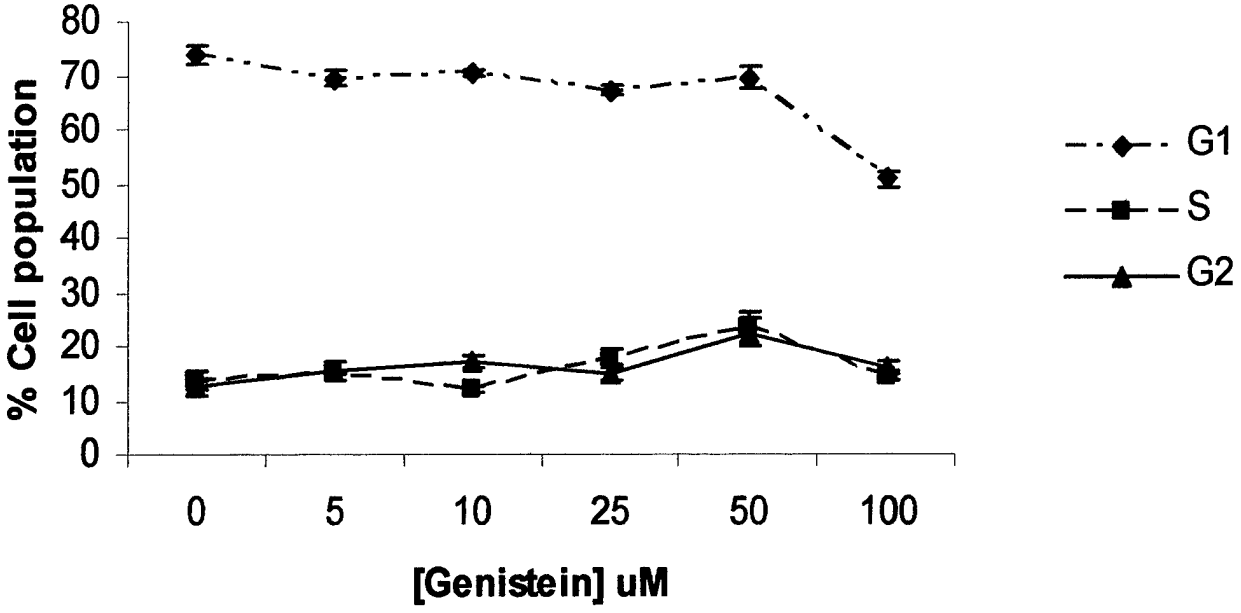
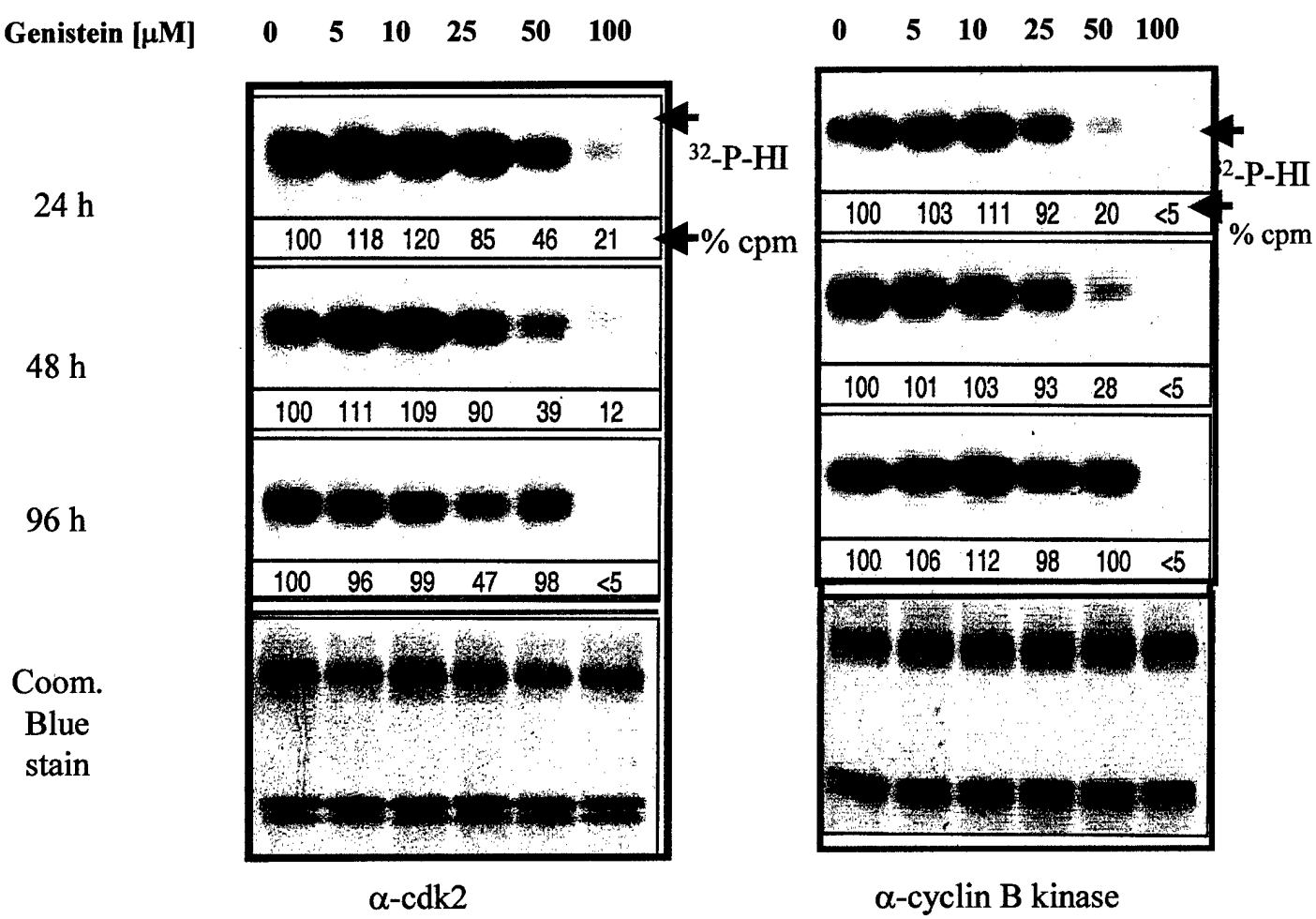


Fig 3. The effects of genistein on cell cycle kinetics in (A) T47D tumorigenic breast epithelial cells and (B) MCF10A nontumorigenic breast epithelial cells. Cells were treated with varying concentrations of genistein, fixed in ethanol, stained with propidium iodide, and analyzed using flow cytometry



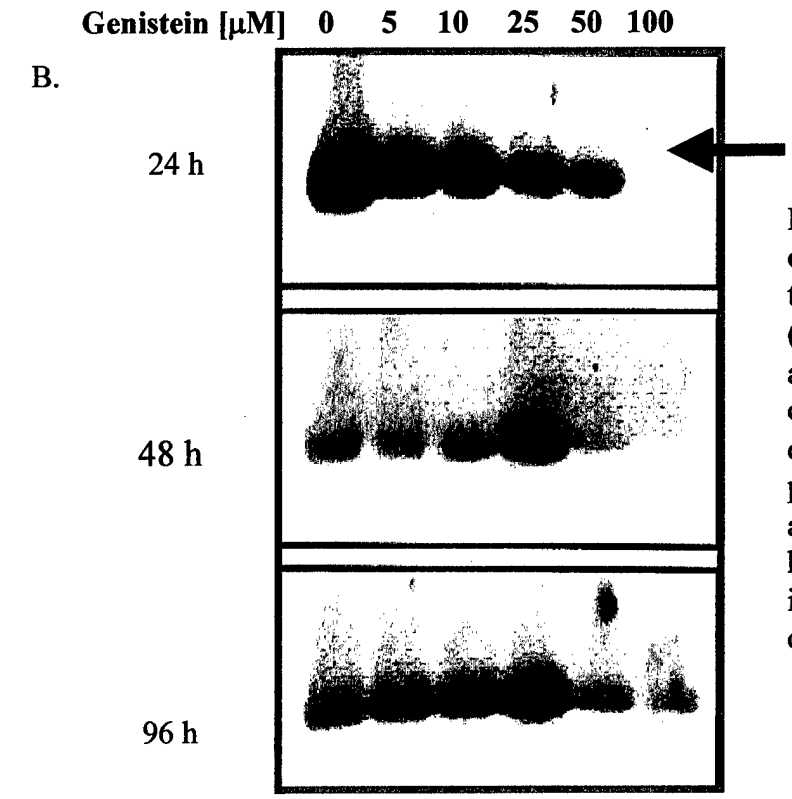
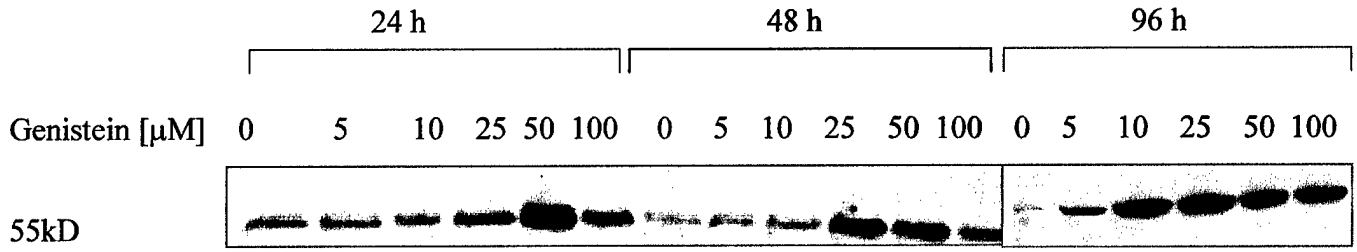


Fig 4. The effects of genistein on the catalytic kinase activities in T47D tumorigenic breast epithelial cells of (A.1) α-cdk2 (A.2) α-cyclin B kinase, and the catalytic kinase activity of (B) α-cdk2 in MCF10A nontumorigenic breast epithelial cells. Aliquots of 100 μg protein were immuoprecipitated with anti- α-cdk2 or α-cyclin B kinase , and histone H1-associated activity of immunopreciptates was evaluated as described.

A.



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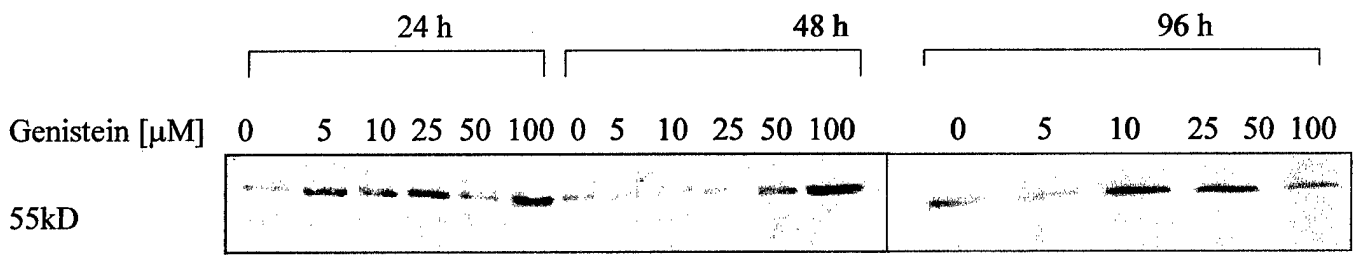

Fig 5. The effects of genistein on cyclin A expression in (A) T47D tumorigenic breast epithelial cells, and (B) MCF10A nontumorigenic breast epithelial cells. A representative of three separate western blots. 30 μg of total protein was separated on a 12% SDS-PAGE gel, transferred to a nitrocellulose membrane, and incubated with cyclin B antibody.

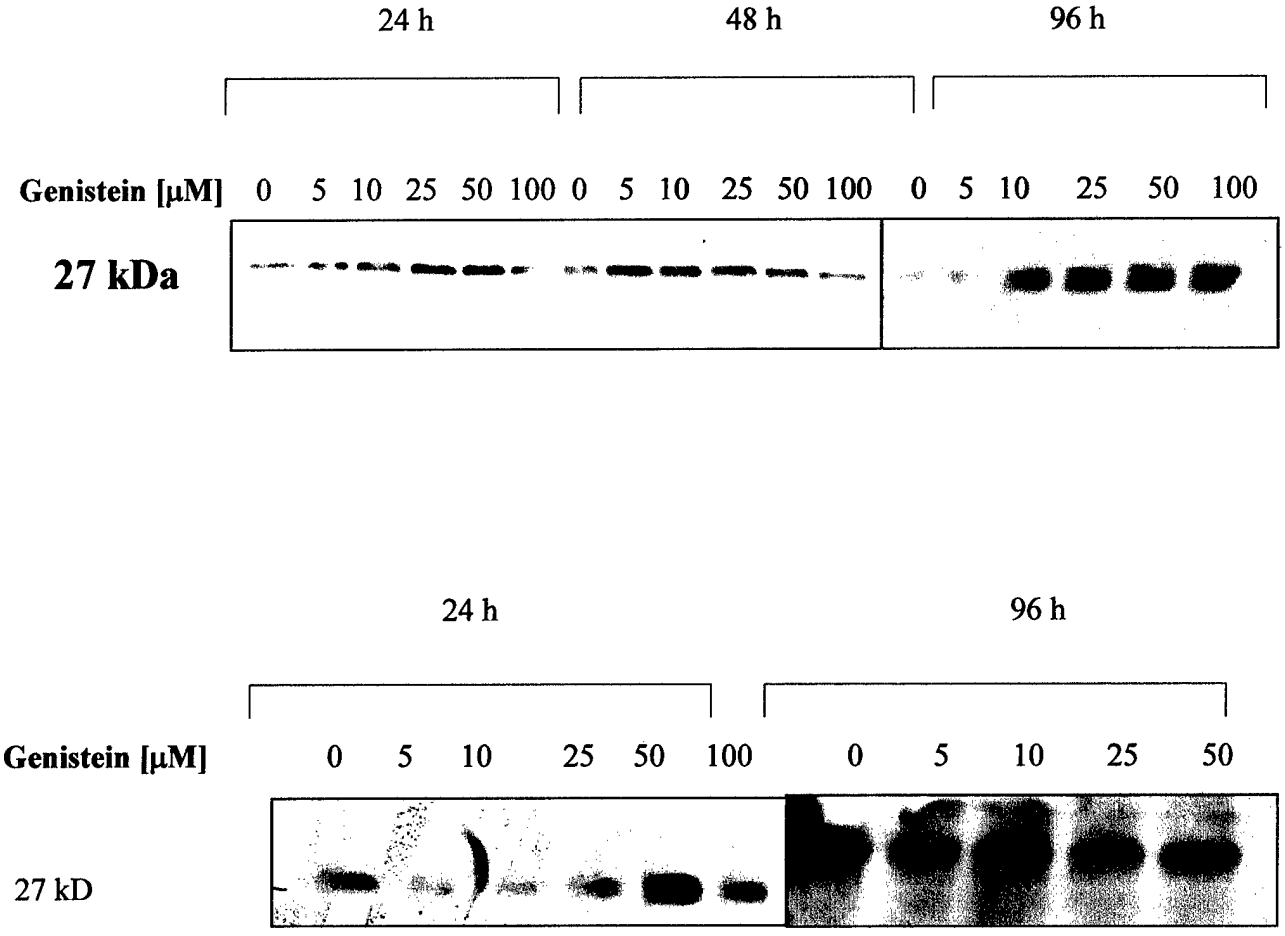

Fig 6. The effects of genistein on p27 expression in (A) T47D tumorigenic breast epithelial cells, and (B) MCF10A nontumorigenic breast epithelial cells. A representative of three separate western blots. 30 μg of total protein was separated on a 12% SDS-PAGE gel, transferred to a nitrocellulose membrane, and incubated with p27 antibody.

Selective cellular association and metabolism of genistein in T47D tumorigenic but not MCF10A nontumorigenic breast epithelial cells: role for CYP450 in formation of a bioreactive metabolite of genistein.

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Summary

Epidemiological studies suggest that consumption of soy isoflavones is associated with a decrease risk of breast cancer. However the mechanisms for their chemopreventive effects have not been elucidated. One isoflavone, genistein, has been shown to prevent tumor formation in several in vivo animal models, and can also exert antiproliferative effects in different cancer cell models, including breast cancer cells. Accumulating evidence suggest that genistein is oxidatively metabolized by the cytochrome P450 family of enzymes, and that its metabolism is specific to transformed breast epithelial cells and not nontumorigenic breast epithelial cells. The CYP450 superfamily of genes encodes a variety of proteins found in most tissues, and these enzymes catalyze the metabolism of endogenous compounds as well as xenobiotics. The increased expression of several CYP450 isoforms has been linked to many types of human cancers. Several lines of evidence suggest that genistein may be an effective prodrug that requires specific CYP450 activation in tumor cells: (1) genistein can be oxidatively metabolized in vitro by human microsomes containing CYP450 (2) tumor cells, including breast cancer cells, express high levels of CYP450 compared to nontumorigenic cells (3) existing studies demonstrate that genistein is not metabolized in nontumorigenic breast epithelial cells to the same extent as in breast tumor cells. However, the possible role of CYP450-mediated oxidation

of genistein in formation of biologically active metabolite(s) of genistein has not been clearly identified. Therefore, the objectives of this study were: (1) to compare the cell-association of isoflavone genistein, and (2) to identify possible biologically active metabolites of genistein in T47D tumorigenic and MCF10A nontumorigenic breast epithelial cells. Our results show that T47D breast cancer cells associated higher levels of genistein than MCF10 breast epithelial cells. Although there were detectable levels of cell-associated genistein in both cell types with low concentrations (<0.5 μM), following 2 hours of exposure, there was no detectable levels of genistein after 24 h treatment with the same low concentrations. This was presumably due to metabolism in both cell types. Furthermore, a genistein metabolite was found to be in higher concentrations in T47D cells than in MCF10A cells. In parallel with this finding, genistein metabolite is also increased in the medium of T47D cells while levels of genistein metabolite in the medium of MCF10A cells were lower. This indicates that genistein is indeed metabolized in cancer cells and not normal cells. The study also probed the effects of CYP450 involvement in the possible formation of a bioreactive form of genistein. Since genistein has been previously shown to cause G2 cell cycle arrest in many cancer cells, the effects of inhibition of cytochrome P450 by cimetidine was investigated. The data demonstrated that cimetidine selectively reversed the genistein-induced G2 cell cycle arrest in T47D

cells. However, genistein did not cause cell cycle arrest in MCF10A cells. In summary, these studies show that genistein exerts distinct effects on cell cycle progression that may be explained by the difference in its selective uptake and metabolism in tumorigenic versus nontumorigenic breast epithelial cells. Furthermore, the bioreactive metabolite is derived from CYP450 oxidation.

Introduction

Epidemiological studies suggest that high dietary soy intake is associated with a decrease risk of breast cancer (Bradlow and Sepkovic, 2002). It is believed that soy isoflavones are the key component that affords chemoprotection. The isoflavone fraction consists primarily of genistein (4',5,7-trihydroxyisoflavone) and daidzein, their 4'-methoxy derivatives biochanin A and formononetin, and their corresponding glycosides, genistin and daidzin. While a specific mechanism of action is not understood, genistein has been shown to inhibit tyrosine kinase (Akiyama et al., 1987), DNA topoisomerase (Constantinou et al., 1995), and angiogeneis (Fotsis et al., 1995), as well as binding to PPAR receptors (Dang et al., 2003), and induce stress response genes (Zhou and Lee, 1998). Although genistein has been reported to exert antiproliferative actions in *in vitro* tumor models, including breast cancer cells (Salti et al., 2000) (Constantinou et al., 1996, Constantinou et al., 1990, Doerge et al., 2000), the relevance of these

studies remain questionable because the high micromolar concentrations used in these studies generally exceed the level of free genistein concentrations in serum (Messina, 1999). Accumulating evidence suggests that serum levels may not reflect tissue or cellular uptake of genistein: (1) The concentration of genistein in endocrine-responsive including rat tissues brain, liver, mammary, ovary, prostate, testis, thyroid and uterus is greater than in serum (Chang et al., 2000). Specifically, the physiologically active aglycone form was present in tissues at fractions up to 100%, and the concentration was always exceeded that observed in serum in which conjugated forms predominated (95-99%). These results for measured amounts of genistein, present as aglycone and conjugates, in putative target tissues provide a link with other studies in which blood concentrations and physiologic effects of genistein are measured. (2) Dietary levels of genistein have been shown to modulate levels of epidermal growth factor and estrogen receptor in rat prostate tissue (Fritz et al., 2002, Dalu et al., 2002, Dalu et al., 1998). Collectively, this suggests that tissue-selective accumulation of genistein may lead to biological responses at the cellular level. Determination of intracellular levels of genistein will provide the relevance of studies using *in vitro* breast cancer models.

The cytochrome P450 (CYP450) superfamily of genes encodes a variety of proteins found in most tissues, and these enzymes catalyze the metabolism of endogenous compounds as well as xenobiotics. These reactions include epoxidation, N-dealkylation, O-dealkylation, S-oxidation and hydroxylation (Guengerich et al., 1998) The expression of several CYP450 isoforms have been linked to many types of human cancers. For example, CYP1B1 is overexpressed in breast tumors as well as lung, liver, gastrointestinal tract, prostate, and bladder (Patterson and Murray, 2002). Anticancer agents can be designed to exploit this feature. Several lines of evidence suggest that genistein may be an effective prodrug that requires specific CYP450 activation in tumor cells: (1) Genistein can be oxidatively metabolized in vitro by human microsomes containing CYP450 (Roberts-Kirchhoff et al., 1999). (2) Tumor cells, including breast cancer cells, express high levels of CYP450 compared to nontumorigenic cells (Patterson and Murray, 2002). (3) Existing studies demonstrate that genistein is not metabolized in nontumorigenic breast epithelial cells to the same extent as in breast tumor cells (Peterson et al., 1996). Furthermore, in transformed breast epithelial cells, genistein metabolism is correlated with a decrease in growth inhibition. This suggested, though not unequivocally, that isoflavone metabolism by transformed breast epithelial cells and not breast cancer cells modulate the growth inhibitory effects of genistein. Because its

effective concentration *in vitro* is in the high micromolar range, it feasible that some metabolites of genistein are the biologically active compound. Although, several studies have demonstrated that genistein is oxidatively metabolized (Kulling et al., 2000, Kulling et al., 2001{Roberts-Kirchhoff, 1999 #95), and sulfated, glucuronidated (Doerge et al., 2000), or hydroylated, and methylated, possible biologically active genistein metabolites remain unidentified. The metabolism of genistein has also been investigated using recombinant human CYP450. Specifically, isoforms 1A1, 1A2, 1B1, 2B6, 2C8, 2E1, or 3A4. can metabolize genistein to form one main hydroylated product, while CYPP450 3A4 produces two different hydroxylated products. These data suggested that different CYP450 isoforms produce different genistein metabolites (Roberts-Kirchhoff et al., 1999). Although some evidence suggests that these hydroxylated and methylated metabolite may be the biologically active, further studies are warranted to directly demonstrate a link between the presence of these metabolites and their biological effect(s).

Based upon these observations, we compared the cellular association of genistein and metabolite(s) of genistein, identified possible biologically active metabolites, and explored the involvement of CYP450 in T47D tumorigenic and MCF10A nontumorigenic breast epithelial cells.

The present study demonstrates that T47D nontumorigenic breast epithelial cells selectively associated a greater amount genistein than MCF10 nontumorigenic breast epithelial cells. Although there were detectable levels of cell-associated genistein in both cell types with low concentrations [0.5-5.0 μM] following 2 hours of exposure, there were no detectable levels of genistein after 24 h treatment with the same low concentrations. This was presumably due to metabolism in both cell types. Furthermore, genistein metabolite was found to be in higher concentrations in T47D cells than in MCF10A cells. In parallel with this finding, genistein metabolite is also increased in the medium of T47D cells. Genistein has been previously shown to cause G2 cell cycle arrest in many cancer cells {Cappelletti, 2000 #109}{Constantinou, 1998 #70}. Inhibition of cytochrome P450 by cimetidine reversed to G2 cell cycle arrest in T47D cells. However, genistein did not cause cell cycle arrest in MCF10A cells.

Materials and Methods

Cell culture- T47D breast cancer cells were cultured in RPMI (Gibco BRL, MD, USA) with 10% fetal bovine serum (Gemini) and 1 % penicillin and streptomycin (Hyclone Laboratories, UT, USA) in 5% carbon dioxide-air at 37^{0}C. Cells will be plated onto 10 cm dishes and grown to 70% confluence. Nontumorigenic breast epithelial MCF10A cells (gift of Dr. Deborah Johnson) were cultured in DMEM/F12; 5% (v/v) Horse Serum (Gemini, USA, 2.5 mM HEPES (Sigma, USA), 2 mM L-glutamine (Sigma, USA), 100 U/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco), 20 ng/ml EGF (Sigma), 100 ng/ml cholera toxin (Sigma), 10 μg/ml bovine insulin (Sigma), 500 ng/ml hydrocortisone (Sigma). All cells were grown to confluence, and were treated with genistein (Sigma, USA) [0.5, 1.0, 5.0, 25, and 50 μM] for 2 and 24 hours. In cell cycle progression studies, cells were also challenged with genistein with or without 250 μM of cimetidine (Sigma), a CYP450 inhibitor, for 96 hours.

Cell cycle analysis- DNA content *per* duplicate will be analyzed using FACStar flow cytometer (Becton Dickinson) as previously described. Cells (stained with propidium iodide containing RNase) will be analyzed by flow cytometry.

Populations of G0/G1, S, and G2/M will be quantitated using Cellquest software {Qiu, 1998 #101}.

Cell-association studies- The cell-associated levels of genistein and possible genistein metabolites from T47D tumorigenic and MCF10A nontumorigenic breast epithelial cells was assessed following 2 and 24 h incubation. The extraction procedure does not distinguish between cytosolic genistein and that which is membrane bound or otherwise cell associated. After exposure, cells were washed 3 times in 40 mL ice cold PBS before lysing with aqueous acidified methanol. The lysed cells were scraped from the plates, collected, vortexed, and centrifuges (1500 rpm for 10 min at 4 degrees celcius). The supernatant was recovered and analyzed by HPLC with photodiodearray detection was previously described. Daidzein was used as an internal standard. Protein concentration of lysate was determined using the Bradford method.

Statistical Analyses

Data were expressed as mean $\pm$ SD. Statistical comparisons were made using an unpaired, two-tailed Student's t-test with a confidence level of 95 %. Significance level was set at $p<0.05$.

Results

Chromatograms

Typical chromatograms obtained from cell lysate of genistein-treated T47D tumorigenic breast epithelial cells showed the presence of both genistein and a genistein metabolite. The retention time of genistein, genistein metabolite, and the internal standard, daidzein, were 59.5, 51.9, and 53.3 min respectively. As expected, the metabolite is a more hydrophilic form genistein (Fig 1).

The cell association of genistein in T47D breast cancer cells and MCF10A nontumorigenic breast epithelial cells

A comparison of the levels of cell-associated genistein and its metabolite in T47D and MCF10A cells was performed. Results were expressed as ng/mg protein in the absence of accurate literature values of volumes for breast epithelial cells. Studies revealed that the T47D breast cancer cells associate more genistein than the MCF10A nontumorigenic breast epithelial cells (Fig 2). Cell-associated levels of genistein in T47D breast cancer cells at both 2 and 24 hours showed a dose-dependent increase in cell-associated concentrations of genistein. In comparison with other dietary flavanoids, cell-associated levels of genistein were relatively

higher in breast cancer cells treated with genistein (5-50 ◎M). The cell-associated levels were similar at 2 hours and maintained at 24 hours. While shorter exposure duration (2 h) and at lower exposure concentrations there are measurable levels of genistein in both cell types (Fig 2A). At the longer 24 h exposure no genistein is detectable at the lower exposure concentrations presumably due to metabolism occurring in both cell types (Fig 2B). Within the range of concentrations of genistein that were tested, T47D cells were generally able to accumulate approximately twice the amount of genistein.

The cell association of genistein metabolite in T47D breast cancer cells and MCF10A nontumorigenic breast epithelial cells

A comparison of the quantitative association of genistein metabolite with the two cell lines revealed that similar to genistein uptake, there is relatively more metabolite (up to 3 fold higher) being formed and accumulated in T47D tumorigenic breast epithelial cells than in MCF10A nontumorigenic cells. An increase in the cell-associated levels of an unidentified metabolite was observed at 2 and 24 hours in T47D cells reaching a plateau at concentrations of 25 μM or higher following 24 hour of treatment. (fig 3A). Contrary to this observation, MCF10A cells did not readily associate with the metabolite. These results suggest that there is a higher concentration of the metabolite being formed or

accumulated in the T47D cells. This may be due to the greater amount of genistein uptake in these cells (Fig 2), or due to a greater amount of metabolism. It does not appear to be due to impaired export of the metabolite form the T47D cells once formed as we measure higher amounts of this metabolite in the medium of these T47D cells also (Fig 3). Therefore, it appears that the T47D cells accumulate and metabolize genistein to a greater extent than the MCF10A cells.

Levels of medium genistein and genistein metabolite in T47D breast cancer cells and MCF10A nontumorigenic breast epithelial cells

At both 2 and 24 h, levels of genistein in media decreased as medium levels of the metabolite increased in T47D cells. In contrast, medium levels of genistein were high while metabolite levels were relatively lower in MCF10A cells. This is consistent with a greater formation of this metabolite by the cancer cells. Furthermore, a steady increase in the cell-associated levels of an unidentified metabolite was observed at 2 and 24 hours (fig 3 & 4). Interestingly, this metabolite was present in the medium at both 2 and 24 hours (fig 5 and 6).

Identification of metabolite in T47D breast cancer cells

In an effort to further identify this metabolite, incubation of this metabolite with two of the phase II enzymes, glucuronidase and sulfatase, was performed. This

resulted in a decrease in the peak at corresponding to the metabolite with a retention time of 51.8 minutes, but did not abolish the peak. Moreover, there was a concomitant appearance of a new peak at a retention time of 53.9 minutes. Therefore, glucuronidation and sulfation alone do not account for this metabolite (Fig 5).

Effects of cimetidine on genistein-induced G2-M phase cell cycle arrest in T47D tumorigenic and MCF10A nontumorigenic breast epithelial cells

The bioreactivity of possible genistein metabolites has not been determined. Although several studies have alluded to possible candidate metabolites, there have been no direct links made between these metabolites and the reported biological actions of genistein. Exisiting studies show that genistein arrests breast cancer cells in the G(2)M phase of the cell cycle (Cappelletti et al., 2000, Santell et al., 2000). Concurrently, it has also been shown that genistein can be oxidatively metabolized by CYP450 family of enzymes (Roberts-Kirchhoff et al., 1999, Kulling et al., 2000, Kulling et al., 2002). Therefore, it is plausible that CYP450 is involved in the formation of a bioreactive metabolite of genistein. Thus, studies were conducted to probe the effects of general CYP450 inhibition by cimetidine on genistein-induced G2 cell cycle arrest. Our results confirmed that genistein induced cell cycle arrest in the G2 phase (65 %) in T47D tumorigenic breast

epithelial cells. Interestingly, cimetidine effectively reversed the G2 cell cycle arrest induced by genistein (Fig 6A). Consistent with the minimal association of genistein and its metabolite in MCF10A nontumorigenic breast epithelial cells, no G2 cell cycle arrest was observed (Fig 6B). These data suggests that there is selective uptake and metabolism of genistein by CYP450 leading to formation of a bioreactive form of genistein in T47D tumorigenic breast epithelial cells.

Discussion

Although some studies suggests that phytoestrogens such as genistein accumulate in breast tissue (Chang et al., 2000), the relative uptake and metabolism of genistein in tumorigenic or nontumorigenic breast epithelial cells is not known. Thus, most of the effects reported based on in vitro experiments cannot be extrapolated to in vivo situations. Moreover, the influence of metabolism on the bioreactivity of genistein is also not well understood. This study assesses for the first time the specific cell association of genistein and its metabolite into both tumorigenic and nontumorigenic breast epithelial cells, and also explores a possible role for CYP450-mediated formation of a bioreacitve metabolite of genistein.

These data suggests that genistein is taken up into the cells, metabolized, and then secreted into the media in T47D tumorigenic breast epithelial cells. The level of cell association of genistein in this tumorigenic cell line is approximately 3-4 times greater than that of other flavanoids with similar structure. Interestingly, there was minimal cell-association of genistein in MCF10A nontumorigenic breast epithelial cells. This difference may be due to the increased level of metabolism associated with tumorigenic cells relative to nontumorigenic cells. Centered upon this concept, it could be surmised that T47D cells are much more metabolically active than MCF10A cells, and can therefore take up more genistein. At this conjuncture, it is unclear whether any differences in possible biochemical markers between the two cells lines could account for this dramatic difference in the cell-association of genistein. Nonetheless, this is an important difference that can be utilized to exploit in the design of drugs that specifically target tumorigenic and not nontumorigenic cells. At the same time, establishing of the cell-association of genistein adds relevance to in vitro models of cancer, as we can better extrapolate the effects to an in vivo situation.

Interestingly, T47D cells accumulated more metabolite than MCF10A cells. Similarly, Peterson et al., described that the basis for the differential sensitivity of

cultured normal human mammary epithelial cells (HME) and a transformed human breast cancer MCF-7 cell line to growth inhibition by the genistein could be accounted for by the observed differences in metabolism. MCF-7 cells extensively metabolized genistein to two metabolites whereas significant genistein metabolism was not observed in HME cells both isoflavones. Of relevance, CYP450 activity and expression is relatively higher also in tumorigenic cells compared with nontumorigenic cells (Patterson and Murray, 2002). Based upon this, it is possible that the CYP450 may be a candidate enzyme that accounts for the difference between the cell-associated levels in T47D tumorigenic versus MCF10A nontumorigenic breast epithelial cells. Of relevance, CYP450 activity and expression is relatively higher also in tumorigenic cells compared with nontumorigenic cells (Patterson and Murray, 2002). Thus, a difference in uptake and overall metabolic capability between the tumorigenic and nontumorigenic cells may be attributed to CYP450 levels.

In T47D cells, the metabolite of genistein was detected, suggesting that once formed, this polar metabolite is excreted from the cells. This parallels the finding that metabolites of genistein have also been detected primarily in the media fraction of MCF7 (Peterson et al., 1996). In our study, medium levels of genistein fall to a greater extent in the mediums of the T47D cells relative to the MCF10A

cells. This would reflect a greater uptake into the cancer cells. Importantly, levels of the new metabolite are much higher in the T47D medium. This is consistent with a greater formation of this metabolite by the cancer cells. In T47D cells, the metabolite of genistein was detected, suggesting that once formed, this polar metabolite is excreted from the cells. This parallels the finding that metabolites of genistein have also been detected primarily in the media fraction of MCF7 (Peterson et al., 1996).

In an effort to further identify this metabolite, incubation of this metabolite with two of the phase II enzymes, glucuronidase and sulfatase, was performed. This resulted in a decrease in the peak at corresponding to the metabolite with a retention time of 51.8 minutes, but did not abolish the peak. Moreover, there was a concomitant appearance of a new peak at a retention time of 53.9 minutes. Therefore, glucuronidation and sulfation alone do not account for this metabolite. Based upon existing studies, this metabolite may also be methylated and hydroxylated (Peterson et al., 1996). Ongoing studies using mass spectroscopy are aimed at identifying this metabolite.

Although one study demonstrated that isoflavone metabolism by transformed breast epithelial cells modulates the growth inhibitory effects of genistein in

transformed cells and not nontransformed cells (Peterson et al., 1996), a role for CY450 oxidation in production of a bioreative metabolite has not been determined. It remains unclear which metabolites of genistein may be pharmacologically active. Under the conditions of the experiments described herein, it was demonstrated the genistein selectively induced cell cycle arrest in the G2-M phase in T47D tumorigenic but not MCF10A nontumorigenic breast epithelial cells. Co-incubation of genistein with cimetidine, a nonspecific inhibitor of CYP450 (Strakowski et al., 2002), resulted in reversal of this observed G2-M phase cell cycle block. However, genistein did not induce cell cycle arrest in MCF10A nontumorigenic breast epithelial cells. Collectively, these results suggest that T47D tumorigenic breast epithelial cells have a selective ability to take up or associate genistein, leading to metabolism of genistein to a bioreactive form via CYP450 oxidation. A clear understanding of the potential mechanisms by which genistein and its metabolites exerts their effects begins by understanding their cellular uptake into cells and tissues.

This is the first study detailing: (1) selective uptake/cell association of genistein (2) selective metabolism of genistein (3) a role for CYP450 oxidation in formation of a bioreactive metabolite. These features are of great relevance in designing a drug that specifically targets tumorigenic cells. Present studies using mass

spectroscopy are aimed at identifying this metabolite, as well as to determine the effects of cimetidine (CYP450 inhibition) on the formation of this metabolite.

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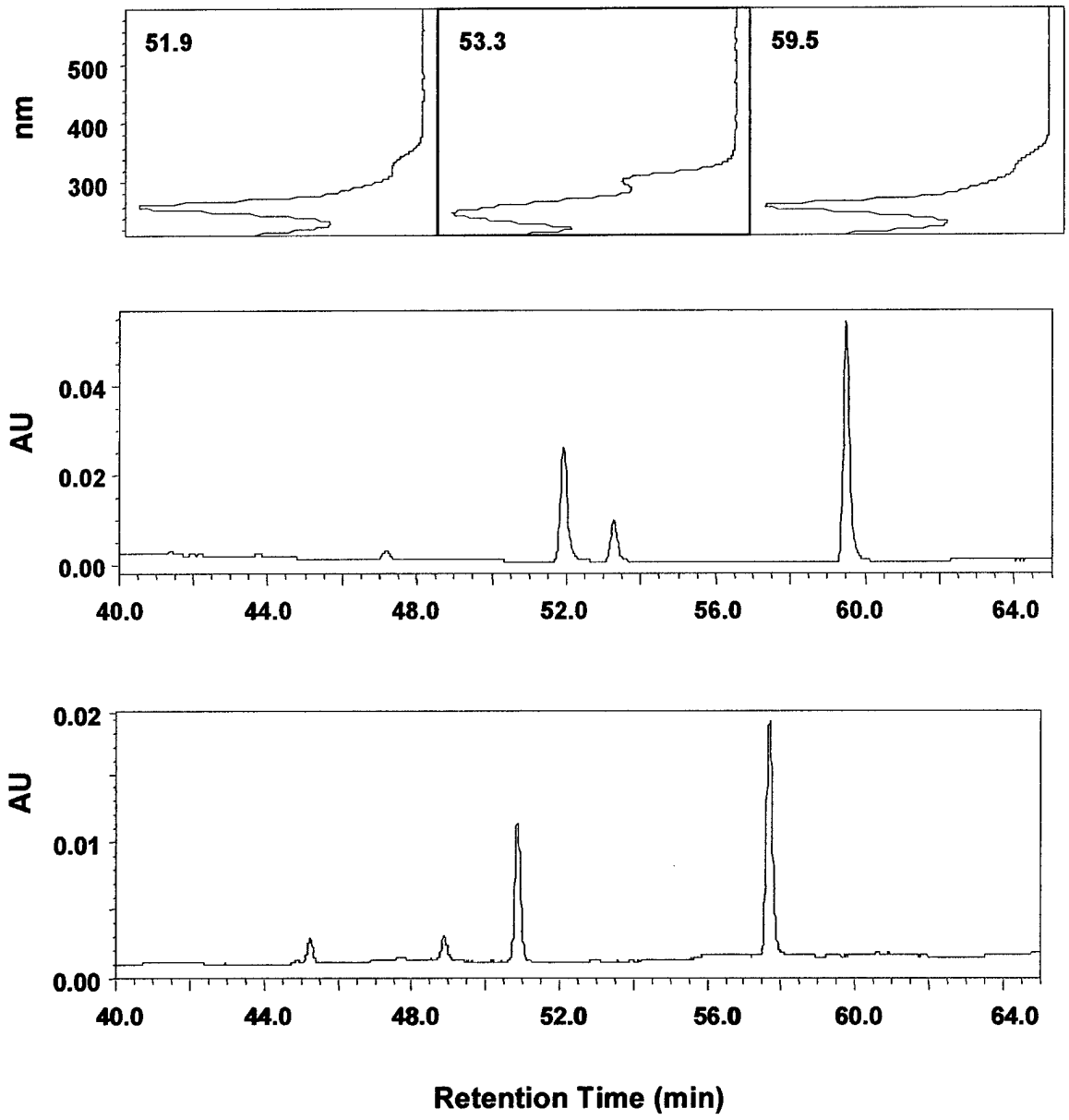


Fig 1. Association of genistein with T47D and MCF10A cells. Typical HLPC traces of cell lysate from cells exposed to genistein (25 μM) for 24 h. Panel A: T47D cells, genistein (RT:59.5 min), daidzein (RT:53.3 min), and metabolite (RT:51.9 min) Panel B: MCF10A cells

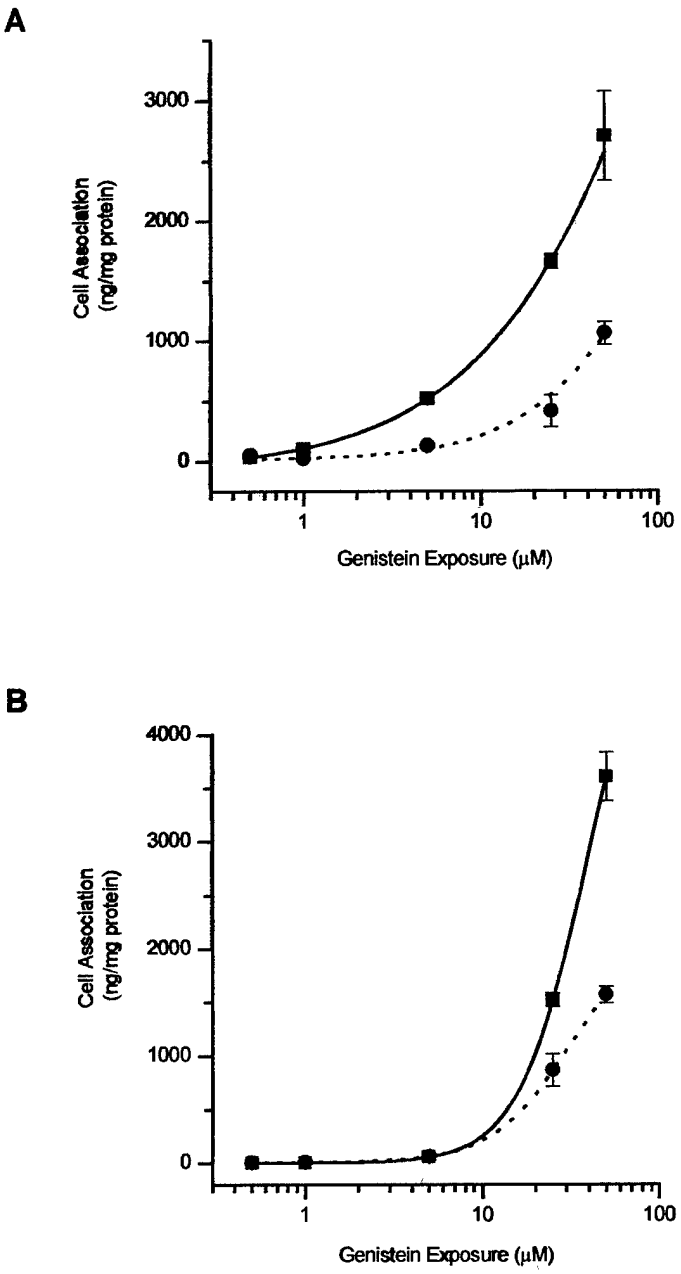


Fig 2.Quantitative association of genistein with T47D and MCF10A cells. Cells were exposed to genistein (0.5-50 μM) for 2 or 24 h after which cells were lysed, deprotonated and analyzed by HPLC-DA. Panel A: 2 h exposure time; Panel B: 24 h exposure time. Solid line: T47D cells.Dashed line: MCF10A cells

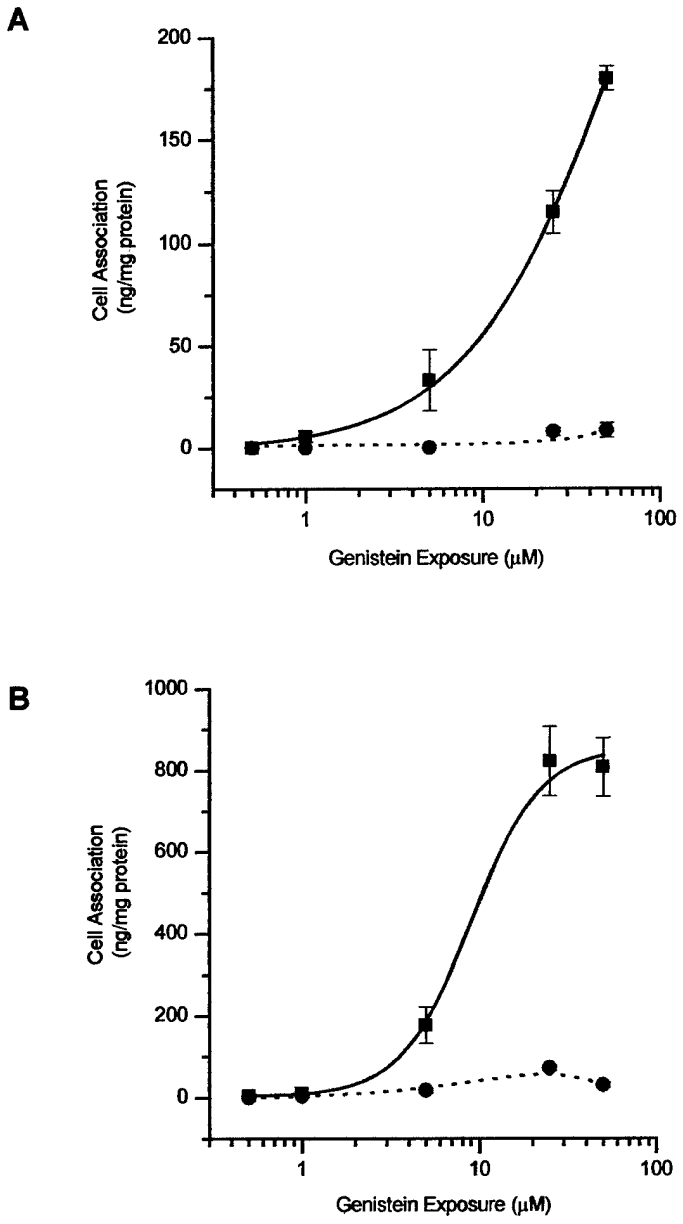


Fig 3. Quantittave association of the genistein metabolite with T47D cells and MCF10A cells. Cells were exposed to genistein (0.5-50 μM) for 2 or 24 h after which cells were lysed, de-protonated, and analyzed by HPLC-DA. Quantitaion of the genistein metabolite was made using genistein as the standard. Panel A: 2 h exposure time, Panel B: 24 h exposure time. Solid line: T47D cells, Dashes line: MCF10A cells

A

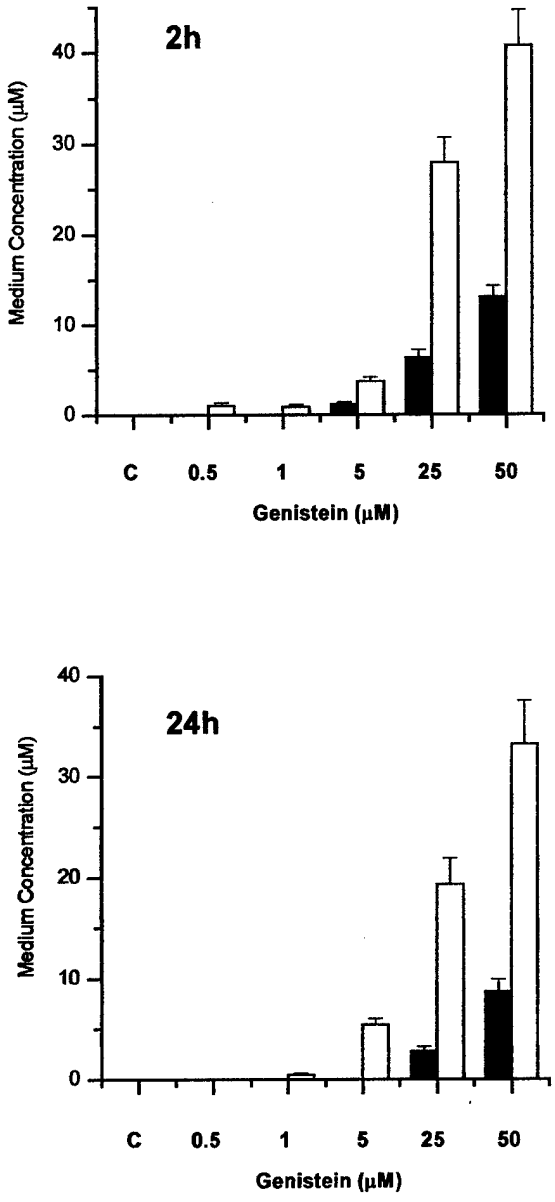


Fig 4. Medium levels of genistein and genistein metabolite following genistein exposure to T47D and MCF10A cells. Cells were exposed to genistein (0.5-50 μM) for 2 and 24 h after which medium was collected and analyzed by HPLC-DA. Panel A: Medium levels of genistein (μM); Panel B: Medium levels of metabolite (μM). Black columns: T47D cells, White columns: MCF10A cells.

B

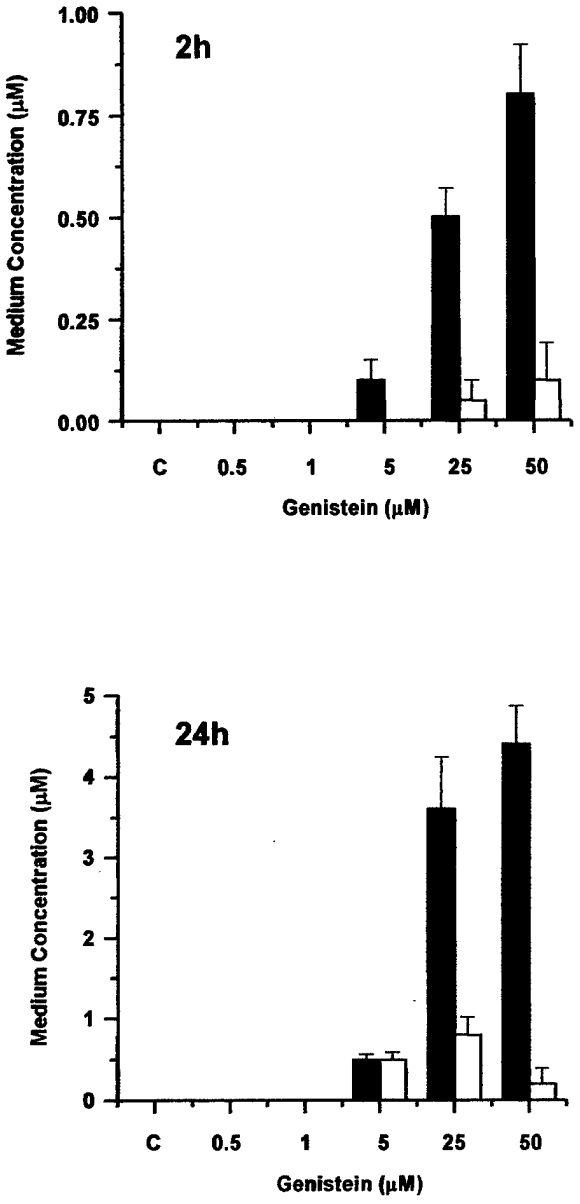
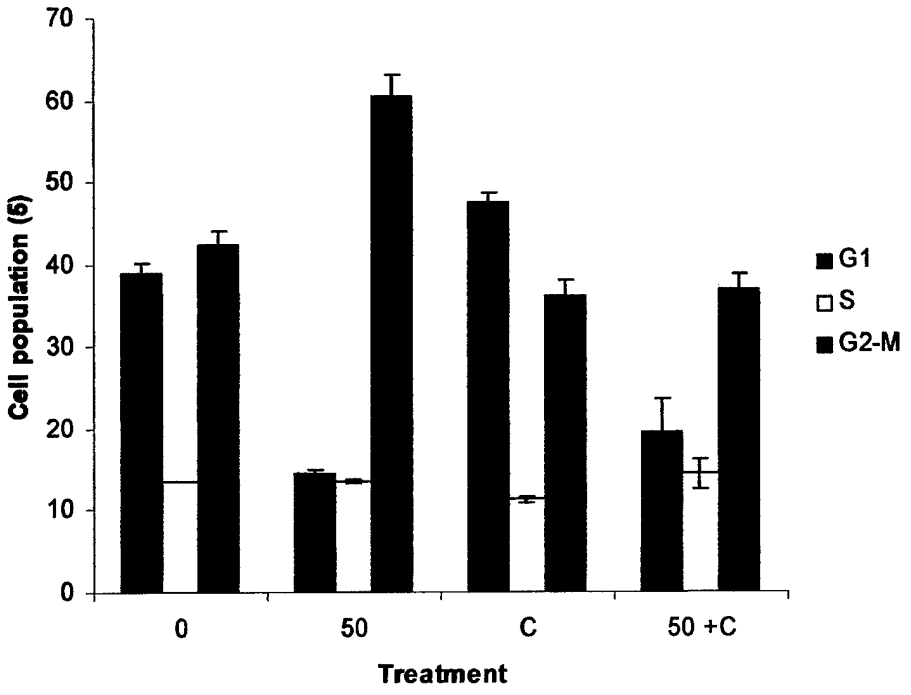


Fig 4. Medium levels of genistein and genistein metabolite following genistein exposure to T47D and MCF10A cells. Cells were exposed to genistein (0.5-50 μM) for 2 and 24 h after which medium was collected and analyzed by HPLC-DA. Panel A: Medium levels of genistein (μM); Panel B: Medium levels of metabolite (μM). Black columns: T47D cells, White columns: MCF10A cells.

A.



B.

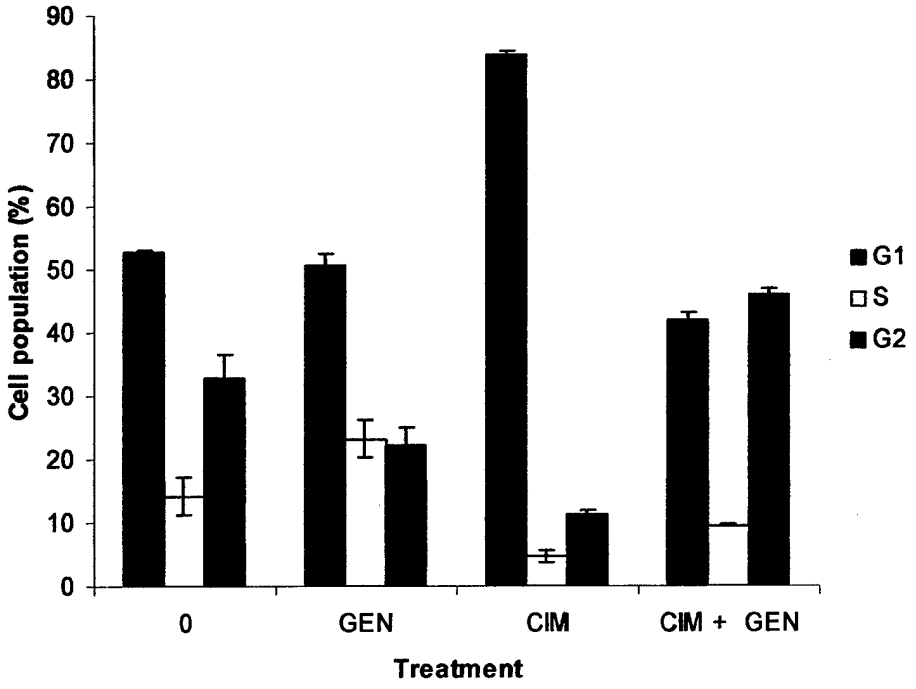


Fig 6. (A) The effects of cimetidine (CYP450 inhibition) on genistein-induced G2 cell cycle arrest in T47D breast cancer cells (B). The effects of CYP450 inhibition on cell cycle progression in MCF10A normal epithelial breast cells